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Sequence variation of captive Malayan Gaur (*Bos gaurus hubbacki*) based on mitochondrial D-loop region DNA sequences

BADRUL MUNIR MD-ZAIN[1,♥], AQILAH ABDUL-AZIZ[1], NOR RAHMAN AIFAT[1], NUR SYAFIKA MOHD-YUSUF[1], ROSLI NORSYAMIMI[2], JEFFRINE JAPNING ROVIE-RYAN[2], KAYAL VIZI KARUPPANNAN[1,2], NADIATUR AKMAR ZULKIFLI[1], SALMAH YAAKOP[1]

[1]School of Environmental and Natural Resource Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. 43600 Bangi, Selangor, Malaysia. Tel.: +60-389-213200, ♥email: abgbadd@ukm.edu.my, abgbadd1966@yahoo.com
[2]National Wildlife Forensic Laboratory, Ex-Situ Conservation Division, Department of Wildlife and National Park (DWNP) Peninsular Malaysia, KM 10 Jalan Cheras, 56100 Kuala Lumpur, Malaysia

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Abstract. *Md-Zain BM, Abdul-Aziz A, Aifat NR, Mohd-Yusof NS, Norsyamimi R, Rovie-Ryan JJ, Karuppannan KV, Zulkifli NA*, *Yaakop S. 2018. Sequence variation of captive Malayan Gaur (*Bos gaurus hubbacki*) based on mitochondrial D-loop region DNA sequences. Biodiversitas 19: 1601-1606.* Malayan gaur (*Bos gaurus hubbacki*) can only be found in Peninsular Malaysia and southern Thailand. The International Union for Conservation of Nature (IUCN) has listed Malayan gaur in the Red List as vulnerable. The main objective of this study was to investigate sequence variation in the mitochondrial D-loop region of *B. g. hubbacki* from two captive centers. We collected 30 DNA samples of Malayan gaur from Jenderak Selatan Wildlife Conservation Center in Pahang and the Sungkai Wildlife Reserve in Perak. Polymerase chain reactions were performed to amplify all the samples. DNA sequences were analyzed using Neighbor-Joining (NJ) and Maximum Parsimony (MP) methods. Based on the 652 base pairs obtained, we found only seven variable characters with a value of 1% and a genetic distance between the two captive centers of 0.001. Haplotype analyses using DnaSP software detected only four haplotypes between these two captive centers. Both NJ and MP trees portrayed all Malayan gaur individuals in Jenderak Selatan and Sungkai captive centers as belonging to the same clade. Genetic variation of Malayan gaur in these centers is considered low due to individuals possibly sharing the same common parent. This sequence variation information is of paramount importance for the proper breeding and conservation management program of Malayan gaur in the future.

Keywords: Malayan gaur, *Bos gaurus hubbacki*, Genetic variation, DNA Sequence, Seladang

INTRODUCTION

Bos gaurus, locally known as seladang, is the largest extant bovine species in the *Bos* genus (Rosli et al. 2016). Malayan gaur has wide shoulders and are brown in color while the lower parts of their legs are pure white as though they are wearing stockings (Figure 1). The three subspecies of *B. gaurus*—namely *B. g. hubbacki*, *B. g. gaurus*, and *B. g. laosiensis* (Rosli et al. 2011) have different distributions: *B. g. hubbacki* is found in Peninsular Malaysia and southern Thailand, *B. g. gaurus* is found in India, southern Nepal, and Bhutan, while *B. g. laosiensis* is found in Myanmar, Laos, Vietnam, and Cambodia (Duckworth et al. 2016).

The International Union for Conservation of Nature (IUCN) has listed *B. gaurus* as vulnerable in its red list (Duckworth et al. 2016). About 500 individuals of Malayan gaur were estimated to inhabit Peninsular Malaysia (Sahir 2001); however, this number has decreased to between 273 and 333 individuals based on statistics provided by the Department of Wildlife and National Parks (DWNP) in 2005. The decreasing number of individuals has put this mammal under the Protected Wildlife Animals Act 76/72 by the DWNP, and conservation effort is needed. Therefore, *B. gaurus* has been placed in captivity as an *ex-situ* conservation program. In Malaysia, a few captive centers are home for *B. gaurus* including Jenderak Selatan Wildlife Conservation Center (Pahang), Sungkai Wildlife Reserve (Perak), the National Zoo, the Taiping Zoo and the Malacca Zoo (Rosli 2012).

Previous studies on Malayan gaur focused on nutrition aspects (Yusof 2009), chromosome evolution (Mamat-Hamidi et al. 2012), semen analysis (Iswadi et al. 2012), phylogenetic relationships (Rosli et al. 2011, 2016), inbreeding (Norsyamimi et al. 2016), and species identification of Malayan gaur, Bali cattle, and Zebu cattle (Romaino et al. 2014). However, little has been done to study the molecular sequence variation of Malayan gaur in captivity. Information gained about the genetic structure within the population can help determine the breeding program and genetic conservation of the species (Xuan et al. 2010). This study of sequence variation in Malayan gaur was conducted to improve conservation efforts and help prevent species extinction. Mitochondrial DNA (mtDNA) of the displacement loop (D-loop) region was chosen because this locus has a potential for molecular studies at the population level (Ang et al. 2011; Ang et al. 2012; Abdul-Latiff et al. 2014a; Md-Zain et al. 2017). The D-loop region has a high mutation rate compared to other mtDNA regions and has the ability to provide high resolution data on phylogenetic relationships (Syed-Shabthar et al. 2013). Thus, the main objective of this study is to infer the sequence variation in the mtDNA D-loop region of *B. g.*

hubbacki in captivity with a focus on the two captive centers.

MATERIALS AND METHODS

Thirty samples of Malayan gaur were obtained from two captive centers in Jenderak Selatan Wildlife Conservation Center, Pahang (14 samples) and Wildlife Reserve Sungkai, Perak (16 samples) (Abdul-Latiff et al. 2017). These noninvasive genetic samples were obtained either from feces or hair follicles. DNA was extracted from these samples using QIAGEN QIAamp DNA Micro Kit and amplified with Polymerase Chain Reactions (PCRs) using an Eppendorf Mastercycler. One pair of specific primers were used to amplify the D-loop region: Walid F (TCA CCG TCA ACT CCC AAA GCT GA) and Walid R (AGG GGG AAG TTT TAT GGA AGG GGG) (Syed-Shabthar et al. 2013). PCR components used in this study were Taq DNA polymerase (5 U/µL), 10X PCR Buffer, 50 mM $MgCl_2$ and 10 mM dNTP. Table 1 lists the PCR components, concentrations, and chemical volumes and Table 2 shows the PCR profile. After amplification, purification was performed using the purification kit GF-1 PCR CLEAN-UP Kit (Vivantis) to remove excessive dNTPs, primers, and buffer. Final purification products were sent for DNA sequencing to First Base Sdn. Bhd. (Shah Alam, Malaysia).

DNA sequences were checked using BLAST software while DNA alignment was completed using BioEdit Sequence Alignment Editor 7.2.0 (Aifat et al. 2016). Using Bioedit, DNA sequences were viewed as chromatograms with different colors for base peaks, and sequences were then compared using ClustalW software (Bakar et al. 2017). All DNA sequences were aligned using MEGA 4.0 (Md-Zain et al. 2018a). Phylogeny trees were constructed based on distance and character approaches using Neighbor-Joining (NJ) and Maximum Parsimony (MP) methods. Bison (*Bison bison*) and water buffalo (*Bubalus bubalis*) were chosen as the outgroup (Syed-Shabthar et al. 2013). The NJ tree was created using the Kimura-2-parameter algorithm (Kimura 1980) supported with 1,000 bootstrap replications (Md-Zain et al. 2018b). MP was executed using a heuristic search of Tree Bisection and Reconnection (TBR) with 1000 random stepwise additions and a 50% consensus majority rule. DnaSP software was used to analyze haplotypes (Rozas et al. 2003). The population genetic structure of Malayan gaur was revealed based on haplotype forms generated from the Arlequin Haplotype List.

RESULTS AND DISCUSSION

Total genomic DNA was isolated from 30 samples of Malayan gaur and three other samples representing the outgroup. The results from DNA sequencing showed that 520 characters (80%) of the 652 characters in the sequences were constant, leaving the remaining 20% as variable characters. Of these 132 variable characters, 63 sites were parsimony informative characters (48%), while the other 52% were parsimony uninformative. However, after the outgroup was removed from the analysis, there were only seven (1%) variable characters. Table 3 shows that there are no parsimony informative characters among the seven variable characters. The nucleotide composition for all the Malayan gaur was also identified. Nucleotide A has the highest frequency of 31.8% while nucleotide G has the lowest frequency of 15.6% only. Nucleotides T and C have a frequency of 28.2% and 24.4% respectively. A genetic distance of 0.001was also calculated between the Jenderak Selatan and Sungkai groups based on the Kimura 2-parameter.

Figure 1. Male Malayan gaur (*Bos gaurus hubbacki*)

Table 1. Final concentrations of the PCR mixture component

PCR component	Final concentration	Volume (µL)
ddH_2O	-	18.8
10X PCR buffer	1X	2.5
dNTP mix (10 mM)	0.28 mM	0.7
$MgCl_2$ (50 mM)	2.4 mM	1.2
Forward primer (10 µM)	0.12 uM	0.3
Reverse primer (10µM)	0.12 uM	0.3
Taq Polymerase (5U/ µL)	1 U	0.2
DNA template	50 ng/uL	1.0
Total	-	25.0

Table 2. PCR cycle profile

Parameter	Temperature (°C)	Duration (sec)	Cycle
Pre-Denaturation	94	180	-
Denaturation	94	60	35
Annealing	58	30	
Extension	72	90	
Post Extension	72	420	-
Cooling	4	∞	-

Table 3. Summary of sequence analyzed

Characters	Base pair (bp)	Percentage (%)
Total characters examined	652	100
Constant characters	520	80
Variation characters	132	20
Parsimony uninformative characters	69	53
Parsimony informative characters	63	47
Variation characters excluding outgroup	7	1

Table 4. Summary of haplotype analysis

Haplotype	Haplotype Sequence	Individual	Location
Hap_1	CTCCCCC	27	14-Jenderak Selatan, 13-Sungkai
Hap_2	TTCCTCC	1	Sungkai (Seladang 3)
Hap_3	CTATCAT	1	Sungkai (Seladang 5)
Hap_4	CACCCCC	1	Sungkai (Seladang 9)

Haplotype analyses were performed using DnaSP version 5 software. The purpose of this analysis was to reveal sequence variation between the two captive centers. Among the 30 individuals, there were only four haplotypes, and each haplotype had only seven base pairs. Haplotype 1 was found in the highest number of individuals (27 individuals) consisting of 14 individuals from Jenderak Selatan and 13 individuals from Sungkai. Haplotypes 2, 3, and 4 were from individuals located in Sungkai. These are Seladang 3, Seladang 5, and Seladang 9 (Table 4). The haplotype diversity value was 0.1931.

The NJ tree (Figure 2) shows the divergence between the ingroup (Malayan gaur) and the outgroup (bison and water buffalo). Malayan gaur from the two captive centers was grouped into the same clade (clade A) with a 100% bootstrap value. The sister clade to the Malayan gaur, composed of bison, was clustered into a different group. Within the Malayan gaur group, Seladang 9 was separated from the other 29 individuals and supported with a 65% bootstrap value. The MP (Figure 3) and NJ tree topologies show little difference: All individuals in the same group in the MP tree are also supported by a 100% bootstrap value. The number of trees produced through the MP analysis was 151. The MP tree analysis has a consistency index (CI) of 0.9747, a homoplasy index (HI) of 0.0253, a retention index (RI) of 0.9773, and a rescaled consistency index (RC) of 0.9525.

Discussion

We conducted a study on the sequence variation within Malayan gaur from two captive centers using the mtDNA D-loop region. The D-loop region is often used for variation studies in either wild animals or animals in captivity (Abdul-Latiff et al. 2014a; Bruford et al. 2003). In this study, only seven sequence variations were observed from a total of 652 base pairs, carrying a value of 1%. This shows that there is low genetic variation among the 30 individuals between the Jenderak Selatan and Sungkai captive centers. This variation is lower than that found by Rosli et al. (2011), who observed a variation of 13.25% by comparing Malayan gaur with other *Bos* genus members using the cytochrome *b* gene. Furthermore, we detected no parsimony informative characters among our seven sequence variations. This suggests that inbreeding has occurred among the individuals of our study and that it is possible that the same common parent has been shared (Norsyamimi et al. 2016). This finding is not surprising as a previous phylogenetic study on Orang Asli and Iban also found similar results with close ties based on a common shared ancestor (Ang et al. 2011).

Our preliminary assumption was that all the Malayan gaur originated from wild populations, captured and protected in these two captive centers. However, an interview with the management officer at Jenderak Selatan Wildlife Conservation Center informed us that only six individuals were originally wild-caught. These six animals consisted of four males (Ahad, Mikael, Isnin, and Jadi) and two females (Biak and Mak Edan) (Abdul-Aziz 2014). These Malayan gaur obtained from wild habitat were used as pioneers for a breeding program (Abdul-Aziz 2014). The reproduction effort started as early as 1982 when the male parent (Ahad) and female parent (Biak) were engaged for a captive breeding program (Sahir 2001). This supports our main finding that a common parent was shared by individuals.

The conservation program of Malayan gaur in the Sungkai Wildlife Reserve was begun in 1998. A few individuals from the Jenderak Selatan Wildlife Conservation Center were brought to Sungkai for a breeding program (Sahir 2001). However, the early lack of experience from the Sungkai Wildlife Reserve management to detect inbreeding in the early stages has led to the genetic consequence that the two captive centers share the same gene pool. Our DNA sequence analysis of Malayan gaur clearly demonstrates that individuals from Jenderak Selatan and Sungkai have low sequence variation with only a 1% difference between them as a result of sharing the same common ancestor.

Haplotype analysis

Previous Malaysian mammal studies also conducted haplotype analyses of the mtDNA D-loop region using the DnaSP software. These haplotype analyses were conducted in the Asian elephant of Peninsular Malaysia (Elliza et al. 2015) and in a population study of otters in Peninsular Malaysia in which a new subspecies of an otter was identified (Rosli et al. 2014). Other loci, such as cytochrome *b*, have also been used, but these results are considered less effective. For example, in a study conducted by Rovie-Ryan et al. (2008), the use of the Cyt *b* region in the haplotype analysis of the Malayan Tapir showed very low haplotype diversity. However, this low variation may be due to the short sequences of the Cyt *b* region.

Based on our haplotype analysis of the D-loop region, only four haplotypes were obtained from the 30 DNA samples. All the Malayan gaur at the Jenderak Selatan Wildlife Conservation Center had one similar haplotype, Hap 1 (5'-CTCCCCC-3'), while the Sungkai captive center had four different haplotypes. Of the 27 individuals with haplotype 1, 13 were from Sungkai. According to the manager at the Sungkai Wildlife Reserve, several individuals of Malayan gaur in this center were taken from Jenderak Selatan Wildlife Conservation Center (Abdul-Aziz 2014), and our haplotype analysis supported this fact.

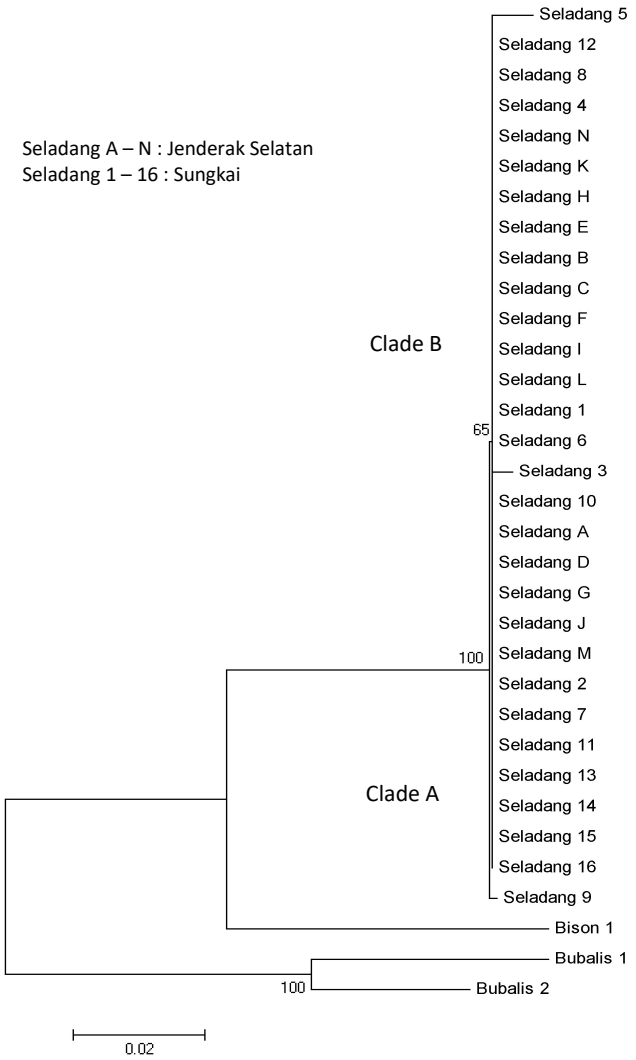

Figure 2. The Neighbor-Joining (NJ) tree of D-loop region

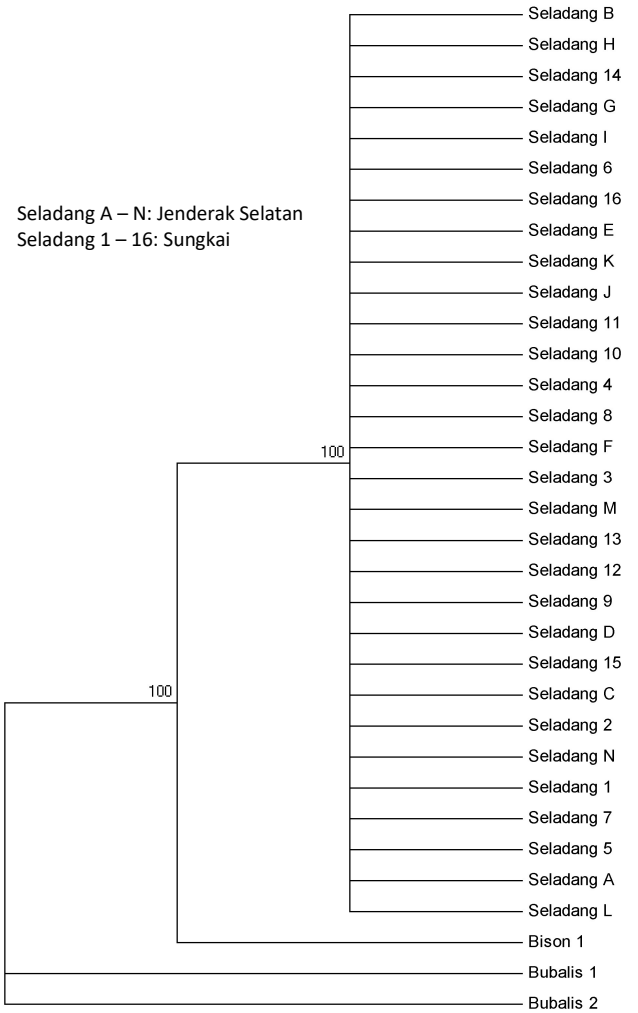

Figure 3. The Maximum Parsimony (MP) phylogenetic tree of D-loop region

Each of the other haplotypes, Hap 2 (5'-TTCCTCC-3'), Hap 3 (5'-CTATCAT-3'), and Hap 4 (5'-CACCCCC -3 '), were found in only one individual gaur each at Sungkai Wildlife Reserve, namely Seladang 3, Seladang 5, and Seladang 9 respectively. Based on the pedigree data at the Sungkai Wildlife Reserve, the breeding program was less managed compared to that at Jenderak Selatan (Abdul-Aziz 2014). Thus, the Malayan gaur population in Sungkai exhibit higher haplotype diversity compared to the gaur in Jenderak Selatan with its single haplotype. However, the haplotype diversity of all samples recorded a low value of 0.1931. According to Hassan et al. (2009), sequence variations characterized the haplotype in their studied sequences. The higher the haplotype diversity, the higher the sequence variation in a population (Liu et al. 2006). The low haplotype diversity value in this study corresponds to the number of haplotypes obtained and the low sequence variation of 1%. This may be due to the samples originating from a captive population in which inbreeding occurred, thereby reducing the variation in the gene pool.

NJ and MP Tree Analysis

All the phylogenetic tree topologies showed a significant separation between the outgroup (bison and water buffalo) and the ingroup (Malayan gaur). The outgroup was selected based on previous studies that also used the *Bos* genus in their studies (Rosli et al. 2011, 2016; Syed-Shabthar et al. 2013). For the NJ tree, clade A was formed by the separation of bison from Malayan gaur, supported with a 100% bootstrap value. Within the Malayan gaur clade A, Seladang 9 is not in the same group as the others (clade B), supported by a 65% bootstrap value, as Seladang 9 is a different haplotype from the other individuals. Seladang 3 and Seladang 5, both individuals in clade B, have a tree length that differs slightly from the other Malayan gaur. The resulting tree is thus equivalent to the results from the haplotype analysis. High haplotype diversity will produce a tree that can distinguish between the populations (Liu et al. 2006).

The MP tree was constructed based on the character method. We produced 151 trees and selected the MP tree

with the least number of changes. The MP tree topology shows a clear separation between Malayan gaur and the outgroup (Rosli et al. 2011, 2016; Syed-Shabthar et al. 2013). The MP tree is slightly different to the NJ tree: All the Malayan gaur are clustered within the same group since there are no parsimony informative characters among the seven variations in the mtDNA D-loop sequence. Parsimony informative characters are important in the formation of MP tree topology (Abdul-latiff et al., 2014a, b; Md-Zain et al. 2014). Thus, the lack of separation between the 30 individuals of Malayan gaur shows that the two captive centers cannot be genetically distinguished from each other.

Both NJ and MP trees show that there is no significant genetic difference in Malayan gaur between Jenderak Selatan and Sungkai. Both captive centers have low genetic variation. The genetic distance analysis, with its value of 0.001, also supports the lack of significant genetic distance between the two captive centers, with all individuals grouped into a single clade. The results of this study support the findings from previous studies of a similar phenomenon of Elliot bird species in captivity (*Syrmaticus ellioti*; Jiang et al. 2005) and the Kangaroo of New Guinea Island (*Dendrolagus matschiei*; McGreevy et al. 2009).

In conclusion, sequence variation of the Malayan gaur was studied to determine the genetic relationships between two Malayan gaur captive centers. The genetic distance among individuals approached zero while haplotype analysis clearly showed no significant genetic differences between Jenderak Selatan and Sungkai captive centers. The findings of this research are of paramount importance for Malayan gaur conservation efforts and its breeding program. Low genetic variation will have a negative effect on this Malayan gaur population which is vulnerable to extinction. Conservation management should avoid the practice of inbreeding among individuals, and the DWNP should increase the number of wild-caught gaur at both captive centers.

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Conservation and selection of plus trees of *Pongamia pinnata* in Bali, Indonesia

NI LUH ARPIWI[1,♥], I GUSTI AYU SUGI WAHYUNI[1], I KETUT MUKSIN[1], SUTOMO[2,♥♥]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Udayana University. Jl. Kampus UNUD Bukit Jimbaran, Badung, Bali Indonesia Tel./fax.: +62-271-663375, ♥email: arpiwi@unud.ac.ic

[2]"Eka Karya" Bali Botanic Gardens, Indonesian Institute of Sciences. Candikuning, Baturiti, Tabanan 82191, Bali, Indonesia. Tel.: +62-368-2033170, 2033170, Fax. +62-368-2033171, ♥♥email: tommo.murdoch@gmail.com

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Abstract. *Arpiwi NL, Wahyuni IGAS, Muksin IK, Sutomo. 2018. Conservation and selection of plus trees of* Pongamia pinnata *in Bali, Indonesia. Biodiversitas 19: 1607-1614. Pongamia pinnata* (L.) Pierre or commonly known as pongamia is a tropical legume tree produces oil seeds for biodiesel feedstock. The aims of present study were mapping growth sites of pongamia in Bali, counting the number of trees and selecting plus trees based on growth parameters such as total height, clear bole height, diameter at breast height, canopy width, and oil content. Method of plus tree selection was comparison tree where one candidate tree was compared with 5 nearby check trees from each village. A total of 126 pongamia trees were found in coastal beach of Bali. The majority of trees were in the north west of the island. Temperature ranges from 26-28°C, humidity ranges from 74-80% and altitude 5-50 meters above sea level. Eight pongamia plus trees were selected from 4 villages namely Kalibukbuk, Pengulon, Pemogan, and Sanur. In conclusion, pongamia was mostly distributed in the northern-west part of the Bali Island of Buleleng District. Lower number of trees was also found in the southern and western part of the island. Trees either scattered or grown in small groups. The number of pongamia tree in Bali is small and this needs further action for conserving the species.

Keywords: Comparison method, conservation, plus tree, *Pongamia pinnata*, selection

INTRODUCTION

Pongamia pinnata (L.) Pierre or commonly known as pongamia is a tropical legume with medium sized perennial tree producing seed oil. The height of the tree is 8-10 meters with trunk diameter about 50 cm and thick spreading branches (Sangwan et al. 2010). The flower is inflorescence long raceme, bisexual and zygomorphic with dark purple-brown calyx, light purple and white corolla (Raju and Rao 2006). The seed oil could be used as raw material for biodiesel (Arpiwi et al. 2013). Biodiesel is fatty acid methyl ester which is derived by reacting vegetable oils or animal fats with alcohol and catalyst known as transesterification reaction (Srivastava and Prasad 2000). The depletion of crude oil reserve due to vary rapid increases in oil consumption has led to the search for alternative renewable energy sources. Pongamia is one of the alternative energy sources that produce non-edible oil for biodiesel feedstock and can be grown on wasteland (Azam et al. 2005).

Pongamia has not been widely cultivated in Indonesia and its population in nature is declining (Jayusman 2017). Broadacre plantations of the species are needed to support biodiesel industry with supplies of feedstock (Scott et al. 2008). These also support the diversification program of renewable energy sources in Indonesia. Selection and improvement of the species is necessary to be studied before raising broad acre plantation with elite trees (Mukta and Sreevali 2010). It needs detailed information about the distribution of the species in nature as sources of breeding material. It is followed by selection of plus trees from natural population to obtain the best phenotype trees, which will be used as source of propagation (Arpiwi et al. 2017).

Data on distribution of pongamia in Indonesia especially in Bali is still very limited. Global Biodiversity Information Facility (GBIF) (https://www.gbif.org/species/2965911) does not have any records of pongamia row in Bali Island. In the red list of IUCN this species is included in the least concern category (Groom 2012). A research by Jayusman (2017) explores pongamia germplasm in Baluran Situbondo National Park of East Java, Alas Purwo Banyuwangi National Park of East Java and Ujung Kulon National Park, Banten Province. In the study, there is no explanation of the number of pongamia trees in each of the National Parks so that no clear picture of the existence of the species in their natural habitat is available. It is mentioned that In Baluran National Park the number of pongamia trees is very small, growing in coastal forest and brackish forest separated 0.5-1 km from each other.

Plus trees are trees that have superior morphological characteristics has advantages over similar tree-like trees, such as growth, height, stem diameter, yields, resistance to disease and oil content of the seeds. Oil content is one of the most important selection criteria because oil is used as raw material for biodiesel (Kesari et al. 2008). Selection of candidate plus tree is an initial and important step for tree breeding for improvement program (Zobel and Talbert 1984). The current research aims at (i) mapping growth sites of pongamia in Bali and; (ii) counting the number of

trees and selecting plus trees based on growth parameters and oil content. This research is also the first to attempt at mapping the distribution of *Pongamia pinnata* in Bali.

MATERIALS AND METHODS

Exploration and mapping of Pongamia

Exploration was carried out along the entire coast of Bali Island, Indonesia to find the growth location of pongamia. The number of trees in each location (village) was counted. Coordinates where each tree grows and the elevation were measured using GPS and weather data including temperature and humidity were recorded. Distribution of pongamia was mapped based on coordinate growth locations. Dried mature pods were taken (2 kg) from each tree for measurement of oil content. Each tree was measured for total height (TH), clear bole height (CBH), diameter at breast height (DBH), canopy width, oil content and observation of pests and diseases affecting the trees.

Oil extraction

Pod samples were dried under the sun for one week. Pods were extracted to obtain the seeds which were then oven dried at 65°C for one week. Dried seeds were ground and then sieved to be homogeneous. Ten (10) grams of fine seed powder was wrapped in a filter paper then placed into a soxhlet for oil extraction. Hexane was used as a solvent and extraction was performed at 65°C for one hour. After the extraction of hexane-mixed oil is separated by distillation. The oil was oven dried at 65°C for one day to evaporate the remaining hexane. The oil yield was expressed as percent dry weight (%w/w).

Selection of plus trees

Selection of plus trees was conducted using comparison trees method. For each village with a minimum of 6 trees, one candidate tree and 5 check trees were selected. The number of pongamia trees in each village varies greatly, ranging from 1 to 30 trees, so that some villages are not represented in the selection of candidate trees because they have less than 4 trees. With respect to candidate and check trees, the following measurements are made: total height, clear bole height, diameter at breast height, canopy width and pest and diseases were measured. Data of check trees and candidate trees were then scored as in Table 1.

Growth parameters

Total height (TH) was measured from the base to the tip of the tree by using software ImajeJ. Clear bole height (CBH) was measured from the base to just under the first branch of a tree. Diameter at breast height (DBH) was measured at 130 cm from the base using a meter tape around the main stem. Canopy width (CW) was measured from the widest distance between two ends of canopy. Pests and diseases were observed on each tree.

The value of each growth parameter for candidate plus tree (CPT) was obtained by dividing the value of CPT by the mean value of check tree (CT) using the following formula:

$$\text{Total Height (TH)} = \frac{\text{THcpt}}{\text{THct}} \times 100$$

$$\text{Clear Bole Height (CBH)} = \frac{\text{CBHcpt}}{\text{CBHct}} \times 100$$

$$\text{Diameter on Breast Height (DBH)} = \frac{\text{DBHcpt}}{\text{DBH ct}} \times 100$$

$$\text{Crown Width (CW)} = \frac{\text{CWcpt}}{\text{CWct}} \times 100$$

$$\text{Oil Content (OC)} = \frac{\text{OCcpt}}{\text{OCct}} \times 100$$

Where
cpt: candidate plus trees
ct : check trees

Data analysis

Growth data and oil content were subjected to cluster analysis with alogarithm paired group and *similarity measure* Euclidean and presented as a dendrogram. Growth data including total height, clear bole height, diameter at breast height and canopy width were correlated with oil content using Statspearman. Weather data including temperature, humidity, and elevation were subjected to analysis of similarity (ANOSIM), non-metric multi-dimensional scaling (NMDS) and principal component analysis (PCA) using Primer 6 software.

Table 1. Scoring CPTs by comparison trees method (Modified from Soeparno 2013)

Characters	Value	Ranges	Score
Total Height (TH)	15	<100%	3
		100-115%	6
		116-131%	9
		132-147%	12
		>147%	15
Clear Bole Height (CBH)	20	<100%	4
		100-140%	8
		141-181%	12
		182-222%	16
		>222	20
Diameter at Breast Height (DBH)	15	<60%	3
		60-85%	6
		86-111%	9
		112-137%	12
		>137%	15
Canopy Width (CW)	15	<69%	3
		69-99%	6
		100-130%	9
		131-161%	12
		>161%	15
Oil Content (OC)	30	<103%	5
		103-105%	10
		106-108%	15
		109-111%	20
		112-114%	25
		>114%	30
Pests and diseases		Absent	5
		Present	0

RESULTS AND DISCUSSION

Distribution of *Pongamia pinnata* in Bali

Pongamia pinnata were found in coastal beach of Bali, Indonesia with a total number of 126 trees and mostly in the north west of the island. Most trees grow naturally. The number of trees in each village varies considerably from 1 to 30 trees. Temperature ranges from 26-28°C, humidity ranges from 74-80% and altitude 5-50 meters above sea level (m asl.). Growth location of *P. pinnata*, the number of trees in each location, temperature, humidity, and elevation were presented in Table 2.

Table 2. Location, weather data and number of *Pongamia pinnata* trees in Bali, Indonesia

Village	Sub-district	District	Number of tress	Temp. (°C)	Humidity (%)	Elevation (m asl)
Penarukan	Buleleng	Buleleng	6	27	75	9
Pemaron	Buleleng	Buleleng	11	28	74	17-20
Kalibukbuk	Buleleng	Buleleng	30	28	74	5-25
Temukus	Banjar	Buleleng	4	28	74	11-28
Dencarik	Banjar	Buleleng	3	28	74	11
Tangguwisia	Seririt	Buleleng	2	28	74	6
Uma Anyar	Seririt	Buleleng	6	27	74	12-14
Kalisada	Seririt	Buleleng	7	27	74	14-20
Celukan Bawang	Gerokgak	Buleleng	4	27	74	13-18
Pengulon	Gerokgak	Buleleng	9	27	74	40-50
Banyu Poh	Gerokgak	Buleleng	1	27	74	17
Sumber Klampok	Gerokgak	Buleleng	14	27	74	8-20
Serangan	Denpasar Selatan	Denpasar	5	28	69	16-23
Pemogan	Denpasar Selatan	Denpasar	11	28	74	5-17
Sanur	Denpasar Selatan	Denpasar	8	27	74	9-12
Kedonganan	Kuta	Badung	1	26	80	9
Tanjung Benoa	Kuta Selatan	Badung	4	28	74	13-18
Total			**126**			

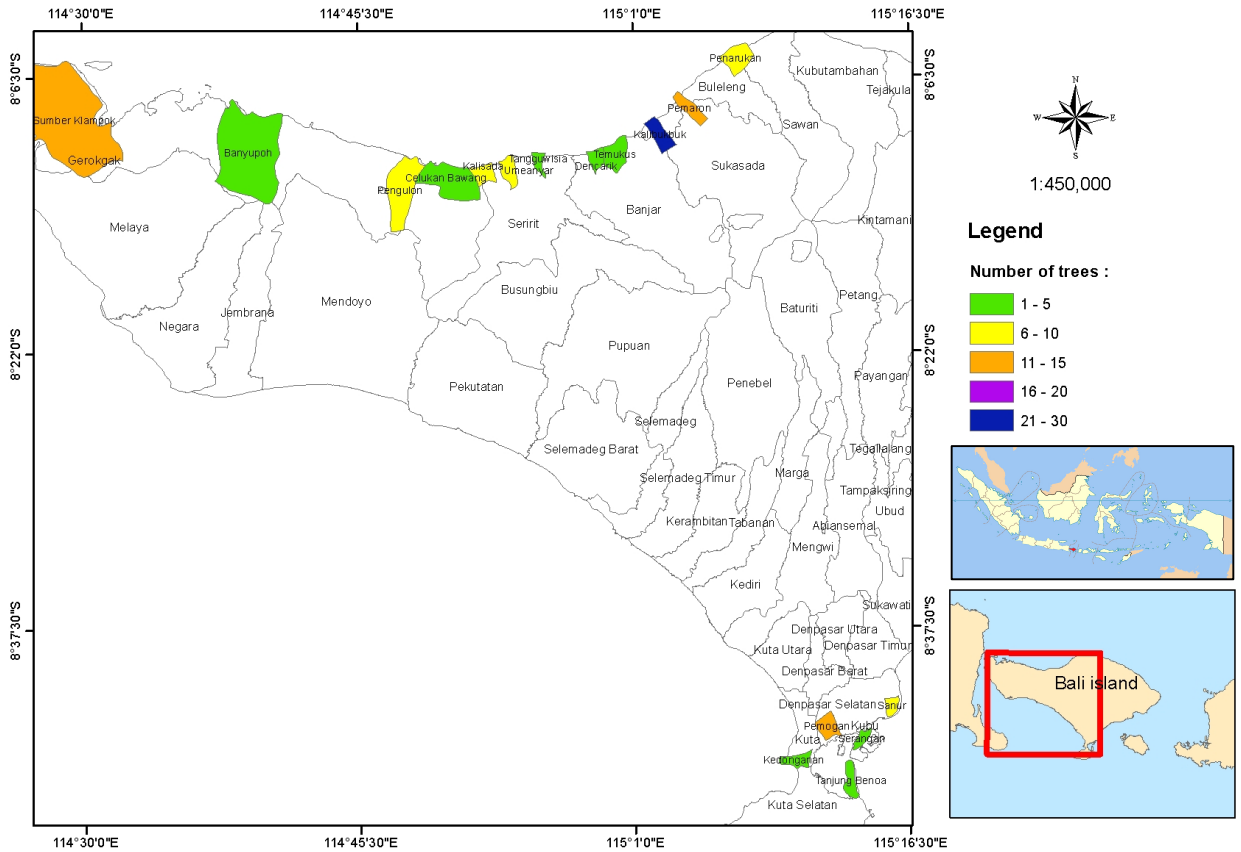

Figure 1. A map of distribution of *Pongamia pinnata* in Bali, Indonesia

Trees were found in 3 districts of Bali, namely Buleleng, Badung and Denpasar. The trees mostly found along the north coast of Buleleng District covering 11 villages, ranging from east to west, namely: Penarukan, Pemaron, Kalibukbuk, Temukus, Tangguwisia, Uma Anyar, Kalisada, Celukan Bawang, Pengulon, Banyupoh and Sumber Klampok Villages. In Badung District, pongamia was found in Kedonganan and Tanjung Benoa Villages. In Denpasar, pongamia was found in the Serangan, Pemogan and Sanur Villages. The map of distribution of pongamia in Bali is presented in Figure 1.

Selection of plus trees

Selection of candidate trees was conducted in villages with a minimum of 6 pongamia trees. Five trees were selected as check trees (CT) and 1-5 as candidate trees depending on the number of trees in each village. From 126 pongamia trees found in Bali, 17 trees were chosen as candidate trees from 9 villages, namely: Penarukan, Pemaron, Kalibukbuk, Uma Anyar, Kalisada, Pengulon, Sumber Klampok, Pemogan, and Sanur. Growth data and oil content of both candidate and check trees are presented in Table 3.

Growth parameters of the 17 candidate trees were rank from the lowest to the highest. Total height was 7.50-13.60 m, clear bole height was 1.40-6.13 m, diameter at breast height was 20.70-63.69 cm, crown width was 6.00-20.00 m, and oil content were 26.00-32.00%. Scores of candidate trees are presented in Table 4.

Tabel 4 showed scores for 17 candidate trees from 9 villages in Bali. Candidate trees with scores more than 60% were selected as plus trees. Eight pongamia plus trees were selected from 4 villages, namely CPT1, 2, 4 and 5 from Kalibukbuk Village, CPT2 from Pengulon Village, CPT1 and 2 from Pemogan Village, and one CPT from Sanur Village.

Cluster analysis

The 17 candidate trees were clustered based on growth parameters and oil content (Figure 2). As can be seen from Figure 2 that the candidate trees were grouped into 2 with a distance of 24. Group A consisted of most of candidate trees while group B consisted of trees from Buleleng District (Pemaron, Kalibukbuk, Kalisada Villages) and Denpasar (Sanur Village). At the distance of 16, group A was divided into 2 subgroups, namely A1 and A2. Group A1 consisted of trees from Buleleng District (Pemaron, Sumber Klampok Kalibukbuk and Kalisada Villages) and Denpasar (Pemogan Village). Group A2 consisted of trees from Buleleng District (Kalibukbuk, Uma Anyar, Pengulon, Penarukan and Sumber Klampok Villages) and Denpasar (Pemogan Village).

Table 3. Growth data and oil content of check (CT) and candidate plus (CPT) trees

Village	Tree code	Total height (m)	CBH (m)	DBH (cm)	Canopy width (m)	Oil content (%)	Pests and diseases
Penarukan	CT	10.14	2.59	55.41	13.20	27.80	A
	CPT	9.32	2.53	46.18	20.00	29.00	A
Pemaron	CT	10.33	3.70	55.10	15.00	28.00	A
	CPT	9.32	4.46	29.30	8.50	28.00	A
Kalibukbuk	CT	11.20	1.46	40.45	10.36	27.90	A
	CPT1	13.60	1.90	41.40	10.00	32.00	A
	CPT2	12.00	2.30	33.44	11.50	32.00	A
	CPT3	9.60	2.69	47.77	7.50	30.00	A
	CPT4	9.55	3.69	63.69	7.50	30.00	A
	CPT5	12.00	2.90	45.54	13.50	30.00	A
Uma Anyar	CT	9.92	2.36	35.22	9.40	30.40	A
	CPT	10.60	3.40	41.40	10.00	32.00	A
Kalisada	CT	12.66	3.23	55.61	14.30	30.20	A
	CPT1	10.70	6.13	61.15	11.00	31.00	A
	CPT2	9.53	2.96	20.70	10.60	32.00	A
Pengulon	CT	10.22	2.00	51.46	10.90	27.60	A
	CPT1	10.90	2.50	40.76	7.00	30.00	A
	CPT2	11.49	5.00	47.77	14.00	30.00	A
Sumber Klampok	CT	8.02	1.54	29.49	9.80	24.20	A
	CPT1	7.50	1.40	29.62	8.00	28.00	A
	CPT2	9.50	1.80	37.90	6.00	26.00	A
Pemogan	CT	7.46	1.68	26.82	7.80	24.00	A
	CPT1	9.30	2.10	28.66	8.00	28.00	A
	CPT2	11.80	1.70	42.99	14.00	27.00	A
Sanur	CT	11.10	2.06	45.86	11.00	26.20	A
	CPT	12.40	3.60	63.06	16.00	29.00	A

Note: A = Absent

Table 4. Sores of candidate trees

Village	CPTs	Total height	CBH	DBH	Canopy width	Oil content	Pests & diseases	Total score
Penarukan	CPT	3	4	6	12	10	5	40
Pemaron	CPT1	9	8	9	9	5	5	45
	CPT2	6	12	3	3	5	5	34
Kalibukbuk	**CPT1**	**9**	**8**	9	**6**	**25**	**5**	**62**
	CPT2	**6**	**12**	6	**9**	**25**	**5**	**63**
	CPT3	3	16	9	6	15	5	54
	CPT4	**3**	**20**	15	**6**	**15**	**5**	**64**
	CPT5	**6**	**16**	12	**9**	**15**	**5**	**63**
Uma Anyar	CPT	6	12	12	9	10	5	54
Kalisada	CPT1	3	16	9	6	5	5	44
	CPT2	3	4	3	6	10	5	31
Pengulon	CPT1	6	8	6	3	15	5	43
	CPT2	**6**	**20**	9	**9**	**15**	**5**	**64**
Sumber Klampok	CPT1	3	4	9	6	30	5	57
	CPT2	9	8	12	6	15	5	55
Pemogan	**CPT1**	**9**	**8**	9	**9**	**30**	**5**	**70**
	CPT2	**15**	**8**	15	**15**	**25**	**5**	**83**
Sanur	**CPT**	**6**	**12**	12	**12**	**20**	**5**	**67**

Note: Bold indicated candidate trees with total scores more than 60% regarded as plus trees.

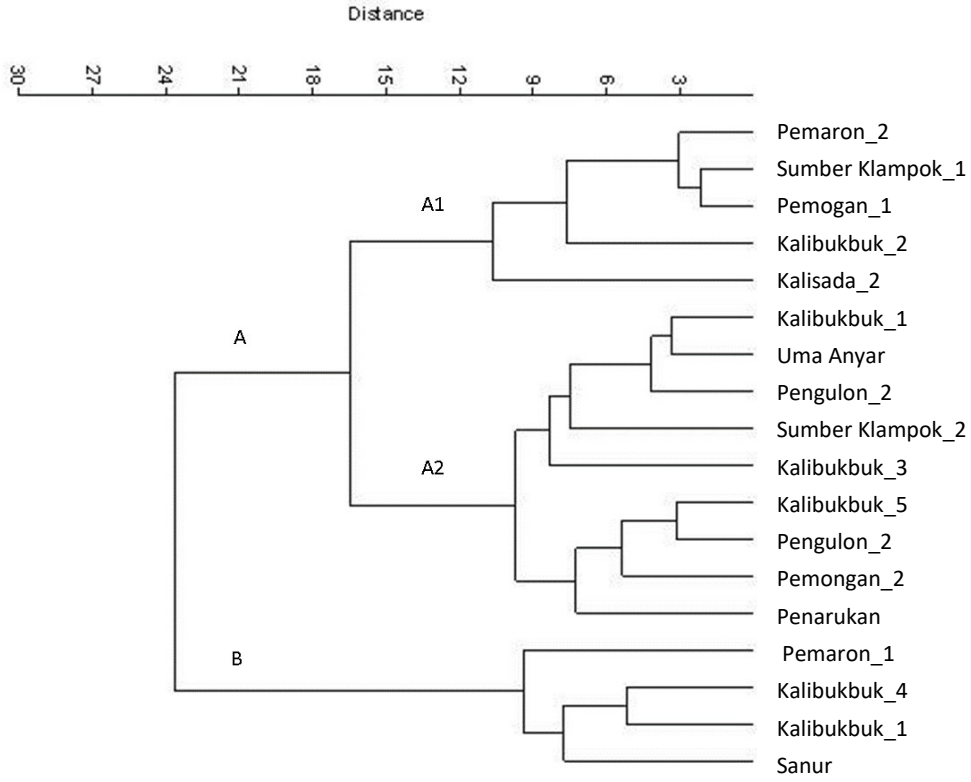

Figure 2. A dendrogram of cluster analysis of CPTs based on growth data and oil content

Table 5. Correlation between oil content and growth parameters

Growth parameters	Oil content		
	Spearman's Rho	df	P value
Canopy width (CW)	0.101	124	0.2594
Diameter at breast height (DBH)	0.204	124	0.022
Total height (TH)	0.267	124	0.00025
Clear bole height (CBH)	0.305	124	0.002516

Note: df = degree of freedom

Correlations

Correlation between oil content and growth parameters including total height, clear bole height, diameter at breast height, and canopy width is presented in Table 5. It is showed that oil is significantly correlated with total diameter at breast height (P = 0.022), total height height (P = 0.00025), clear bole height (P = 0.002516).

Weather data and elevation

Multivariate analysis of the environmental factors including temperature, relative humidity and elevation indicated that there were differences in those factors among sites. Analysis of similarity (Figure 3) showed global R ANOSIM of 0.6-0.7 indicating significant difference in environmental factors among sites (villages).

Sites were clustered based on the environmental factors using Nonmetric Multidimensional Scaling (NMDS) (Figure 4). There are 4 cluster sites, namely Pengulon, Serangan, Sumber Klampok and mixture of sites.

Principal component analysis (PCA) of the sites based on temperature, relative humidity and elevation (Figure 5) indicating most sites had similar temperature and relative humidity respectively. The difference among sites was due to elevation. The only one site had differences in both temperature and RH was Serangan Village.

Discussion

Pongamia were mostly distributed in the northern-west of Bali Island in Buleleng District (Figure 1). Trees mostly grow in small group of 2-14 trees and vary rarely in a big group. The only big group of 30 trees was found in Kalibukbuk Village which is a tourism area for Dolphin attraction. Pongamia in Kalibukbuk Village was grown in the parking area, between art shop, and along the beach for shading. Total of 126 pongamia trees was found in Bali and mostly distributed in the north-west of the Island. This is relatively small number of pongamia trees compared to population of the species in other islands. For example, population of pongamia in Kutai Kartanegara District of Kalimantan Island is about 121.000 trees with high density (Sidiyasa et al. 2012).

The existence of pongamia in Kutai Kartanegara District of Kalimantan Island is supported by biotic and abiotic factors. Pongamia grows well on sandy beaches either directly face the sea or bordered by muddy mangrove trees. Pongamia is mostly found in undisturbed habitats such as area with no abrasion and no human disturbance (Sandiyasa et al. 2012). In the case of the small number of pongamia in Bali probably due to human activities such as development of residential areas, development of tourism facilities, hotels, and restaurants in most beaches in Bali. These may have largely contributed to habitat loss of pongamia and threatening the population of the species. Jayusman (2017) stated that population of pongamia is relatively stable in conservation areas, such as Ujung Kulon, Baluran and Alas Purwo National Parks. While in the areas with bad sea abrasion such as in the southern beaches of West Java, population of pongamia is small. Many big pongamia trees have killed due to intrusion of sea water.

Bali Island which located on the west of Wallacea line has abundance species of flora as part of its biodiversity. At the moment, an array of threats are thought to threaten this diversity whether it is natural such as climate change, natural disasters or anthropogenic such as land conversion from forests to agricultural fields, settlements, roads, mining, etc. On the other hand, humans are relying on plants for timber, foods, medicines, etc. In order to sustain the availability, the practice of plant conservation efforts is needed. Plant conservation basically can be grouped as in-situ and ex-situ.

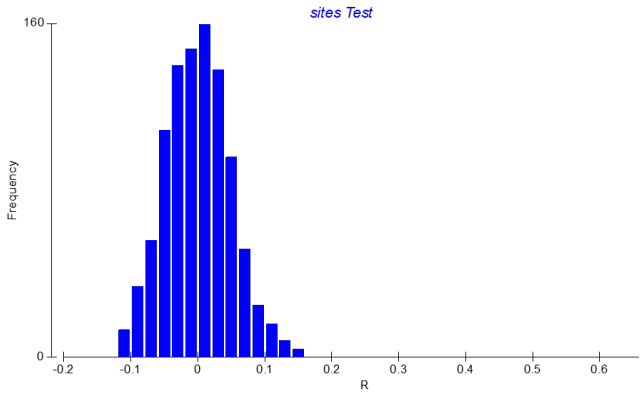

Figure 3. Analysis of similarity (ANOSIM) based on temperature, humidity, and elevation of site studied

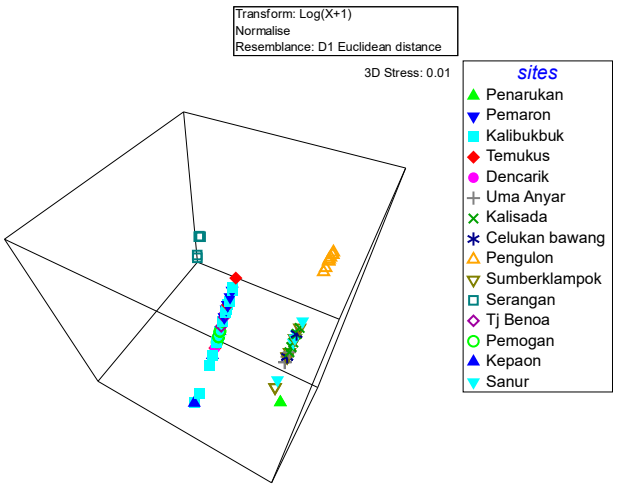

Figure 4. Nonmetric Multidimensional Scaling of sites based on temperature, relative humidity, and elevation

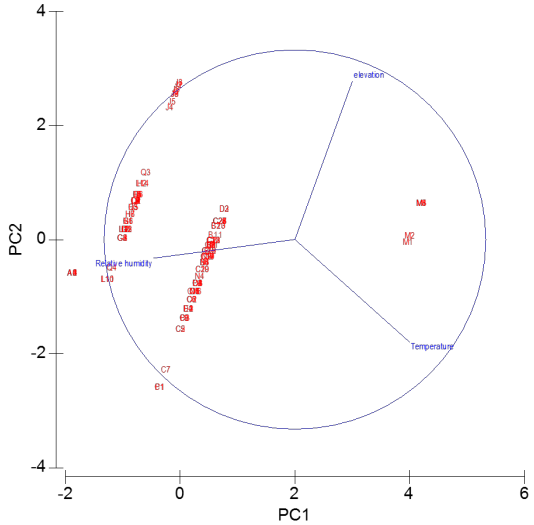

Figure 5. Principal component analysis (PCA) of the sites based on temperature, relative humidity, and elevation

Conservation of pongamia in Bali is urgently needed to conserve the threatened genetic material of the species. Both in-situ and ex-situ conservations should be done to rescue the existence pongamia trees. For the purpose of ex-situ conservation where plant species are research, propagate and planted inside a botanical garden, the research on its ecology (relationship with environmental factors in its native habitat) and also selection of plus tree species of pongamia is needed. Through this selection, its hoped that the acclimatization and propagation processes can be done effectively so that the final goal of ex-situ conservation (which is reintroduction or enrichment planting to its natural habitat) can be achieved.

From 126 pongamia trees counted and observed in Bali, 17 candidate trees were selected from 9 villages based on growth characters and oil content. Selection criteria of plus trees depend on the purpose of selection. Oil content is one of important criteria for selecting biodiesel crops (Kaushik et al. 2007; Kesari et al. 2008; Raut et al. 2010). In the present study range of oil content of candidate trees was 26-32%. The highest oil content of 32% was found on candidate trees from Kalibukbuk, Uma Anyar dan Kalisada. There were 8 pongamia plus trees selected out of 17 candidate trees from 4 villages in Bali, namely Kalibukbuk, Pengulon, Pemogan, and Sanur. Oil content of plus trees ranged from 27 to 32%. Oil content of the present study was lower than those CPTs selected from Haryana, India with 32-44% oil (Kaushik et al. 2007). Variability in oil content is observed among trees within the same location and between locations (Arpiwi et al. 2013). The existence of variability in character studied can be utilized for selection of elite trees especially CPTs with high oil content can be used for developing high oil-yielding line (Mukta et al. 2009).

Total height and diameter at breast height of the CPTs rank from 7.50-13.60 m and 20.70-63.69 cm respectively indicating differences in age of the trees (Simbolon et al. 2003). Selection of CPTs from natural pongamia populations in Bali is proven hard due to limited number of trees. The nature of scattering grow instead of colony grow was another limiting factor in choosing base population for selection of pongamia plus trees in Bali. Therefore, some villages with less than 6 pongamia trees were not included in selection of plus trees, for example, Temukus, Dencarik, Tangguwisia, Celukan Bawang, Banyu Poh, Tanjung Benoa and Kepaon (data not shown).

The dendrogram of individual trees from different locations grouped them into 3 at the taxonomic distance of 12 based on growth parameters such as, total height; clear bole height, diameter on breast height, canopy width and oil content. Each group consisted of individuals from different locations in Bali indicating narrow morphological variations. Trees were very closely related or they might come from less and very closely related mother trees, however, this needs further study on molecular genetic diversity.

Correlation between growth parameters and oil content indicated that the most significant factor influencing oil content was clear bole height ($P = 0.002516$ and Spearman's Rho = 0.305) followed by total height ($P = 0.00025$ and Spearman's Rho = 0.267). In terms of pongamia, it is observed in our field sampling and laboratory analysis that the highest oil content was found in trees which are tall and have high clear bole height. Clear bole height is associated with total tree height. These two parameters (beside diameter) are indicator of the maturity of tree (Simon, 1996). Significant positive correlation between oil content and tree height are found in *Madhuca latifolia* Macb (Divakara 2014) and sunflower (Kaya et al. 2007). However, another study by Rao et al. (2011) do not find significant correlation between oil content and plant height in pongamia. The discrepancy between the present study and thus by Rao et.al. (2011) on the correlation between oil content and plant height probably due to the difference in the age of trees. Rao et al (2011) clearly restricts the age of trees in their study above 10 years old while in the present study the age of trees varied as indicated by variable tree height among samples. Local environmental parameters that we observed in our sites fall within the normal description as to where pongamia is usually found. *Pongamia pinnata* is found in coastal areas, often along beaches or rivers and in thickets close to sea level (Groom 2012). Orwa et al. (2009) wrote that pongamia has biophysical limit. It grows only at an altitude range of 0-1200 m asl with mean annual temperature of 1-16°C to 27-38°C and mean annual rainfall of 500-2,500 mm.

In conclusion, this research is the first to attempt at mapping the distribution of *Pongamia pinnata* in Bali. *Pongamia pinnata* was mostly grown in the north-west part of the Bali Island of Buleleng District. Lower number of trees was also found in the southern and western part of the island. Trees either scattered, grown in small groups and very rarely grown in big groups. The number of pongamia tree in Bali is small and this needs further action for conserving the species. Eight trees were selected as pongamia plus trees from 4 villages, namely Kalibukbuk and Pengulon (Buleleng District), Pemogan (Denpasar) and Sanur (Badung District).

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Short Communication: The species diversity and composition of roadside trees in five cities in Sumatra, Indonesia

WIRYONO♥, YANSEN♥♥, ADITYA, DAVID JUARA LAMHOT, JURIAH HUTAHAEAN
Department of Forestry, Faculty of Agriculture, Universitas Bengkulu. Jl. WR Supratman, Bengkulu, Indonesia. Tel./fax.: +62-736-21170.
♥email: wiryonogood@unib.ac.id, wiryonogood@yahoo.com, ♥♥ yansen@unib.ac.id

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Abstract. *Wiryono, Yansen, Aditya, Lamhot DJ, Hutahaean J. 2018. Short Communication: The species diversity and composition of roadside trees in five cities in Sumatra, Indonesia. Biodiversitas 19: 1615-1621.* Roadside trees make living in a city more enjoyable due to their aesthetic values and ecosystem services. The ecological benefits of roadside trees may be enhanced by increasing the species diversity and the proportion of native species. The objective of this study was to know the species diversity and composition of roadside trees in the cities of Palembang, Bengkulu, Curup, Pematang Siantar and Argamakmur, in Sumatra Island, Indonesia, varying in size and altitude. Data of trees were collected from selected streets and analyzed to determine the species richness (S), Shannon-Wiener index of diversity (H′) and Ellenberg index of community similarity (IS_E). The results showed that the species richness in a city ranged from 7 to 26, with the diversity index between 1.05 and 2.08. The large and medium cities had higher S and H′. More introduced species were found, both in number of species and number of individuals, than the native ones. The similarity among cities in species composition ranged from 47 to 82%. Overall, *Swietenia macrophylla*, an introduced species, was the most abundant species. The S and H′ values of all cities were considered low and the composition of tree species did not support the conservation of native species. It is, therefore, essential to increase the species diversity of street trees, especially by planting native species.

Keywords: Biodiversity, Indonesia, Sumatran cities, urban forest

INTRODUCTION

Urban trees, including roadside trees, not only make a city beautiful, but also provide many ecosystem services beneficial to urban community, such as sequestering carbon (Zhao and Sander 2015) and other pollutants (Jim and Chen 2008), ameliorating temperature (Fan et al. 2015), and controlling stormwater (McPherson et al. 2005). Living in cities with trees is enjoyable because people experience positive thinking from direct contact with nature (Chiesura 2004), so the presence of trees managed beautifully in a city can increase the rental price of apartment and office in that city (Laverne and Wilson-Geideman 2003; Donovan and Butry 2011)

To get the maximum benefits from road trees, city planners must consider many things when selecting trees for streets. The ability of a tree to cope with harsh environment of the street may be the most important criterion (Sjöman and Nielsen 2010), but they also need to consider the aesthetic value of the tree, the landscaping objective, and the health of the urban street tree population, the seedling availability, the site condition, the planting techniques, and the maintenance management (Li et al. 2011). Some arboriculturist proposed that many species must be selected for roadside trees, so they proposed the maximum percentage of a single species planted in streets for a city. Barker (1975) proposed that any single species of trees categorized as liberal use should not exceed 5% of the total trees in streets, while Miller and Miller (1991) increased the limit of liberal use to 10%. Richards (1993), however, argued that this simple numerical limits had no scientific basis and he believed that species equity was a poor standard for selecting street trees.

Although there may be no scientific basis for setting maximum percentage of a given species to be planted in streets, there have been concerns that street trees as well urban parks must be used for promoting diversity because many natural ecosystems have been degraded (Alvey 2006; Nielsen et al. 2014). This concern is relevant to the situation of Sumatra, the second largest island in Indonesia which used to have extensive natural tropical rainforest harboring a great variety of tree species. However, in the last five decades, much of the natural forest in Sumatra has gone due to logging industries, conversion into oil palm plantation, mining and illegal clearing (FWI 2011; Saxon and Roquemore 2011; Abood et al. 2015). Increasing species diversity in roadside trees in Sumatra will help conserve the biodiversity on this island. Also, studies show that increasing biodiversity in urban ecosystems can have a positive impact on the quality of life (Chivian and Bernstein 2004; Fuller et al. 2007).

Not only for promoting diversity, street trees may also be used for conserving native species. So far, more exotic species than the native ones are found in streets and urban parks in many cities in the world, such as in Brazil (Moro and Castro 2015), in Santiago, Chile (Figueroa et al. 2016), in Bangalore, India (Nagendra and Gopal 2010), in ten Nordic cities (Sjöman et al. 2012), and in Halifax, Canada

(Turner et al. 2005). The presence of exotic species may bring deleterious effects to the local ones. The exotic chestnut imported from Japan to the US in the 19th century almost brought the native American chestnut (*Castanea dentata*) to extinction due to chestnut blight (*Cryphonectria parasitica*) which came with the exotic species (The American Chestnut Foundation 2017). The use of native species for roadside trees not only help conserve the species but also help introduce local people to the local plant species (Savard et al. 2000). The rare presence of native species in roadside trees will alienate people of their local species and may reduce local botanical knowledge and support biodiversity conservation (McKinney 2006; Dearborn and Kark 2010).

The objective of this paper was to report the results of four independent studies to know species diversity and species composition of roadside trees in the cities of Palembang, Bengkulu, Curup, Argamakmur and Pematang Siantar in Sumatra Island, Indonesia. As a whole, this is considered a preliminary study, because the number of streets sampled was low. To get a more comprehensive results, bigger samplings need to be done.

MATERIALS AND METHODS

Study area

This study reported four independent studies. The first one was conducted in Palembang City, South Sumatra Province, the second one in Bengkulu City, Bengkulu Province, the third one in Curup and Argamakmur Cities, Bengkulu Province, and the fourth one in Pematang Siantar, North Sumatra Province. Bengkulu City is located at the west coast of Sumatra (3° 47' 59" S/102° 16' 1" E), with altitudes of 0-20 m, Palembang at the east coast of Sumatra (2° 55' 0" S/104° 44' 45" E) with altitudes of 0-20 m. The other three sites are inland cities with higher elevation. Argamakmur lies at 3° 25' 24" S/102° 11' 21" E, with altitudes of 200-250 m, Pematang Siantar at 2° 57' 34" N/99° 4' 7" E, with altitudes of 400-500 m, and Curup at 3° 28' 13" S/102° 31' 15" E with altitudes mostly between 500 m to above 1000 m.

Palembang is the capital city of South Sumatra Province, a large city, with an area of about 400 km², and a population of about 1.6 million (Central Bureau of Statistics of Palembang City 2017). Bengkulu is the capital

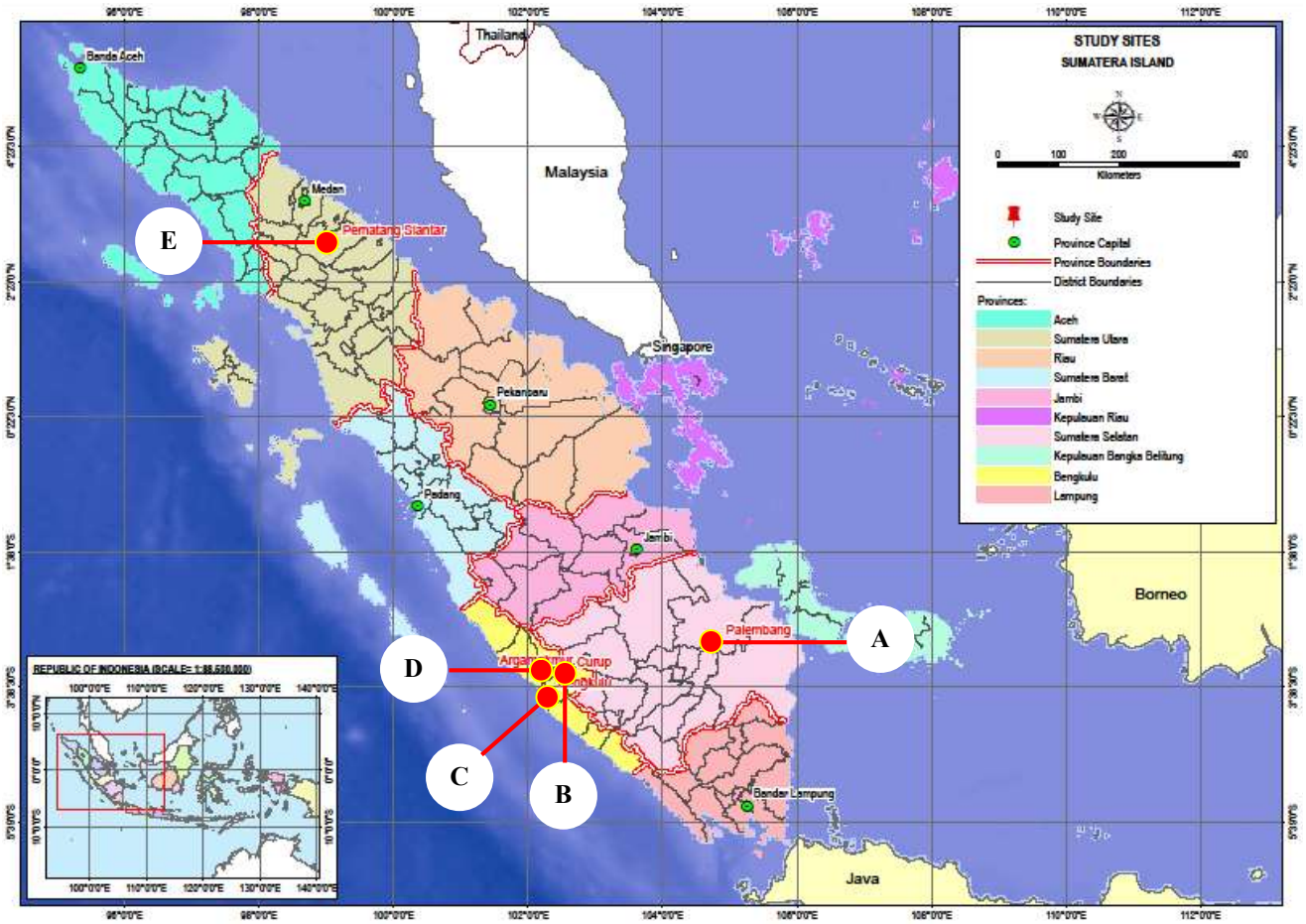

Figure 1. Study sites in five cities of Sumatra, Indonesia, i.e., A. Palembang, B. Curup, C. Bengkulu, D. Argamakmur, and D. Pematang Siantar

city of Bengkulu Province, a medium-size city, 144.5 km² in area, with a population of about 350 thousand (Central Bureau of Statistics of Bengkulu City 2017). Pematang Siantar is a small-medium city, about 80 km² in area with a population of 249 thousand (Central Bureau of Statistics of Pematang Siantar 2017). On the other hand, Curup City (about 3.95 km² area) and Argamakmur City (about 32 km² area) are small cities with about 29 thousand and 42 thousand inhabitants respectively (Central Bureau of Statistics of Rejang Lebong 2017; Central Bureau of Statistics of North Bengkulu 2017).

All five locations fall into *Af* climate classification according to Köppen dan Geiger, which is a tropical rainforest climate. Coastal cities, i.e. Bengkulu and Palembang, have slightly higher air temperature than inland cities. In general, all cities have high rate of rainfall (https: //id.climate-data.org).

Tree sampling

Each researcher conducted an independent study with sampling intensity based on the species diversity of each street and available time. In Palembang, 12 streets were selected, and trees in those streets were recorded with 100% sampling intensity (SI); in Bengkulu 20 streets with 10% SI; in Curup and in Argamakmur, 8 streets with 50% SI respectively, and in Pematang Siantar 8 streets with 10% SI. This inequality of sampling intensity was one of the weaknesses of this article. For a better result, the same sampling intensity should have been done for every city.

Data analyses

The species, number, and percentage of trees in each location were tabulated to show the species composition. The species were categorized into native and introduced. Native species are those whose natural distribution includes Sumatra Island, and so, those which do not meet this criterion were classified as introduced. The natural distribution of species was searched from the literature on the internet.

The diversity of tree species in each city was determined by calculating the number of species of species richness (S) and the Shannon-Wiener diversity index (H′). The distribution of individuals among species was determined using species evenness index (E), and the similarity in species composition among cities were determined using Ellenberg. The formulas for these indexes were taken from Mueller-Dombois and Ellenberg (1974).

Shannon-Wiener index:

$$H' = -\sum_{i=1}^{s} (pi)(\ln\ pi)$$

Where:

H′ : Species diversity index

s : Total species

pi : Proportion of species i = ni/N = (total individual of species i/total species).

Species evenness index (E) = H′/ H'_{max}

H'_{max} = Ln S, where S is the number species

The similarity of species composition between two locations was determined using Ellenberg index. Ellenberg is a modification of Jaccard index, with the integration of species abundance in the equation. While Sørensen and Jaccard index treats every species equally, Ellenberg index gives more weight for species having higher abundance. For community having low evenness index, Ellenberg is more appropriate than index of Sørensen or Jaccard.

$$IS_E = \frac{Mc : 2}{Ma + Mb + (Mc : 2)} \text{ x } 100\ \%$$

Where:

Mc: Biomass or other quantitative measures of common species;

Ma: Biomass or other quantitative measures of species found only in the first community;

Mb: Biomass or other quantitative measures of species found only in the second community

The similarity of species composition in street trees was also compared with that of natural forest from literature (Kusumo et al. 2016).

RESULTS AND DISCUSSION

Species diversity

The species diversity of road trees in five sites was low, as indicated by the low species richness (S) and diversity index (H′), while the evenness index was considered medium (Table 1). The largest number of trees was found in Palembang and Bengkulu, which was only 26. These two cities also had the highest H′. In term of diversity, the roadside trees in five urban areas in this study did not represent the natural vegetation of Sumatra which has very high diversity (Whitten et al. 1984; Laumonier 1997). In other cities of Sumatra, the total species of roadside trees was also low, such as in Medan, the capital of North Sumatra Province, which was only 33 species (Purwasih et al. 2013) and in Pekanbaru, the capital of Riau Province, which was only 13 species (Nursal et al. 2005).

The species diversity in this study was affected by the size of the city (Table 1). Palembang and Bengkulu, a large and a medium-sized cities, had more species and higher species diversity index than the three smaller cities, Curup, Argamakmur and Pematang Siantar. In general, the diversity of roadside size increases with increasing size of the city. The number of street tree species in Medan (Purwasih et al. 2013), the largest city in Sumatra, was higher than that in five cities in this study and that in Pekanbaru (Nursal et al. 2005), while Jakarta, the largest city in Indonesia, had 119 tree species along the roadside (Nasrullah and Suryowati 2009).

Unlike the natural forest in Sumatra which has no single dominant species, the roadside trees in three cities in this study, namely Bengkulu, Curup and Pematang Siantar, was dominated by *Swietenia macrophylla*, constituting more than 40% of the total roadside trees in each city, while the roadside trees in Argamakmur was dominated by *Polyalthia*

longifolia, constituting more than 65% of the total trees. In every city in this study, only six or fewer species constituted more than 80% of all the trees, and in Argamakmur, 88.5% of the trees was composed only by two species, namely *P. longifolia* and *Mimusops elengi* (Table 2). In Medan, five species constituted 90% of all the trees (Purwasih et al. 2013), while in Jakarta, 10 species comprised 79% of all the trees (Nasrullah and Suryowati 2009).

Some experts have set a limit on the maximum percentage of a single species planted on roadside. Barker (1975) proposed that any single species of trees categorized as liberal use should not exceed 5% of the total trees in streets, while for trees categorized as limited use, the maximum number was 3%, and for those categorized as candidate use the maximum number was 2%. Miller and Miller (1991) increased the limit of liberal use to 10%. Richards (1993), however, said that this simple numerical limits had no scientific basis and he believed that species equity was a poor standard for selecting street trees. Instead, he stated that Street tree diversity should relate to

While there are no scientific or legal bases for setting the limit of single species proportion in street tree population, or the ideal value of species richness or species diversity index for a city park, it is believed that high diversity of species and genera in urban forest is essential for the health and sustainability of urban trees (Raupp et al. 2006). In addition, with the continuing decline of natural forest, urban forest, including roadside trees, should be utilized to promote species diversity (Alvey 2006; Nielsen et al. 2014). It is, therefore, important to increase the diversity of street tree species, especially for Sumatra nbIsland, where much of its natural forest has been converted into single-species plantation, mainly oil palm (Saxon and Roquemore 2011), and even conservation forest areas have also been illegally cleared (FWI 2011). Increasing species diversity in roadside trees in Sumatra will help conserve the biodiversity on this island. Also, studies show that increasing biodiversity in urban ecosystems can have a positive impact on the quality of life (Chivian and Bernstein 2004; Fuller et al. 2007).

Species composition

Of the 44 species found in 5 cities, 27 were introduced, and only 17 were native, and the top five dominant species were all introduced (Table 4). The species composition of roadside trees in all sites had zero similarity with natural forest in Sumatra (Kusumo et al. 2016). Among the sites, however, the similarities in species composition were high, with scores between 57%-82% using Ellenberg index, despite differences in altitude (Table 3). Argamakmur which is located in lowland had 82% similarity with Curup and Pematang Siantar which are located in high altitude. Likewise, Palembang a lowland city had 72% similarity with Pematang Siantar. High similarity among five cities despite differences in altitude confirms the statement of McKinney (2006) that urbanization homogenizes the ecosystems.

Among 44 species, *S. macrophylla*, an introduced species, had the highest percentage in this study. The same species was also found the most dominant in Jakarta (Nasrullah and Suryowati 2009) and the second most dominant in Pekan Baru (Nursal et al. 2005). The next four most dominant species in this study were *Mimusops elengi, Polyalthia longifolia, Roystonea regia* and *Samanea saman*, all of which are introduced. Only in Bengkulu, a native species *Casuarina equisetifolia* had a large percentage (10.6%), because one of the streets studied was located along the natural beach forest dominated by *C equisetifolia*.

Table 1. Species richness (S), index of species diversity (H′) and evenness (E)

City	S	H’	E
Palembang	26	2.03	0.62
Bengkulu	26	2.08	0.64
Curup	19	1.80	0.61
Pematang Siantar	10	1.46	0.63
Argamakmur	7	1.05	0.54

Table 2. The dominant species of roadside trees in three small cities, a medium-size city, and a large city

Species	Abundance (%)
Small city	
Curup	
Swietenia macrophylla	47
Mimusops elengi	22
Samanea saman	7
Hura crepitans	5
Total	81
Argamakmur	
Polyalthia longifolia	65
Mimusops elengi	24
Total	89
Pematang Siantar	
Swietenia macrophylla	45
Mimusops elengi	25
Roystonea regia	11
Samanea saman	9
Total	90
Medium-size city	
Bengkulu	
Swietenia macrophylla	43
Casuaria equisetifolia	10.6
Diallum indum	10.4
Tectona grandis	8.8
Polyalthia longifolia	5.2
Angsana	4.8
Total	82
Large city	
Palembang	
Mimusops elengi	24
Pterocarpus indicus	21
Swietenia macrophylla	18
Roystonea regia	15
Samanea saman	10
Total	88

Table 3. The list of species, its family, abundance and origin

Species	Family	Abundance (%)						Origin
		Palembang	Bengkulu	Curup	Arga-makmur	Pematang Siantar	Average	
Swietenia macrophylla King	Meliaceae	18.15	43	46.76	45.40	2.80	31.22	Introduced
Mimusops elengi L.	Sapotaceae	24.20	3.2	22.25	24.69	23.50	19.57	Introduced
Polyalthia longifolia Sonn	Annonaceae	1.99	5.2	1.13	6.56	65.00	15.98	Introduced
Roystonea regia (Kunth) O.F.Cook	Arecaceae	14.88	2.4	2.54	10.62	1.40	6.37	Introduced
Samanea saman (Jack) Merr.	Fabaceae	10.03		7.32	9.37		5.34	Introduced
Pterocarpus indicus Wild	Fabaceae	20.83	4.8				5.13	Native
Casuarina equisetifolia L.	Casuarinaceae	0.02	10.6			0.70	2.26	Native
Hura crepitans L.	Euphorbiaceae	1.28	0.6	4.51		0.60	1.40	Introduced
Elaeis guineensis Jack	Arecaceae	1.89				4.50	1.28	Introduced
Terminallia catappa L.	Combretaceae	0.32	3.4		0.62		0.87	Native
Cerbera odollam Gaerth	Apocynaceae	1.21	0.6	0.28			0.42	Native
Delonix regia (Boj. ex Huff.) Raf.	Fabaceae	1.77	0.2				0.39	Introduced
Cocos nucifera L.	Arecaceae	0.02	1.8			0.10	0.38	Native
Syzygium polyanthum (Wight) Walp.	Myrtaceae	0.69	0.8				0.30	Native
Juniperus chinensis L.	Cupressaceae	0.15				1.30	0.29	Introduced
Tabebuia aurea (Silva Manso) Benth. & Hook.f. ex S.Moore	Bignoniaceae	1.13					0.23	Introduced
Ficus benjamina L.	Moraceae	0.05	0.2	0.28		0.10	0.13	Native
Artocarpus heterophyllus Lam.	Moraceae	0.66	0.4				0.08	Introduced
Acacia mangium Willd.	Fabaceae	0.15	0.2				0.07	Introduced
Melaleuca cajuputi Powell	Myrtaceae	0.10					0.02	Native
Terminallia mantally H.Perrier.	Combretaceae	0.12					0.02	Introduced
Artocarpus altilis (Parkison) Forsberg.	Moraceae	0.12					0.02	Introduced
Erythrina crista-galli L	Fabaceae	0.05					0.01	Introduced
Acacia auriculiformis A. Cunn. ex Benth.	Fabaceae	0.05					0.01	Introduced
Syzygium aqueum (Burm f.) Alston	Myrtaceae	0.05					0.01	Native
Dialium indum L	Fabaceae		10.4				2.08	Native
Mangifera indica L.	Anacardiaceae		0.8				0.16	Introduced
Leucaena leucochepala (Lam) de Wit.	Fabaceae		0.6				0.12	Introduced
Averrhoa carambola L	Oxalidaceae		0.4				0.08	Native
Maesopsis eminii Engl.	Rhamnaceae		0.2				0.04	Introduced
Durio zibethinus L	Malvaceae		0.2				0.04	Native
Lagerstroemia floribunda Jack 1820	Lythraceae		0.2				0.04	Introduced
Lannea coromandelica (Houtt.) Merr.	Anacardiaceae		0.4				0.08	Introduced
Muntingia calabura L.	Muntingiaceae		0.4				0.08	Introduced
Tectona grandis L.f.	Lamiaceae		8.8	0.28			1.82	Introduced
Felicium decipiens (Wight & Arn.) Thwaites ex Hook.f.	Sapindaceae		0.2	2.54			0.55	Introduced
Duranta erecta L.	Verbenaceae			2.54			0.51	Introduced
Pinus merkusii Jungh. & de Vriese	Pinaceae			2.25			0.45	Native
Cinnamomum burmannii (Nees & T.Nees) Blum	Lauraceae			2.82			0.56	Native
Alstonia scholaris L. R. Br.	Apocynaceae			1.69			0.34	Native
Psidium guajava L.	Myrtaceae			0.85			0.17	Introduced
Nephelium lappaceum L.	Sapindaceae			0.56			0.11	Native
Areca catechu L.	Arecaceae			0.56			0.11	Native
Annona muricata L.	Annonaceae			0.56	2.18		0.55	Introduced

Table 4. Similarity in species composition among locations (using Ellenberg index)

	Similarity index (%)				
	Palembang	Bengkulu	Curup	Arga makmur	Pematang Siantar
Palembang	100				
Bengkulu	70	100			
Curup	62	47	100		
Argamakmur	58	66	77	100	
Pematang Siantar	72	57	82	82	100
Primary forest	0	0	0	0	0

The top five introduced species have characteristics that make them suitable to be planted along roadsides. *Swietenia macrophylla* has an umbrella-shaped crown, makes it suitable for a shade tree. In addition, this species can tolerate a wide range of soil and environmental conditions (Krisnawati et al. 2011). Like *S. macrophylla, S. saman* has an umbrella-shaped crown. It thrives in tropical and subtropical climate regimes, ranging from wet to seasonal dry climate and can adapt to a wide range of soil types and pH (Staples and Elevitch 2006). *Mimusops elengi* ,in addition to having medicinal value, also has an elegant

shape and fragrant flowers (Gami et al. 2012) *Polyalthia longifolia* var *pendula* with its columnar-shaped crown can be used to frame the view and the structure of a landscape (Mitra et al. 2013). *Roystonea regia* a native of Cuba is valued as an ornamental palm and has fragrant flowers (Connor 2002).

The higher number of introduced or exotic species in roadside trees and in urban ecosystem, in general, was also found in many parts of the world. Exotic species constituted 72% of roadside trees in Fortaliza, Brazil (Moro and Castro 2015), 85.1% of vegetation in public places in Santiago, Chile (Figueroa et al. 2016), and 77% of urban parks in Bangalore, India 77% (Nagendra and Gopal 2010). More exotic species were also found in ten Nordic cities (Sjöman et al. 2012), in Halifax, Canada (Turner et al. 2005). In Beijing, however, the percentage of exotic species was only 53%, slightly higher than the native ones (Zhao et al. 2010). The focus on ornamental value is one of the reasons why exotic species dominate urban vegetation (Turner et al. 2005).

For biodiversity conservationists, the presence of exotic species is undesirable because the exotic species may bring deleterious impacts on native species through competition and introduction of pest and diseases. The exotic chestnut imported from Japan to the US in the 19th century almost brought the native American chestnut (*Castanea dentata*) to extinction due to chestnut blight which came with the exotic species (Jordan 2008). In Oregon, exotic grass species caused the decline of plant community diversity and abundance of native grass species (Davies 2011). To prevent the detrimental effect of exotic species, many parties have advocated for increasing native plant species in urban ecosystems (Wilde et al. 2015). The use of native species for roadside trees not only help conserve the species but also help introduce local people to the local plant species (Savard et al. 2000). The rare presence of native species in roadside trees will alienate people of their local species and may reduce local botanical knowledge and support biodiversity conservation (McKinney 2006; Dearborn and Kark 2010).

Increasing native species and conserving biodiversity can be done simultaneously if every city plants mostly its native species. However, the city planner should also consider the aesthetic value of the tree, the landscaping objective, and the health of the urban street tree population, the seedling availability, the site condition, the planting techniques, and the maintenance management (Li et al. 2011). These desired traits for roadside trees may be conflicting with each other (Wilde et al. 2015), so it will be hard to find a single species having those traits. The most important criterion for a road tree species may be its ability to cope with harsh environment of street (Sjöman and Nielsen 2010), because the trees must survive first before they can provide ecosystem services. Pioneer native species may be good choices, since they are able to live in harsh condition, so they do not need intensive care. To get mass seedlings of pioneer native species, plant tissue culture can be used.

In conclusions, the roadside trees in five cities of Sumatra Island, Indonesia, had relatively low diversity. More introduced species than the native ones were found, both in number of species and number of individuals. The diversity and composition of species did not represent the native forest of Sumatra. Therefore, a great variety of native species should be planted for roadside trees.

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Short Communication: Novel Single Nucleotide Polymorphisms (SNPs) in the 5'UTR of Bovine Heat Shock Protein 70 ($bHSP_{70}$) Gene and its association with Service per Conception (S/C) of Pasundan cattle

SYAHRUDDIN SAID♥, WIDYA PINTAKA BAYU PUTRA♥♥
Research Center for Biotechnology, Indonesian Institute of Sciences. Jl. Raya Bogor Km. 46, Cibinong, Bogor 16911, West Java, Indonesia
Tel.: +62-21-8754587 , Fax.: +62-21-8754588. ♥email: syahruddinsaid01@gmail.com. ♥♥ widya.putra.lipi@gmail.com

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Abstract. *Said S, Putra WPB. 2018. Short Communication: Novel Single Nucleotide Polymorphisms (SNPs) in the 5'UTR of Bovine Heat Shock Protein 70 ($bHSP_{70}$) Gene and its association with Service per Conception (S/C) of Pasundan cattle. Biodiversitas 19: 1622-1625.* Heat stress in the livestock reduces reproductive traits, including of service per conception (S/C). The influence of heat stress in livestock can be handled through molecular selection. One of the candidate genes that is affecting heat stress tolerance is Bovine Heat Shock Protein 70 ($bHSP_{70}$) gene. This research was carried out to detect the single nucleotide polymorphism (SNPs) in the 5'UTR of $bHSP_{70}$ gene from 44 heads of Pasundan cows at breeding station (BPPIBT-SP Ciamis, West Java). Research showed that 18 SNPs were detected with sequencing method. The insertion mutation was detected in all animal studied and occurred at position between g.1112 and g.1113. The moderate of polymorphic informative content (PIC) were detected in two SNPs of g.1117G/A and g.1125A/C. Preliminary analysis showed that haplotype of two homozygote genotype combination from both SNPs had better of S/C value of than the others ($P<0.01$). Moreover, the average of S/C value in heterozygote genotype seems higher than homozygote genotype. However, the further research for clarification of this research results through large number of observation is important. It was concluded that two SNPs of g.1117G/A and g.1125A/C are potential as marker-assisted selection (MAS) for reproductive traits in Pasundan cows because of moderate PIC value.

Keywords: $bHSP_{70}$ gene, Pasundan cattle, SNP, PIC, S/C

INTRODUCTION

Pasundan cattle is one of Indonesian cattle that spread out at West Java Province. These cattle were chosen as Indonesian native cattle based on the decision of Ministerial Decree No: 1051/Kpts/SR.120/10/2014 (Ministry of Agriculture of the Republic of Indonesia 2014). Said et al. (2017) reported that Pasundan cattle has a combination of phenotypic characterizations from Madura (*Bos indicus*) and Bali (*Bos javanicus*). As a tropical breed, Pasundan cattle has heat tolerance traits and capable to adapt well mainly in Ciamis Regency (Putra et al. 2016). Heat stress occurs when animals are exposed to temperature beyond the upper critical level, high humidity and low air movement causing an increase in heat production in the animal body (Banks et al. 2009). In addition, reducing the heat stress effect through management improvement, e.g., close house system and fan application, are too expensive for most farmers in Indonesia. Heat stress in cattle can redux reproductive traits (Das et al. 2016), including the service per conception (S/C). High number of S/C indicates that the cow is not productive and it increases the artificial insemination (AI) cost (Mwatawala and Kifaro 2009). However, heat stress in cattle can be reduced through molecular selection. One of the candidate gene that affecting heat stress tolerance is bovine Heat Shock Protein 70 ($bHSP_{70}$) gene. Moreover, heat stress tolerance is heritable trait and can be used for genetic improvement in the future (Ravagnolo and Misztal 2000).

The $bHSP_{70}$ gene is important for producing heat shock protein 70 (HSP_{70}) that affecting heat stress (Mohanarao et al. 2014). Heat shock protein 70 is one of HSPs family that has molecular weight of 68-73 kDa (Pockley et al. 2008) with length consisted of 641 amino acids (Gade et al. 2010). The coding region of $bHSP_{70}$ gene in cattle (*Bos taurus* and *Bos indicus* and buffaloe (*Bubalus bubalis*) is similar in length 1,926 bp (Sodhi et al. 2013). The HSP_{70} is present in all cells of the body. It increases in numbers when an animal is subjected to various stressors such as heat, cold, and oxygen deprivation. In addition, the HSP_{70} is well correlated with the development of thermotolerance in many cell types (Li and Max 1989). The $bHSP_{70}$ gene can be used as a candidate gene for selection of cattle based on heat tolerance traits (Archana et al. 2017). Heat adaptability is a complex phenomenon that depends on the integrity and proper coordination of various systems like respiratory, circulatory, excretory, nervous, endocrine and enzymatic systems of animal body (Mishra and Tapan 2014).

The genetic polymorphism of $bHSP_{70}$ gene explains the difference between individuals in the stress condition. Several polymorphisms were reported in previous studies in the 5'UTR (Cai et al. 2005; Rosenkrans et al. 2010; Basirico et al. 2011; Turner et al. 2013; Gafer et al. 2015;

Ramesha et al. 2016; Oner et al. 2017), 3'UTR (Adamowicz et al. 2005; Basirico et al. 2011; Oner et al. 2017) and coding regions (Brown et al. 2010; Habib et al. 2017). In addition, the bHSP$_{70}$ gene polymorphisms were affected by reproductive traits in Deoni cattle (Ramesha et al. 2016), cellular thermotolerance traits in Tharparkar cattle (Bhat et al. 2016), peripheral blood mononuclear cells (PBMC) response in Holstein Friesian and Sahiwal cows (Basirico et al. 2011; Parmar et al. 2015), sperm motility in Qincuan and Egyptian bulls (Zhang et al. 2015; Gafer et al. 2015) and horn-fly infestation response in Brahman and Angus cows (Turner et al. 2013). There are no studies on genetic characterization of bHSP$_{70}$ gene in Pasundan cattle as well as environmental resistance of the breed.

This research was conducted to detect SNPs in the 5'UTR of bHSP$_{70}$ gene in Pasundan cows, reared at breeding station and its association with S/C. The result of this cattle can be used as early information for developing molecular selection program to improve reproductive traits in the future.

MATERIALS AND METHODS

Blood sample and DNA extraction

The blood samples were collected from 44 Pasundan cows kept at the breeding center (BPPIBT-SP Cijeungjing, Ciamis, West Java) using vacutainer containing K_2EDTA about 3 mL for each cattle through jugular veins. The DNA of blood samples were extracted using Genomic DNA Mini Kit (Geneaid Biotech Ltd., Taiwan), following the procedures instruction. The extracted DNA was appropriately labeled and stored at -20 °C for the next analysis.

PCR amplification and DNA sequencing

The PCR reaction was performed in a Mastercycler® gradient (Eppendorf, Germany) with a pair of primer according to Basirico et al. (2011), i.e., Forward: 5'-GCCAGGAAACCAGAGACAGA-3' and Reverse: 5'-CCTACGCAGGAGTAGGTGGT-3'. This primer amplified the bHSP$_{70}$ gene from nucleotide position 749 to 1287 bp (329 bp) according to GenBank: M98823.1. The PCR reagent was performed in the 30 µL total volume, comprising 7.8 µL of ddH_2O, 0.6 µL of primer forward and reverse (1 µM), 15 µL of PCR kit (Mytaq^{TM} HS Red Mix, USA), and 6 µL of DNA genome. DNA amplification was performed using Mastercycler® (Eppendorf, Germany) and performed with predenaturation temperature at 94°C for 2 minutes and following 39 cycles of denaturation at 94°C for 30 seconds, annealing at 58.2°C for 1 minutes, extension at 68°C at 1 minutes, and final extension at 68°C for 10 minutes. The 30 µL of PCR products were directly sequenced by commercial laboratory service, i.e., First BASE Laboratories Sdn. Bhd. (Malaysia) using ABI Prism 96-capillary 3730xl DNA analyzer (Applied Biosystems, USA). Sequence analysis began with a contig analysis which combines both the sequencing results (forward and reverse) to obtain a complete single sequence of each sample. The sequence obtained were aligned with the reference sequence and analyzed by using BioEdit ver 7.2.0 software (Hall 1999).

Statistical analysis

Statistical analysis for the bHSP$_{70}$ gene sequence consisted of genotype frequency, allele frequency, expected heterozygosity (H_e), observed heterozygosity (H_o), number of effective allele (n_e) and polymorphic informative content (PIC) and Hardy-Weinberg equilibrium were calculated based on Nei and Kumar (2000). Data of S/C were analyzed using general linear model (GLM) at the level of probability 0.01 to test the significance of the differences between the averages studied and based on the formula of the mathematical model:

$$Y_i = \mu + S_i + e_i$$

Where:
Y_i = the value of S/C
μ = means of S/C
S_i = effect of i^{th} SNPs
e_i = error term

RESULTS AND DISCUSSION

The bHSP$_{70}$ gene of Pasundan cattle was successfully amplified using a pair of primer (Figure 1). The result indicated that amplification fragment had good specificity and could proceed directly to sequencing analysis. The size of the PCR product was in accordance with the reference sequence. A total of 17 SNPs and one insertion/deletion (indel) were detected in the 5'UTR of bHSP$_{70}$ gene in Pasundan cattle (Tabel 1). Most of the mutation types that occurred in this study are transversion (61.11%) and followed by transition (33.33%). Ten SNPs of g.1045G/A; g.1069C/T; g.1096A/G; g.1117G/A; g.1125A/C; g.1128G/T; g.1134T/C; g.1164G/T; g.1204T/C and g.1255C/T so far are detected in many cattle breeds such as Brahman (Rosenkrans et al. 2010), native Turkish breeds and Friesian Holstein (Oner et al. 2017). The moderate PIC value (0.30<PIC<0.50) was found in two SNPs of g.1117G/A and g.1125A/C (Table 2). Oner et al. (2017) reported that SNP of g.1117G/A in Boz Irk breed has moderate PIC value (0.31), which is similar to this study. Three SNPs of g.1125A/C; g.1128G/T and g.1204T/C have a moderate PIC value in Brahman (Rosenkrans et al. 2010), Friesian Holstein and native Turkish breeds (Oner et al. 2017). In contrast, two SNPs of g.1128G/T and g.1204T/C in this study had a low PIC value (PIC<0.30). Banks et al. (2009) reported that two SNPs of g.1125A/C and g.1128G/T have significant association with calving percentage of Brahman cows with genotype AA (g.1125A/C) and GG (g.1128G/T) as the best genotype. Meanwhile, Turner et al. (2013) reported that SNP of g.1128G/T has significant association with horn-fly infestation response in Brahman and Angus cows with GG or TT as the best genotype. In addition, Genotype GT in SNP of g.1128G/T and it suggested the best genotype for

PBMC responses in Friesian Holstein (Basirico et al. 2011). Research showed that most of animal studied had GG genotype (0.93) in SNP of g.1128G/T and suggested that Pasundan cattle has potential traits for high calving percentage and horn-fly infestation resistance and important to investigate with large number of observation.

Preliminary analysis with limited number of samples (13 heads of Pasundan cows) showed that two SNPs of g.1117G/A and g.1125A/C were not affected to S/C (Table 3). However, the homozygote genotype had lower of S/C value than heterozygote genotype in both SNP. Banks et al. (2009) obtained the homozygote genotype (AA) as the best genotype for reproductive traits in Brahman cows based on SNP of g.1125A/C. This result is similar to this study. Singe nucleotide polymorphism of g.1125A/C under Hardy-Weinberg equilibrium (χ^2<5.99), revealed that the distribution of A and C alleles on this SNP were randomly distributed and no genetic drift effect (Table 2). The highest n_e value was detected in SNP of g.1125A/C (1.98) and indicated that this SNP is polymorphic. Therefore, molecular selection based on SNP of g.1125A/C in regarding improve reproductive traits of Pasundan cattle, can be performed in the future. Haplotyping based on two SNP of g1117G/A and g.1125A/C were significantly affected the S/C in the animal studied (P<0.01). In addition, animal with homozygote genotype of GG/AA (g.1117G/A) and AA/CC (g.1125A/C) has lower of S/C value than the other genotype combinations (P<0.01). The further research to investigate this finding is important regarding obtain marker-assisted selection (MAS) for reproductive traits in the future through large number of sample.

In conclusion, the 5'UTR of $bHSP_{70}$ gene of Pasundan cattle is polymorphic with 18 mutation sites. Two SNPs of g.1117G/A and g.1125A/C have moderate PIC value (PIC>0.30) and it is potentially as the marker-assisted selection (MAS) for reproductive traits of Pasundan cows. Preliminary analysis reveals that homozygote genotype animals have lower S/C value than other genotypes.

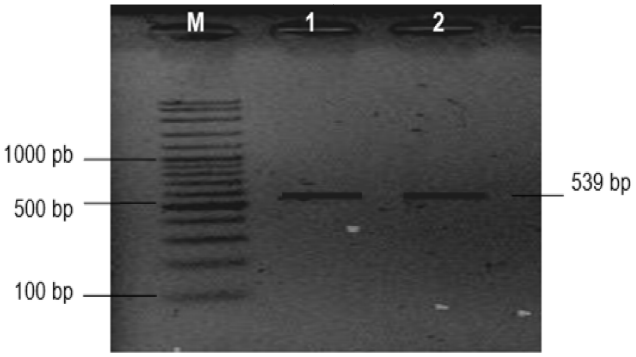

Figure 1. The amplification of bHSP gene (539 bp) in Pasundan cows separated on 1% agarose gel. M: marker (DNA ladder 100 bp); line 1-2: number of samples

Table 1. Identification of SNPs in the 5'UTR of $bHSP_{70}$ gene in Pasundan cows

Position	Nucleotide change	Mutation type	N	Frequency
862	A→T	Transversion	2	0.05
1017	C→G	Transversion	4	0.09
1019	T→G	Transversion	2	0.05
1036	C→T	Transition	1	0.02
1045	G→A	Transition	6	0.14
1050	T→C	Transition	3	0.07
1058	A→G	Transition	2	0.05
1069	C→T	Transition	6	0.14
1096	A→G	Transition	4	0.09
1112/1113	ins./del.C	Insertion	44	1.00
1117	G→A	Transition	24	0.55
1125	A→C	Transversion	10	0.23
1128	G→T	Transversion	3	0.07
1134	T→C	Transition	12	0.27
1164	G→T	Transversion	6	0.14
1204	T→C	Transition	34	0.77
1255	C→T	Transition	5	0.11
1262	C→T	Transition	3	0.07

Note: N: number of observation

Table 2. Results of statistical analysis in the SNPs at 5'UTR of $bHSP_{70}$ gene in Pasundan cows

SNP	Genotype frequency			Allele frequency		H_e	H_o	n_e	PIC	χ^2
g.862A/T	AA (0.96)	AT (0.02)	TT (0.02)	A (0.97)	T (0.03)	0.07	0.02	1.07	0.06	18.87
g.1017C/G	CC (0.91)	CG (0.09)	GG (0.00)	C (0.95)	G (0.05)	0.09	0.09	1.10	0.08	0.10*
g.1019T/G	TT (0.95)	TG (0.05)	GG (0.00)	T (0.98)	G (0.02)	0.04	0.05	1.05	0.04	0.02*
g.1036C/T	CC (0.98)	CT (0.02)	TT (0.00)	C (0.99)	T (0.01)	0.02	0.02	1.02	0.02	0.01*
g.1045G/A	GG (0.86)	GA (0.05)	AA (0.09)	G (0.89)	A (0.11)	0.20	0.05	1.25	0.18	26.38
g.1050T/C	TT (0.93)	TC (0.07)	CC (0.00)	T (0.97)	C (0.03)	0.07	0.07	1.07	0.06	0.06*
g.1058A/G	AA (0.95)	AG (0.05)	GG (0.00)	A (0.97)	G (0.03)	0.04	0.05	1.05	0.04	0.02*
g.1069C/T	CC (0.86)	CT (0.00)	TT (0.14)	C (0.86)	T (0.14)	0.24	0.00	1.31	0.21	44.00
g.1096A/G	AA (0.91)	AG (0.09)	GG (0.00)	A (0.95)	G (0.05)	0.09	0.09	1.10	0.08	0.10*
g.1117G/A	GG (0.45)	GA (0.25)	AA (0.30)	G (0.58)	A (0.42)	0.49	0.25	1.95	0.37	10.44
g.1125A/C	AA (0.34)	AC (0.43)	CC (0.23)	A (0.56)	C (0.44)	0.49	0.43	1.98	0.37	0.69*
g.1128G/T	GG (0.93)	GT (0.07)	TT (0.00)	G (0.97)	T (0.03)	0.07	0.07	1.07	0.06	0.06*
g.1134T/C	TT (0.73)	TC (0.16)	CC (0.11)	T (0.81)	C (0.19)	0.31	0.16	1.45	0.26	10.55
g.1164G/T	GG (0.86)	GT (0.14)	TT (0.00)	G (0.93)	T (0.07)	0.13	0.14	1.15	0.12	0.24*
g.1204T/C	TT (0.05)	TC (0.18)	CC (0.77)	T (0.14)	C (0.86)	0.24	0.18	1.31	0.21	2.29*
g.1255C/T	CC (0.89)	CT (0.11)	TT (0.00)	C (0.94)	T (0.06)	0.11	0.11	1.12	0.10	0.16*
g.1262C/T	CC (0.93)	CT (0.00)	TT (0.07)	C (0.93)	T (0.07)	0.13	0.00	1.15	0.12	44.00

Note: SNP: single nucleotide polymorphism; H_e: expected heterozigosity; H_o: observed heterozigosity; n_e: number of effective allele; PIC: polymorphic informative content; χ^2: chi-square value; *under Hardy-Weinberg equilibrium (χ^2<5.99)

Table 3. Preliminary analysis for investigating the effect of two SNPs with moderate PIC value to service per conception (S/C) in Pasundan cows

SNP	Genotype	N	S/C
g.1117G/A	GG	3	1.67±1.15
	AG	7	3.29±1,25
	AA	3	1.00±0.00
g.1125A/C	AA	3	1.33±0.58
	AC	7	3.14±1.46
	CC	3	1.67±1.15
Haplotype	Hom./Hom.	4	1.00±0.00^{a}
	Hom./Het.	4	2.25±0.96^{ab}
	Het./Het.	5	3.60±1.34^{b}

Note: N: number of observation; Hom.: homozygote genotype; Het.: heterozygote genotype. Means in the same column with different superscript differ significantly (P<0.01)

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Carbon storage variability in seagrass meadows of Marine Poton Bako, East Lombok, West Nusa Tenggara, Indonesia

FIRMAN ALI RAHMAN[1], IBNUL QAYIM[2], YUSLI WARDIATNO[3,4,♥]

[1]Graduate School, Institut Pertanian Bogor. Kampus IPB Dramaga, Bogor 16680, West Java, Indonesia.
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Pertanian Bogor. Kampus IPB Dramaga, Bogor 16680, West Java, Indonesia.
[3]Department of Aquatic Resources Management, Faculty of Fisheries and Marine Sciences, Institut Pertanian Bogor. Kampus IPB Dramaga, Bogor 16680, West Java, Indonesia. Tel.: +62-251-8622909, ♥email: yusli@ipb.ac.id
[4]Center for Coastal and Marine Resources Studies, Institut Pertanian Bogor. Kampus IPB Baranangsiang, Bogor 16143, West Java, Indonesia

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Abstract. *Rahman FA, Qayim I, Wardiatno Y. 2018. Carbon storage variability in seagrass meadows of Marine Poton Bako, East Lombok, West Nusa Tenggara, Indonesia. Biodiversitas 19: 1626-1631.* The increase of atmospheric CO_2 concentration in the last decades leads to global warming, having an adverse effect on the environment condition on the Earth. One of the natural mechanism as an effort to reduce the impact of global warming is carbon absorption and storage through photosynthesis mechanism of seagrass vegetation. Research conducted at Poton Bako, a district in East Lombok was aimed to reveal the composition of seagrass species, density, seagrass coverage, the biomass of seagrass tissue, content of carbon storage in seagrass tissue (above and below substrates), carbon content in seagrass sediments, and estimation of carbon stock in the area. The research included observation of species composition, and the sample was collected from 0.5 m × 0.5 m plot area. The total plot area was 36 on six lanes with the space between plots 25 m and between lanes 100 m. Six species from two families were found in the seagrass meadows, i.e., *Cymodocea rotundata, Enhalus acoroides, Halophila minor, Holodule uninervis, Thalassia hemprichii* and *Thalassodendron ciliatum*. The three highest total densities were *C. rotundata* 214.67±110.469 stands m^{-2}, *T. hemprichii* 85.11±41.471 stands m^{-2}, and *H. minor* 42.22±44.204 stands m^{-2}. Species with the highest coverage value at all observation plots was *C. rotundata* (33.47±26.748 %), and *T. ciliatum* had the lowest value (2.12±5.071 %). The total biomass was 676.32 g DW m^{-2} with biomass above substrate 329.94±57.725 g DW m^{-2} and below substrate 654.88±81.199 g DW m^{-2}. The carbon content of substrate ranged from 0.11% to 0.51% with the average of 0.35±0.081%, which was categorized low. The total average of carbon storage in seagrass was 447.92 g C m^{-2} comprising 142.77 g C m^{-2} of their tissue above substrate and 305.15 g C m^{-2} below substrate. Regarding the area, the total carbon stored in seagrass meadows with 56.65 ha area was 249.27 t C ha^{-1}.

Keywords: Biomass, carbon dioxide emission, carbon storage, seagrass bed, substrate

INTRODUCTION

The increase of atmospheric CO_2 concentration in the last decades leads to global warming, having an adverse effect such as the increase in earth temperature, drought, the rise of sea level, and ocean acidity. Generally, global warming does not only provide adverse impacts to the environment but also to the human life, and it will affect metabolisms of terrestrial and marine biota (Goel and Bhatt 2012; Brath et al. 2015).

One of the natural mechanisms reducing the increase of CO_2 concentration is CO_2 absorption through photosynthesis mechanism of seagrass vegetation (Sunquist et al. 2008; Bala 2014). Seagrass meadows in Indonesia is one of the widest in the world, which is 30000 km^2 consisting of thirteen species (Romomohtarto and Jumana 1999; Green and Short 2003). The ecological role of seagrass meadows is not only as habitat for various marine biota but also as a part of vegetation that can absorb and store the carbon as the implementation of blue carbon concept of coastal area. The potential of carbon storage in seagrass meadows is 2-4 times greater (4 t C ha^{-1} yr^{-1}) than that in the tropical forest (1.8-2.7 t C ha^{-1} yr^{-1}) (Lewis et al. 2009; Kennedy et al. 2010; Murray 2011).

West Nusa Tenggara is one of the provinces having the potential of seagrass meadows with a total area of 9379 ha (Imran et al. 2015). The primary metabolism processes (photosynthesis) in seagrass ecosystem might be affected by 60% of the organic and inorganic carbon from the sediment of river flow running to the ocean (Triatmodjo 1999; Bouillon and Connoly 2009; Rustam et al. 2014). Tt is expected, therefore, that it will also affect the storage of carbon content in seagrass biomass and carbon content in seagrass sediments.

The objective of the study was to dtermine number of seagrass species, its density, percentage of seagrass coverage, biomass of seagrass tissue, carbon content of seagrass tissue (above substrate and below substrate), and carbon content in seagrass sediments as well as estimation of carbon stock area in the coastal area of Poton Boko, East Lombok, West Nusa Tenggara, Indonesia.

MATERIALS AND METHODS

This research was performed in the coastal areas of Poton Bako, East Lombok - West Nusa Tenggara with the area of seagrass bed, was 55.65 ha. The site is affected by two river flows, and there are also mangrove forests so that the water condition of seagrass is turbid. This study was carried out from September to December 2017, including site observation and laboratory analysis. Site observation included identification of seagrass species following den Hartog (1970) and Azkab (1999). In the field, the seagrass coverage was estimated by using the Seagrass-Watch method (McKenzie et al. 2001) and by counting the number of stands in each observation plot (0.25 m^2 area). The number of observation plot was 36 in six lanes with the distance between plots was 25 m and between lines was 100 m.

Data collection and analysis

The data was collected by taking the entire stands to the depth of root penetration in each plot (0.25 m^2) as the sample of tissue biomass and carbon content of seagrass tissue that were above substrate (leaf sheaths and blades) and below substrate (rhizomes and roots). The calculation of top and below substrate was performed using oven drying method at a temperature of 60°C until dry weight stable was achieved (Kaldy and Dunton 2000). Meanwhile, the calculation of the carbon content of seagrass tissue was performed using Loss On Ignition method Helrich (1990). The sediment sample was collected in each plot to a depth of root penetration of 30 cm with the slope of 30° using a pipe having a diameter of 5 cm and length of 35 cm. The carbon content in seagrass sediments was analyzed using Kurmis method (Helrich 1990). Analysis of seagrass carbon and sediments was performed at the Soil Laboratory of Assessment Institute for Agricultural Technology, West Nusa Tenggara.

Analisis data

Density

Seagrass density is the sum of all seagrass individuals per unit area (Brower and Zar 1977). Value of seagrass ecosystem density was calculated using the following formula:

$$D = \frac{Ni}{A}$$

Where:

D : density of seagrass, species *i* (stands m^{-2})
Ni : number of seagrass with species *i*
A : area of observation plot (m^2)

Biomass of seagrass species

Biomass is an organic material produced through photosynthesis process, either product or waste. Seagrass biomass (g DW m^{-2}) is a result of oven drying method calculated by using Azkab (1999) formula:

$$\text{Biomass (g DW m}^{-2}) = \frac{\text{Dry Weight (g DW)}}{\text{Wide area of observation area (m}^2)}$$

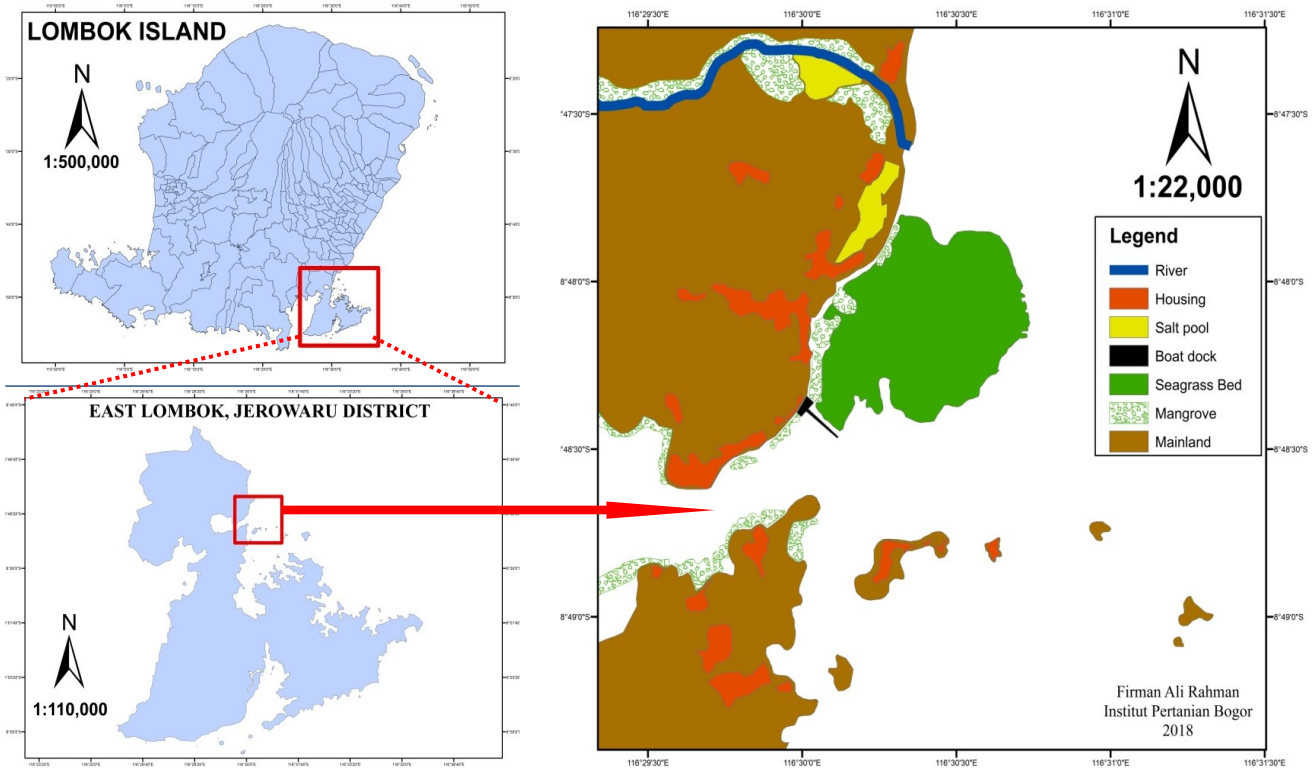

Figure 1. The study location at marine Poton Bako, East Lombok, Indonesia

Seagrass tissue carbon

Analysis of seagrass tissue carbon was calculated using Helrich (1990) formula:

$$\text{Ash content (\%)} = \frac{c - a}{b - a} \text{ x } 100\%$$

Where:

a : Cup weight

b : Cup weight + dry weight of seagrass tissue

c : Cup weight + ash weight of seagrass tissue

Organic carbon material as a result of weight reduction while digestion process using Helrich (1990) formula:

$$\text{Organic Material Content (\%)} = \frac{[(b - a) - (c - a)]}{[b - a]} \text{ x } 100\ \%$$

Where:

a : Cup weight

b : Cup weight + dry weight of sample

c : Cup weight + ash

Value of organic carbon content of seagrass tissue was calculated using Helrich (1990) formula and the value as result of the carbon content was then calculated as the value of carbon content of seagrass tissue.

$$\text{Organic Carbon Content (\% C)} = \frac{\text{Organic material content [\%]}}{1.724}$$

Where:

1.724 : Constant value of the organic material

Carbon content in seagrass sediments

Carbon content in seagrass sediments was calculated using Sulaeman et al. (2005) formula:

$$\text{Organic carbon content in sediments (\%)} = \frac{\text{ppm curve x } 10}{500 \text{ x correction factor}}$$

Where:

ppm curve : Sample content obtained from the relationship curve between the standard serial content and the reading after correction

Correction factor : 100/ (100 - % water content)

Total carbon stock area

The calculation of carbon stored (g C m^{-2}) of seagrass tissue was performed using the approach of seagrass biomass weight (g DW m^{-2}) using Barron et al. (2004) formula:

$$\text{Carbon stored (g C m}^{-2}) = \frac{\text{Carbon content (\% C) x Biomass of species (g DW m}^{-2})}{100}$$

Then, the estimation of the total carbon stock area was calculated using Sulaeman et al. (2005) formula:

$$Ct = \sum (Li \text{ x } Ci)$$

Where:

Ct : total carbon (t C)

Li : area of seagrass bed ecosystem (ha)

Ci : the average of seagrass carbon content (g C m^{-2})

RESULTS AND DISCUSSION

Seagrass density and coverage

Density is a type of structure that can be used to estimate the production capability of a primary seagrass based on the number of individuals in the research sites. Based on the result of observations and calculations, *C. rotundata* (214.67±110.469 stands m^{-2}) was the species having the highest density found in coastal habitats; this was in line with Hartati et al. (2012) that there was a single highly associated species in the coastal area. Besides, *T. hemprichii* had the second highest density because it was able to adapt well in the Indonesian ocean environment (Larkum et al. 1989). This result was different from the density of *E. acoroides* (20.44±12.217 stands m^{-2}) that was lower than *C. rotundata* and *T. hemprichii*, but it had a wide variety of species in Poton Bako. This was because the morphology of *E. acoroides* was big and each of them required a wider space area. The lowest density value belonged to *H. uninervis* (16.67±25.000 stands m^{-2}) and *T. ciliatum* (5.56±8.333 stands m^{-2}) since these species were found only on one observation plot with low distribution value.

The percentage of seagrass coverage was related to the level of species capability and distribution. *C. rotundata* had the highest coverage value of 33.47±26.748%, and it had a positive correlation on high density value while the lowest seagrass coverage value belonged to *T. ciliatum* 2.12±5.07% (Table 1).

Seagrass tissue biomass

Biomass is the product of plant metabolism stored in its morphological part. The total biomass of six seagrass species in Poto Bako was 984.82±138.940 g DW m^{-2}. Overall, seagrass biomass of below substrate (654.88±81.199 g DW m^{-2}) had greater biomass content than the above substrate (329.94±57.725 g DW m^{-2}) (Table 2). *E. acoroides* had the highest biomass (628.57±57.67 g DW m^{-2}), and the lowest biomass belonged to *H. uninervis* (22.56±2.476 g DW m^{-2}) and *H. minor* (11.54±0.269 g DW m^{-2}) due to its small morphology type; whereas the low biomass of *T. ciliatum* (23.08±4.242 g DW m^{-2}) was caused by low species distribution and the low number of stands.

Table 1. Density and seagrass coverage at marine Poton Bako, East Lombok, Indonesia

Seagrass species	Density (stands m^{-2})	Seagrass coverage (%)
C. rotundata	214.67±110.469	33.47±26.748
T. hemprichii	85.11±41.471	23.07±18.161
H. minor	42.22±44.204	7.78±12.833
E. acoroides	20.44±12.217	31.07±16.241
H. uninervis	16.67±25.000	2.48±5.833
T. ciliatum	5.56±8.333	2.12±5.071

Table 2. Seagrass biomass at marine Poton Bako, East Lombok, Indonesia

Lane	Species	Number of plot	Seagrass biomass (g DW m^{-2})			
			Leaf sheaths and blades	Rhizomes	Roots	Total biomass
1	*C. rotundata*	4	34.80±10.886	33.64±10.796	16.27±4.156	84.72±25.840
	E. acoroides	1	9.85±0.990	13.73±0.134	3.56±2.121	27.15±3.250
	T hemprichii	1	6.69±0.509	7.25±1.485	4.12±1.549	18.06±3.540
2	*C. rotundata*	2	15.88±7.616	9.10±1.796	7.12±1.782	32.10±11.190
	T. hemprichii	5	28.65±4.158	24.48±3.413	16.06±1.930	69.19±9.500
3	*C. rotundata*	1	5.55±0.877	5.12±1.563	4.12±1.393	14.78±3.830
	E. acoroides	3	35.70±6.390	94.94±7.313	13.42±2.622	144.06±16.33
	T. hemprichii	3	17.55±3.563	15.60±4.625	9.20±2.149	42.35±10.340
4	*C rotundata*	2	9.52±2.503	9.68±0.870	7.12±1.782	26.32±5.160
	E. acoroides	4	61.92±3.802	123.99±5.308	16.56±0.758	202.47±9.870
5	*C. rotundata*	1	3.97±1.414	4.57±2.121	3.00±1.344	11.54±4.880
	E. acoroides	2	25.60±8.577	50.81±4.059	9.60±1.393	86.02±14.03
	H. uninervis	1	8.03±0.778	9.59±0.849	4.95±0.849	22.56±2.480
	T. ciliatum	1	7.95±0.707	9.14±2.121	6.00±1.414	23.08±4.240
6	*E. acoroides*	6	54.30±4.835	92.28±8.335	22.29±1.020	168.88±14.190
	H. minor	2	3.97±0.120	4.57±0.078	3.00±0.071	11.54±0.27

Table 3. Percentage of carbon content in all parts of seagrass at the coastal area of Poton Bako, East Lombok, Indonesia

Species	Leaf sheaths and blades carbon stored		Rhizomes carbon stored		Roots carbon stored	
	(% C)	(g C m^{-2})	(% C)	(g C m^{-2})	(% C)	(g C m^{-2})
C. rotundata	49.05±1.369	34.20	45.77±14.097	28.43	44.79±9.443	16.85
E. acoroides	41.27±3.699	77.33	49.09±4.444	184.46	44.98±5.329	29.43
T. hemprichii	46.33±4.696	24.50	48.73±6.955	23.06	35.80±4.583	10.52
T. ciliatum	36.27±0.000	2.88	46.70±0.000	4.27	36.46±0.000	2.19
H. uninervis	35.00±0.000	2.81	32.10±0.000	3.08	28.70±0.000	1.42
H. minor	26.40±0.000	1.05	19.87±0.000	0.91	17.93±0.000	0.54

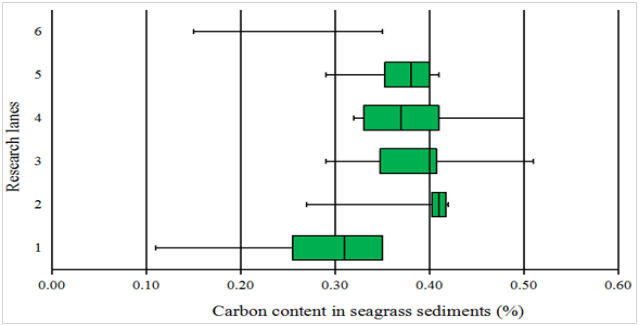

Figure 2. Carbon content in seagrass sediments at marine Poton Bako, East Lombok, Indonesia

Carbon content in seagrass sediments

The carbon content in seagrass sediments was the result of animals and litter corrosion decomposed by microorganisms (Purnama 2013). Carbon content in seagrass sediments in Poton Bako ranged from 0.11% to 0.51%, with an average of 0.35±0.081% (Figure 2). The carbon content in seagrass sediments of sandy clay and clay containing sand were relatively higher than sand substrate. In general, the value of carbon content in seagrass sediments of Poton Bako marine was categorized as very low because it was < 1% (Sulaiman et al. 2005).

Seagrass tissue carbon

The carbon content of seagrass tissue (leaf sheaths and blades, rhizomes and roots) was able to show the seagrass potential as blue carbon in each part of its morphology. *E. acoroides* had the highest carbon content (291.22 g C m^{-2}), while *H. minor* (2.50 g C m^{-2}) had the lowest carbon content. The level of seagrass carbon content can be related to the species biomass by considering the research sites. The carbon content stored in biomass of below substrate (rhizomes and roots) was higher than the biomass of the above substrate (leaf sheaths and blades) in the six types of seagrass with a ratio of 2:1, which was a good potential for seagrass as blue carbon because biomass of below substrate can be stored for thousands of years (Mateo et al. 1997).

Discussion

East Lombok is one of the areas where the seagrass bed area of 784.3 ha and it has the potential to be blue carbon area of Indonesia (Imran et al. 2015). Based on

observations, there were six species of seagrass (two families, 6 genera) of the thirteen Indonesian seagrass species in Poton Bako, including *Cymodocea rotundata, Enhalus acoroides, Halophila minor, Holodule uninervis, Thalassia hemprichii* and *Thalassodendron ciliatum*. The composition of seagrass in Poton Bako was lower than the composition of nine species in Tanjung Luar East Lombok (Syukur et al. 2017), eight species in Sanur Bali (Graha et al. 2015), eight species in Menjangan Kecil Island, eight species in Pintok Karimunjawa Archipelago (Hartati et al. 2017), seven species in Tanjung Lesung, Miskam Bay Banten (Rustam et al. 2014), seven species in Kotania Seram Tenggara Bay (Wawo et al. 2014), seven species in Pari Island (Husodo etal. 2017), and seven species in West Bali National Park (Purnomo et al. 2017). The low composition of seagrass in Poton Bako was suspected because the effect of two river flow causing turbid water so that it became the factor why the growth and development, especially in photosynthesis process as well as the distribution of seagrass became limited.

The composition and structure of the seagrass were related to the density value, distribution, and percentage of seagrass coverage in the five seagrass species, except *E. acoroides*, which had the fourth highest density with the second highest coverage value because *E. acoroides* had a big morphological size but with a low number of stands. This supported Short and Coles (2003) that the size of the species morphology can affect the coverage and individual values having small morphology size such as *H. minor* having a low coverage value. Composition value and structure of the seagrass can be affected by the depth, substrate type, light intensity, current, temperature, pH, turbidity, nutrients and salinity (McRoy and McMillan 1977; Zieman and Wetzel 1980).

Overall, there was no seagrass species having a 'rich' status based on the Decree of the Minister of Environment No. 200 of 2004 on six types of Poto Bako seagrass because they had coverage value that was less than 60%, and there were only two species with less rich status: *C. rotundata* (33.47±26.748%) and *E. acoroides* (31.07±16.241%); while the other four seagrasses were classified as poor (< 29.9%).

Biomass was related to density value in Poton Bako. The present research result revealed that total biomass of *C. rotundata* (169.47 g DW m^{-2}) was higher than that of *T. hemprichii* (129.60 g DW m^{-2}), although *T. hemprichii* had bigger morphology size than *C. rotundata*. Azkab (1999) saying that biomass may be affected by morphological and density factors. The biomass content of below substrate had a higher value than that of above substrate on all seagrass species in Poton Bako with a ratio of 2:1, because the biomass material of below substrate was generally more solid and larger morphological sizes.

Carbon content in seagrass sediment can be affected by the characteristics of the substrate fraction, the growing seagrass species, the environmental factors and the activity of the littering organisms. Large diameter sand substrate allowed the occurrence of oxidation mechanisms which can lead to the detached organic material content and low carbon storage, whereas higher carbon content can be found in clay or fine substrate fractions (Azkab and Kiswara 1999; Yunitha et al. 2014). The low carbon content of the low substrate in Poton Bako was because the lather of seagrass was not drowned and decomposed but was carried away to the coastal area. Besides, the anaerobe condition and high pH can affect the low corrosion activity and mineralization of organic material by microorganisms in the substrate (Poljakoff-Mayber and Gale 1975; Tangketasik et al. 2012).

Overall, *E. acoroides* had the highest carbon content because it had the highest biomass with a larger morphological size than other species. Kennedy and Bjork (2009) and Rahmawati (2011) reported that seagrass with large morphology size could accumulate larger carbon such as *E. acoroides*, that was about 40% of its biomass, and vice versa. Björk et al. (2008) reported that *Halophila sp* had a low carbon content that was suspected to be pioneering species with small morphological size. In addition to morphological factors, high density factor can affect the value of biomass species having implications on carbon content such as in *C. rotundata* (79.48 g C m^{-2}) that was the second highest species in Poton Bako.

In general, the total seagrass carbon content stored in tissue on the top and below substrate was 447.92 g C m^{-2}; above substrate was 142.77 g Cm^{-2} and below substrate was 305.15 g C m^{-2}. The estimation of carbon stock in Poton Bako seagrass beds was 249.27 t C or equivalent to 4.48 t C ha^{-1}. The higher total carbon content was dominated on the below substrate (169.82 t C) than the above substrate (79.45 t C). The total amount of this storage was much higher than that found by Graha et al. (2015) at Sanur Beach Bali with a total carbon stock of 66.60 t C on an area of 322 ha or equivalent to storage of 0.21 t C ha^{-1}. Similarly, the result of research performed by Supriadi (2012) in Baranglompo Island area of 58.05 ha revealed that E. acoroides also dominated the *is*land with the total carbon storage of 52.06 t C or equivalent to 0.9 t C ha^{-1}.

To conclude, there were six types of seagrass (two families, six genera) at Poton Bako, East Lombok, including *Cymodocea rotundata, Enhalus acoroides, Halophila minor, Holodule Uninervis, Thalassia hemprichii* and *Thalassodendron ciliatum*. The highest density value and coverage percentage were found in *C. rotundata* with the respective value of 214.67±110.469 stands m^{-2}, and 33.47±26.748%. The average of above substrate total biomass was 329.94±57.725 g DW m^{-2}, and the below substrate was 654.88±81.199 g DW m^{-2}. The average carbon content in seagrass sediments of 0.35±0.081% was included in the category of very low because it was less than 1%. The total carbon stock storage in seagrass ecosystem of 55.65 ha was 249.27 t C or equivalent to 4.48 t C ha^{-1}.

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Short Communication: Habitat characterization of *Aristolochia baetica* L. in Tessala Mount, Western Algeria

HAKEMI ZAHRA, MEHDADI ZOHEIR, LATRECHE ALI, CHERIFI KOUIDER, AISSET AMEL
Laboratory of Plant Biodiversity: Conservation and Valorization, Faculty of Natural and Life Sciences, University of Djillali Liabes. Sidi Bel Abbes 22000, Algeria. ♥email: zahra.hakmi.biologie@gmail.com

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Abstract. *Zahra H, Zoheir M, Ali L, Kouider C, Amel A. 2018. Short Communication: Habitat characterization of* Aristolochia baetica *L. in Tessala Mount, Western Algeria. Biodiversitas 19: 1632-1641.* The aim of the current investigation was to study the characterization of the habitat of *Aristolochia baetica* L., an Ibero-Mauretanian species, present in Tessala Mount (West of Algeria). Twenty-four phytoecological surveys were realized on eight stations (St1-St8) in which the species is present. The floristic inventory allowed us to identify 68 species which are part of the floristic of *A. Baetica*, distributed in 31 botanical families; 6 biological and 19 biogeographical types. The assessment of edaphic and plant data using correspondence factor analysis (CFA) and hierarchical ascending classification (HAC) showed that *A. baetica* is a member of plant training of scrublands and grows especially in stations at low heights with a high rate of limestone.

Keywords: *Aristolochia baetica*, habitat, Ibero-Mauretanian, Tessala Mount

INTRODUCTION

The Mediterranean region forms a climatic and biogeographical entity enriched with floral elements and very contrasted climates (Gamisans 1991; Quézel 1995). This area is characterized by an exceptional biodiversity too (Cowling et al. 1996) and a raised richness of rare vegetation, mainly concentrated in large plant families (Dominguez Lozano and Schwartz 2005). The reason for what this region was classified as one of the five regions of the world where the environmental issues are the most important (Ramade 1993; Beaulieu et al. 2005) insofar where flora is suffering from strong anthropogenic pressures. The human influence on the Mediterranean vegetation is antique (Pons and Quézel 1985), which result in fragmentation of habitat, disappearance of species and populations (Grenon and Batisse 1989; Ramade 1990; Heywood 1995).

Algeria has an important richness of Mediterranean flora and fauna. Basing on Quézel and Santa's flora (1962; 1963), Zeraia (1983) count 289 rare species, 647 rare, 675 very rare, 35 extremely rare, where 1611 rare species, which represent approximately more than half of the National Algerian flora.

These rare species were the object of several privileged studies. They have a considerable value in term of preservation too, either for patrimonial reasons, or for their great risk of extinction (Pimm et al. 1988; Gaston 1994). In this context, we select *Aristolochia baetica* L. among the endemic species in Algeria (Maire 1961*)*. This species is rather common one according to Quézel and Santa (1962, 1963). *A. baetica* is known by the vernacular name *Bereztem* (Bellakhdar 1997; Bammi and Douira 2004), and belonging to the Aristolochiaceae family (Heywood 1995). *A. baetica* L. is a climbing plant, perennial, characterized by persistent foliage and a long period of flowering extending from winter until spring (Berjano et al. 2011). *A. baetica* was used in phytotherapy in the whole world as anti-poison and a childbirth facilitator by stimulating uterine contractions (Bellakhdar 1997). The roots were used in case of bites of snakes and scorpion stings as antidotes. Furthermore, it was used to treat malaria, abdominal pains as anti-helminths and anti-worm causing their expulsion outside (Heinrich et al. 2009). The methanol extract of *A. baetica* is used as insecticide against *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) (Jbilou et al. 2006). *A. baetica* is a Mediterranean species represented by a reduced number of localized populations and low numbers (Schemske et al. 1994; Colas 1997), which was found at the level of our study site where this species is seldom found. Those characteristics make this species an appropriate model primarily to study the lines of life history of the species growing in small populations, and to appreciate the issues of preservation of rare species. These two problematics are associated by combining an ecological approach, which is uncommon in most studies concerned by preservation of rare species.

The purpose of the current study consists on characterization of the habitat of *A. baetica* in Tessala Mount (north-west of Algeria), through the description of its edaphic substrate and floristic association.

MATERIALS AND METHODS

Study area

Our study site is located at the level of Tessala Mount, limited by the mounts of Berkèche on the west and on south by the plain of Sidi-Bel-Abbes, Algeria. This area is oriented by the southwest to northeast, characterized by summits with average heights of 600 m; the highest point rises to 1061m of height (Bouzidi et al. 2009; Saidi et al. 2016). The study area is characterized by a Mediterranean climate semi-arid with fresh winter. The annual average pluviometry is 335.16 mm and maximum average temperatures of 26.3°C in August and a minimum of 9.5°C in January (Cherifi et al. 2017) (Figure 1).

Procedures

Vegetation analysis

The various floristic reviews conducted at the level of the study area, allowed to select eight sampling stations (St1-St8) according to the presence of *A. baetica* as shown in Figure 1, where GPS coordinates are represented in Table 1. Aiming to characterize the species habitat, we examined the floristic and edaphic substrate of each station. For the analysis of the vegetation, 24 floristic reviews were performed on all stations, with an average of 3 reviews per station. These floristic reviews constituted qualitative and quantitative inventories of the vegetation using Braun-Blanquet (1951) method. For each surface statement with 25 m² (5m x 5m), were recorded the geographical localization, the height, the exposure, the slope as well as the present species by strata (tree layer - shrub layer - shrubby and herbaceous layer), affected by abundance-dominance coefficients (Coefficient 5: species covering more than 3/4 of the area; Coefficient 4: 3/4 to 1/2 of the area, Coefficient 3: 1/2 to 1/4 of the area; Coefficient 2: abundant species but covering - 1/4 of the area; Coefficient 1: species represented but covering - 1/20 of the area; Coefficient +: Present but not quantifiable species), sociability (1: isolated individuals; 2: individuals in small groups; 3: individuals in vast groups; 4: individuals in small colonies; 5: individuals in vast and dense populations) and their frequency of occurrence (F) (Class I: F= - 20% , very rare species; Class II: 40%>F>20%, rare species; Class III: 60%>F>40%, frequent species; Class IV: 80%>F>60%, abundant species; Class V: F> 80%, Constant species) in the various conducted floristic reviews. The inventoried species were classified according to their taxonomic families too (Quézel and Santa 1962-1963), biological spectrum (Raunkiaer 1934; Ellenberg and Mueller 1968), and biogeographical spectrum (Quézel and Santa 1962-1963; Ozenda 1985; Bonnier 1990).

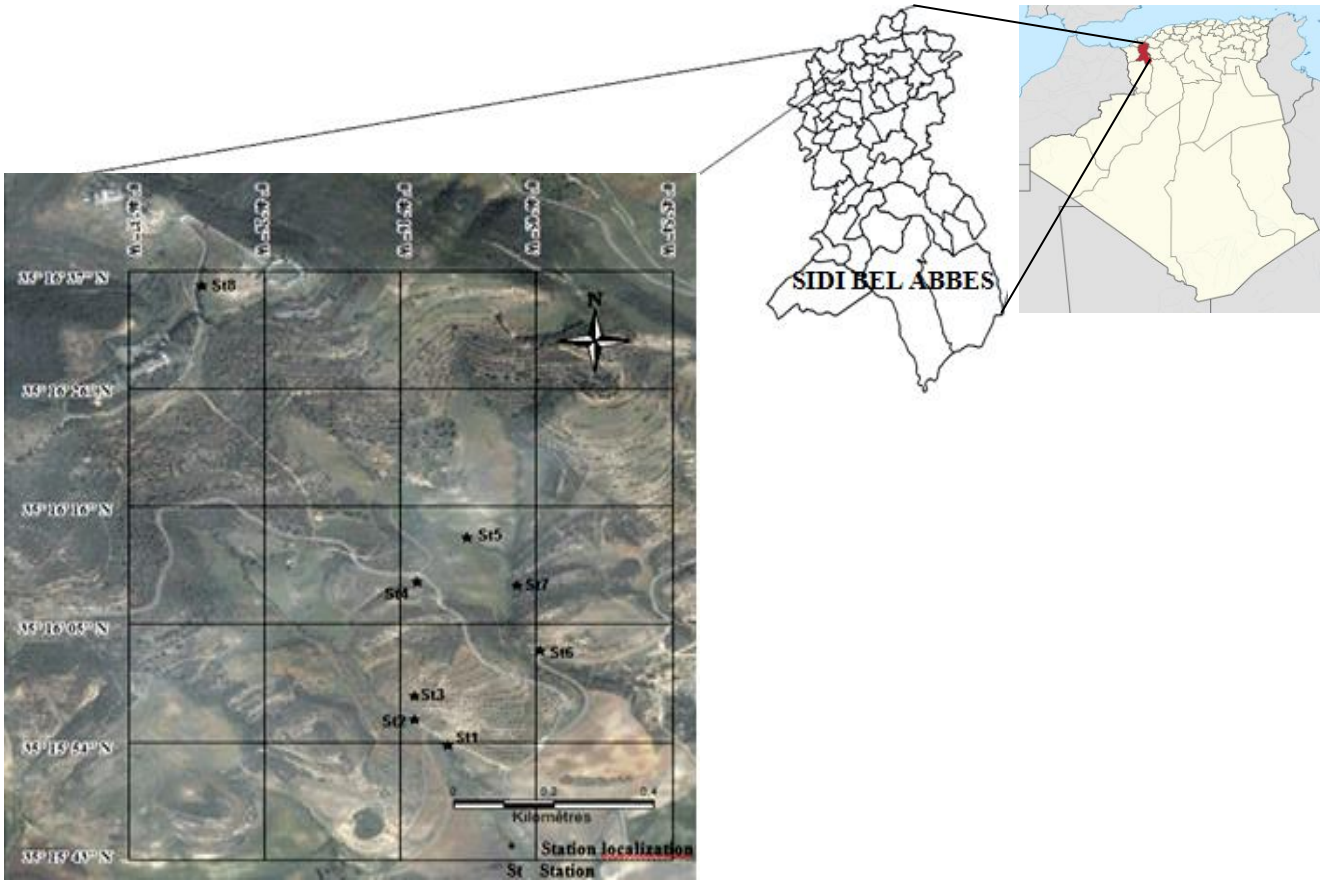


Figure 1. Positioning sampled stations in in Tessala Mount, Western Algeria (prepared by the MapInfo Professional ver. 8.0)

Table 1. Floristic surveys

Stations	**St1**			**St2**			**St3**			**St4**			**St5**			**St6**			**St7**			**St8**			
Exposure	SW			SW			SW			S			SE			SE			SE			NW			
Longitude	- 0°46'12''			- 0°46'15''			- 0°46'15''			- 0°46'15''			- 0°46'10''			- 0°46'04''			- 0°46'06''			- 0°46'31''			
Latitude	35°15'54''			35°15'56''			35°15'59''			35°16'09''			35°16'13''			35°16'02''			35°16'08''			35°16'35''			
Altitude (m)	726			728			771			800			750			747			680			935			
Slope (%)	25			12			25			12			12			10			60			50			
	Floristic surveys																								
	FS1	**FS2**	**FS3**	**FS4**	**FS5**	**FS6**	**FS7**	**FS8**	**FS9**	**FS10**	**FS11**	**FS12**	**FS13**	**FS14**	**FS15**	**FS16**	**FS17**	**FS18**	**FS19**	**FS20**	**FS21**	**FS22**	**FS23**	**FS24**	**F (%)**
Treelayer																									
Ficus carica L.	-	-	-	-	1.2	2.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8.33
Nerium oleander L.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.1	-	-	-	-	-	4.16
Olea europea L.	-	-	-	1.1	-	1.1	-	-	-	-	-	-	3.3	4.3	4.3	-	-	-	1.2	1.2	1.2	-	-	-	33.30
Pinus halepensis L.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.2	1.2	1.2	-	-	-	12.50
Pistacia terebinthus L.	-	-	-	1.1	1.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	08.33
Quercus coccifera L.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	4.3	4.3	12.50
Quercus ilex L.	-	-	-	-	-	-	-	-	-	-	-	-	4.3	3.3	3.3	-	-	-	-	-	-	4.3	3.3	3.3	25.00
Shrublayer																									
Olea europea var. oleaster L.	-	1.1	-	-	-	-	-	-	-	-	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	08.33
Shrubby and herbaceous layer																									
Ajuga iva (L) Schreb	-	1.1	1.1	-	-	-	-	-	-	-	-	-	-	-	-	1.2	1.2	-	-	-	-	-	-	-	16.70
Allium roseum L.	-	-	-	-	-	-	-	-	-	-	-	-	1.2	-	1.2	-	-	-	-	-	-	-	-	-	08.33
Ammi visnaga L.	-	2.1	1.1	-	-	-	-	-	-	-	-	-	1.2	1.2	-	-	-	-	1.2	-	-	-	-	-	20.80
Ampelodesmos mauritanicus Bir.	1.1	1.1	1.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12.50
Anacyclus clavatus (Desf.) Pers.	-	-	-	-	-	-	-	-	-	-	-	-	-	1.1	-	-	-	-	-	-	-	1.1	1.1	-	12.50
Anagallis monelli L.	-	-	-	-	-	-	-	-	-	-	-	-	1.1	1.1	1.1	-	-	-	-	-	-	1.1	1.1	1.1	25.00
Aristolochia baetica L.	3.3	3.3	2.3	3.2	2.3	2.3	1.2	2.2	1.2	1.2	1.2	1.2	3.2	2.2	3.2	2.2	1.2	1.2	1.1	1.1	2.1	2.2	1.2	1.2	100.00
Asparagus acutifolius L.	2.2	2.2	1.2	3.2	3.2	3.2	-	-	-	1.1	1.1	1.2	1.2	1.2	1.2	1.2	1.2	2.2	-	-	-	1.1	1.1	1.1	75.00
Asparagus albus L.	+	1.1	-	-	-	-	-	-	-	-	-	-	1.2	1.1	-	-	-	-	-	-	-	-	-	-	16.70
Asphodelus microcarpus Salzm et Viv.	1.1	1.2	1.2	2.2	2.2	1.2	-	1.1	1.1	1.2	2.2	-	1.2	1.2	1.2	-	1.2	1.2	1.1	-	-	1.1	1.1	1.1	79.20
Asteriscus maritimus L.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.1	-	-	-	-	-	-	-	04.16
Avena sterilis L.	-	-	1.1	1.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8.33
Ballota hirsuta Benth	1.1	-	1.2	-	-	-	1.2	-	-	2.2	1.2	-	1.2	-	1.2	-	-	-	1.2	1.2	-	-	-	1.1	41.70
Bellis annua L.	-	-	-	-	-	-	-	-	-	-	-	-	1.1	1.1	1.1	-	-	-	-	-	-	-	-	-	12.50
Bromus rubens L.	-	-	-	1.1	-	1.1	-	-	-	-	-	-	1.2	1.2	-	1.1	-	1.1	-	-	-	1.1	1.1	-	33.30
Bromus lanceolatus Roth	1.2	1.1	1.2	-	-	-	1.1	-	1.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	20.80
Calendula arvensis L.	-	-	-	-	-	-	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	-	-	-	-	-	-	1.1	1.1	1.1	50.00
Calycotome spinosa L.	2.3	3.3	3.3	2.2	2.2	2.2	3.3	3.3	3.3	3.2	2.2	2.2	1.2	1.2	1.2	2.3	3.3	2.3	2.2	2.2	1.2	1.1	1.1	1.1	100.00
Carduus pycnocephalus L.	-	-	-	-	-	-	-	-	-	1.1	1.1	-	-	-	-	-	-	-	-	1.2	-	-	-	-	12.50
Centaurea acaulis L.	-	-	-	1.1	1.1	1.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12.50
Centaurea pullata L.	-	-	-	-	-	-	-	-	-	-	-	-	1.1	1.1	-	-	-	-	-	-	-	1.1	1.1	-	16.70

Chamaerops humilis L.	3.3	2.3	3.3	2.2	2.2	1.2	3.3	3.3	3.3	2.2	2.2	2.2	2.2	1.2	2.2	3.3	2.3	2.3	4.3	3.3	3.3	1.1	1.1	2.1	100.00
Convolvulus althaeoides L.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+.+	-	-	-	1.1	1.1	1.1	-	-	1.1	20.80
Daphne gnidium L.	-	-	-	1.2	2.2	1.2	1.2	-	1.2	2.2	2.1	-	1.1	1.1	1.1	-	-	-	2.2	1.2	-	1.1	1.1	1.1	62.50
Daucus carota L.	-	-	-	-	-	-	1.1	1.1	-	1.1	1.1	1.1	-	-	-	-	-	-	-	-	-	-	-	-	20.80
Eruca vesicaria L.	-	-	-	-	-	-	-	-	-	-	-	-	1.2	1.2	-	-	-	-	-	-	-	1.1	1.1	-	16.70
Evax pygmaea Pers	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	04.16
Foeniculum vulgare Mill.	-	-	-	-	-	-	-	-	-	-	-	-	1.1	-	-	-	-	-	-	-	-	1.1	-	-	08.33
Hedera helix L.	1.1	1.1	1.1	-	-	-	1.1	1.1	1.1	-	-	1.1	-	-	-	-	-	-	-	-	-	-	-	-	29.20
Helianthemum appeninum L.	-	-	-	-	-	-	-	-	-	-	-	-	1.2	-	1.2	-	-	-	-	-	-	1.1	-	-	12.50
Iris xiphium L.	-	-	-	-	-	-	-	-	-	+	1.1	-	-	-	-	-	-	-	-	-	-	-	-	-	08.33
Lobularia maritima L.	1.1	1.1	-	-	-	-	1.1	1.1	-	1.1	1.1	1.2	-	-	-	1.1	-	1.1	-	-	-	-	-	-	37.50
Malva sylvestris L.	-	-	-	-	-	-	1.1	1.1	-	1.2	-	-	-	-	-	+	+	+	-	-	-	-	-	-	25.00
Marrubium vulgare L.	-	-	-	1.1	1.1	1.1	-	1.1	1.2	-	-	-	2.2	1.2	1.2	-	-	-	-	-	-	-	1.2	1.2	41.70
Ornithogalum umbellatum L.	-	-	-	-	-	-	-	-	-	1.1	-	-	-	-	-	1.2	1.2	1.2	-	-	-	-	-	-	16.70
Oxalis corniculata L.	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	04.16
Papaver rhoeas L.	-	-	-	-	-	-	-	-	-	-	-	1.2	-	-	-	-	-	-	-	1.2	1.2	-	-	-	12.50
Paronychia argentea Lam.	-	-	-	-	-	-	-	-	-	1.1	1.1	1.1	1.2	-	-	-	1.1	1.1	-	-	-	1.1	-	-	29.20
Pistacia lentiscus L.	-	-	-	-	-	-	-	-	-	-	-	-	1.1	-	-	-	-	-	-	-	-	1.2	-	-	08.33
Plantago albicans L.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.2	-	1.2	-	-	-	08.33
Plantago lagopus L.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.1	1.1	1.1	-	-	-	-	-	-	12.50
Ranunculus arvensis L.	-	-	-	-	-	-	-	-	-	1.1	-	-	-	-	-	-	-	-	-	1.2	1.2	-	-	-	12.50
Raphanus raphanistrum L.	-	-	-	-	-	-	-	-	-	-	-	-	-	1.1	1.1	-	-	-	-	-	-	-	1.1	1.1	16.70
Reseda alba L.	-	-	-	-	-	-	-	-	-	-	-	-	1.2	1.2	1.2	-	-	-	1.1	1.1	-	1.1	1.1	1.1	33.30
Rosmarinus officinalis L.	-	1.2	1.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	08.33
Rumex bucephalophorus L.	-	-	-	-	-	-	+	-	-	1.1	1.2	1.2	-	-	-	1.1	1.1	1.1	-	-	-	-	-	-	29.20
Ruta chalepensis L.	-	-	-	-	-	-	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	-	-	-	-	-	-	-	1.2	1.2	-	41.70
Ruta montana (Clus.) L.	1.2	1.2	-	1.1	1.1	1.1	-	-	-	1.1	1.1	1.1	1.1	1.1	1.1	-	-	-	1.1	-	1.1	1.2	1.2	1.2	66.70
Salvia argentea L.	-	-	-	-	-	-	+	-	-	1.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	08.33
Salvia officinalis L.	-	-	-	-	-	-	-	-	-	-	-	-	1.1	1.1	1.1	-	-	-	-	-	-	-	-	-	12.50
Scolymus hispanicus L.	-	-	-	-	-	-	-	-	-	1.1	1.1	-	1.1	1.1	1.1	-	-	-	-	-	-	-	-	-	20.80
Scolymus sp.	-	-	-	1.1	1.1	1.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12.50
Silene colorata Poiret.	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	04.16
Silybum marianum L.	-	1.1	1.1	1.1	1.1	-	1.2	1.2	1.2	-	-	-	-	-	-	-	-	-	1.1	1.1	1.1	1.1	-	1.1	50.00
Sisymbrium officinale L.	-	-	-	-	-	-	-	-	-	1.1	1.2	1.2	-	-	-	-	-	-	1.1	-	1.1	-	-	-	20.80
Stipa tenacissima L.	-	-	-	-	-	-	+	-	-	+	+	-	-	-	1.1	1.1	1.1	-	-	-	-	+	-	-	29.20
Teucrium polium L.	1.2	1.2	1.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	16.70
Thymus ciliatus Desf.	-	-	-	-	-	-	-	-	-	1.1	1.1	-	1.2	-	-	-	-	-	-	-	-	1.1	-	-	16.70
Torilis nodosa L.	-	-	-	-	-	-	-	-	-	-	1.1	1.1	-	-	-	-	-	-	-	-	-	-	-	-	08.33
Trifolium stellatum L.	-	1.1	1.1	-	-	-	-	-	-	1.1	1.1	1.1	-	-	-	-	-	-	1.1	1.1	-	-	-	-	29.20
Urginea maritima (L.) Baker.	1.2	1.1	1.2	1.1	1.1	1.1	1.1	-	1.1	1.2	-	1.2	1.2	1.2	1.2	1.2	1.2	-	-	-	-	1.2	1.2	1.2	75.00
Number of species by survey	15	19	17	16	14	14	17	12	12	28	23	18	30	25	23	14	15	14	17	14	12	25	20	18	/
Number of species by station	23			17			19			31			34			17			20			29			/
Total number of species	68																								

Note: St: Station; FS: Floristic surveys; SW: South-West; S: South; SE: South-East; NW: North-West; F: frequency of occurrence

Soil analysis

Concerning soil analysis, three samples were taken from each station of the topsoil to a depth from 10 to 20 cm. Soil samples were then dried in the open air for 15 days. Once dried, we analyzed the following parameters: pH (the principle consists in measuring the electromotive force of an aqueous solution of the soil (water/soil ratio) using a pH meter); organic matter content (the carbon of the organic matter is oxidized by bichromate of potassium in the presence of sulfuric acid. Knowing the amount of bichromate necessary for this oxidation, the percentage of organic carbon and humus in the soil was calculated using this formula: % humus /% COx = 1.724 (Baize 1988);

The texture of soil revealed by its granulometric analysis whose principle is based on the rate of sedimentation of particles separated and dispersed by destruction of their cement (limestone and organic matter). The fractionation of these particles took place via the Robinson pipette, which allows the determination of clay and silt fractions (Baize 1988). These results were reported according to the percentages of clays, silts, and sands in the textural triangle, to determine the texture. The electrical conductivity was measured with a conductimeter as a function of the concentration of electrolytes in a 1/5 aqueous extraction solution (Richards 1954). The cationic and anionic compositions of the soil extract were carried out according to the method described by Jackson (1962). The total limestone ($CaCO_3$) measure that is based on the characterized reaction of carbonate of calcium (CaCO3) with hydrochloric acid (HCl) was carried out with the calcimeter of Bernard (Baize 1988). The active limestone was carried out with a specific reagent (ammonium oxalate), which only attacks a fraction of the total limestone. The extracted calcium was then dosed (Baize 1988). The analytical methods used were those set by Aubert in its manual soil testing (1978).

Data analysis

Plant and soil data were assessed through correspondence factor analysis (CFA), a suitable approach to phytoecological studies, since it allows to treat jointly floristic variables and soil variables (Djebaili 1984; Cherifi et al. 2011; Bouterfas et al. 2013). Moreover, CFA was used to bring out the floristic cortege of *A. baetica*, as well as the characteristics of the substrate on which it evolved. In addition to the CFA, we used the hierarchical ascending classification (HAC) to better individualize the limits between the different groups (Benzécri 1984; Cherifi et al. 2014, 2017). These two techniques indicated the degree of similarity (homogeneity) or disparity (diversity) of the species composition in the different investigated stations (Pearson 1982).

RESULTS AND DISCUSSION

Vegetation analysis

Table 1 summarizes the floristic reviews conducted at the sampling stations. The floristic inventory allowed us to identify 68 species making part of floristic cortege of *A. baetica*. The floristic composition presented in Table 1 revealed a heterogeneous diversity between 17 species (St2 and St6) and 34 species (St5). This heterogeneity reflects the influence of different environmental parameters and even anthropogenic action on the vegetation distribution. The assessment of the frequency of occurrence of the inventoried species on all floristic surveys revealed the presence of very rare species (52.94%), rare (26.47%), common (8.82%), abundant (7.35%), and constants species (4.42%) as illustrated in Figure 2.

Among the common species which are associated with *A. baetica* in most surveys, we have *Calycotome spinosa*, *Chamaerops humilis*, *Urginea maritima*, *Asparagus acutifolius*, and *Asphodelus microcarpus* that constitutes its floristic cortege.

Families' characterization

The inventoried species belong to 31 botanical families (Figure 3). Asteraceae represent the highest rate in St2 (17.65%); St5 (14.7%); St7 (10%) and St8 (13.79%). Afterward come Lamiaceae in St1 (17.39%); St3 (15.79%); St4 (12.5%), and St6 (16.67%), then Liliaceae in St6 (16.67%), and Poaceae in St1 (13.05%). The other families are poorly represented. Generally, Asteraceae and Lamiaceae represented the most dominant families in the 8 stations, with rates of 15.95% and 13.04% respectively.

Biological spectrum of identified species

Analysis of the biological spectrum of the inventoried species on all stations is represented on Figure 4. Species belonging to the floristic cortege of *A. baetica* were represented mainly by therophytes and hemicryptophytes. The therophytes were represented essentially at St7 with a rate of 35%. The hemicryptophytes dominated in St3 with a maximum of 31.58%. Then come the chamaephytes with a maximum rate of 26.08% in St1. In the fourth position come the geophytes with a maximum rate of 22.22% in St6. The phanerophytes and nanophanérophytes were

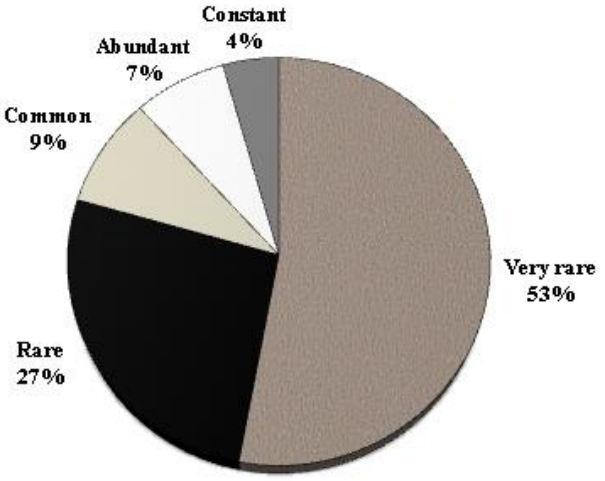


Figure 2. Distribution of species inventoried according to their frequency of occurrence

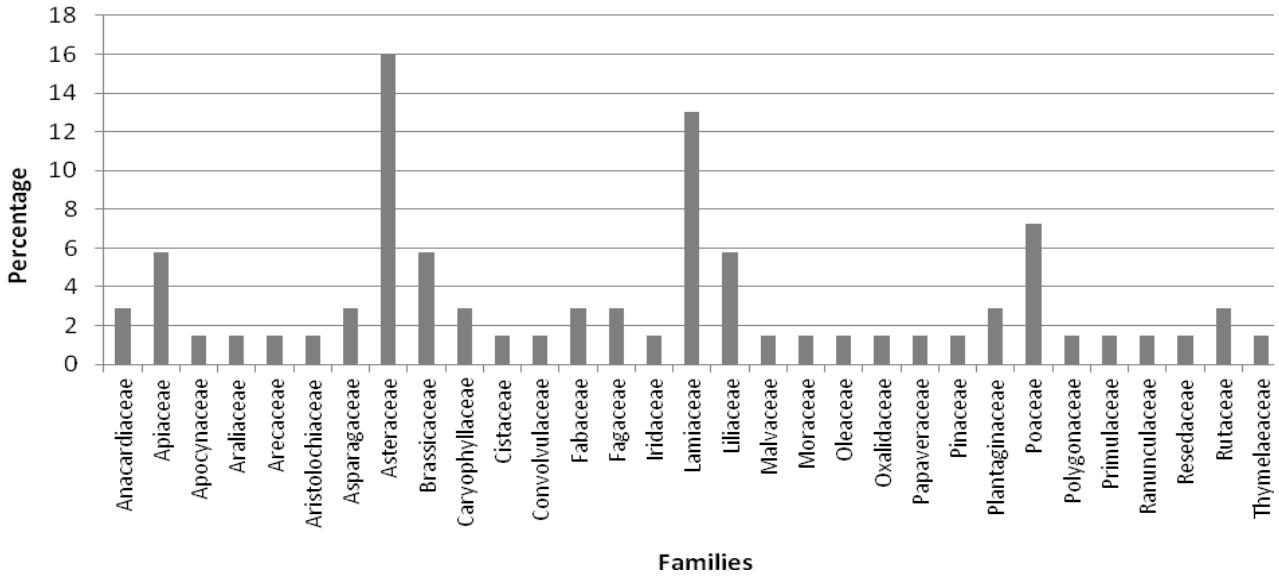
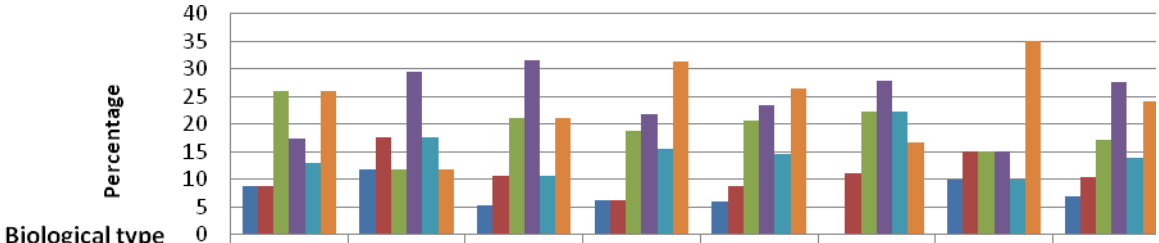


Figure 3. Distribution of the percentage of botanical families



Biological type	Station 1	Station 2	Station 3	Station 4	Station 5	Station 6	Station 7	Station 8
Phanerophytes	8,7	11,76	5,26	6,25	5,88	0	10	6,9
Nanophanerophytes	8,7	17,65	10,53	6,25	8,82	11,11	15	10,34
Chaméphytes	26,08	11,76	21,05	18,75	20,59	22,22	15	17,24
Hemicryptophytes	17,4	29,42	31,58	21,87	23,53	27,78	15	27,59
Geophytes	13,04	17,65	10,53	15,63	14,71	22,22	10	13,79
Therophytes	26,08	11,76	21,05	31,25	26,47	16,67	35	24,14

Figure 4. Biological spectrum of species inventoried

poorly represented. The analysis of the biological spectrum of all the inventoried species on the 8 stations indicated the predominance of therophytes (33.34%) and the hemicryptophytes (24.63%). The therophytisation is a result of unfavorable weather conditions, particularly drought and anthropogenic pressure (Benaradj et al., 2015; Mostefai et al. 2015, 216; Saidi et al. 2016; Chalane et al. 2017; Cherif et al. 2017), and edaphic conditions. The abundance of hemicryptophytes is explained by the richness of organic matter and altitude which is the case in our study area (Cherifi et al. 2011, 2014, 2017).

Biogeographical type

Analysis of the biogeographic type showed the dominance of Mediterranean type species which represented almost half of the recorded species (34 species) with a rate of 49.27%, followed by Eurasian type species (7 species with a rate of 10.13%). The other types, despite their low participation, contribute to the diversity and richness of plant genetic potential of the studied area (Figure 5). More than 15 biogeographical types were observed, which indicated a high phytodiversity in the studied area. The dominance of the Mediterranean element was confirmed in Mount of Tessala in some work on the assessment of plant diversity (Cherifi et al. 2011; Bouterfas et al. 2013; Cherifi et al. 2014; Bouzidi et al. 2012; Benchiha et al. 2014) and in several regions of western Algeria as north of Tlemcen (Ghezlaoui et al. 2009; Belhacini and Bouazza 2012; Hachemi et al. 2012).

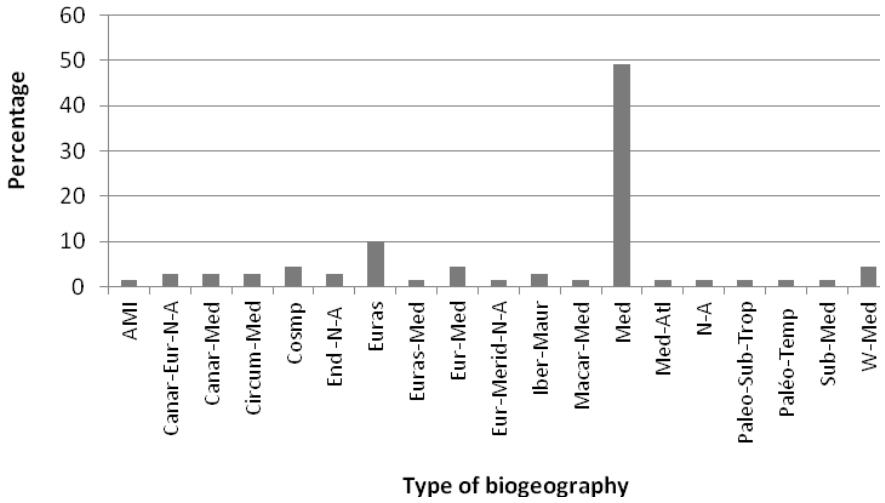


Figure 5. Biogeographical spectrum of species inventoried. AMI: Algéria, Morocco and Iberian-Peninsula; Canar-Eur-N-A: Canarien-Europeen-North-African; Canar-Med: Canarian-Mediterranean; Cosmp: Cosmopolitan; End-N-A: Endemic North African; End-Alg-Tun: Endemic Algeria-Tunisia; Euras: Eurasian; Euras-Med: Eurasian-Mediterranean; Eur-Merid-N-A: Southern Europe-North African; Iber-Maur: Ibero-Mauretanien; Macar-Med: Macaronésien-Mediterranean; Med: Mediterranean; Med-Atl: Atlantic Mediterranean; N-A: North African; Paleo-Sub-Trop: Paléo-Sub-Tropical; Paléo-Temp: paleo-Temperate; Sub-Med: sub-mediterranean; W-Med: West Mediterranean.

Table 2. Soil analysis

Stations	St1	St2	St3	St4	St5	St6	St7	St8
pH	7.37	7.48	7.74	7.87	7.38	7.96	7.72	7.65
OM (%)	4.20	6.20	7.44	7.54	4.40	0.50	6.35	6.65
Sand (%)	40	45	45	40	50	38	43	40
Limon (%)	40	20	30	35	27	30	37	35
Clay (%)	20	35	25	25	23	32	20	25
Al (%)	3.00	1.88	2.38	5.38	2.00	3.75	5.50	2.87
Tl (%)	20.00	17.62	6.00	18.80	5.20	12.00	34.80	6.40
Co (ms/cm)	0.15	0.08	0.09	0.13	0.09	0.14	0.13	0.07

Note: OM: organic matter; Al: Active limestone; Tl: Total limestone; Co: conductivity

Soil analysis

The physicochemical analysis results of soil samples taken from each experimental site are summarized in table 2. We observed that *A. baetica* grew in different textures (silty texture: St1, St4, St7, and St8; sandy clay loam texture: St3 and St5; clay loam texture: St6 and sandy clay texture: St2). The pH, in the majority of samples, varied between 7.96 and 7.37. However, the electrical conductivity was low and did not exceed 0.6 ms/cm, which indicated that our soil was slightly alkaline and unsalted. These two parameters depend on the nature of the vegetation and climatic conditions (Dajoz 1982). Furthermore, pH is related to the quantity of calcium present in the soil and depends on the clay-humus complex too (Arshad & Cohen 1992). The rate of soluble salts in the soil depends on depth, texture, evapotranspiration, and profile moisture (Bendaanoun 1981; Bouterfas 2015). The pH value determines the physical behavior of the soil (structural stability, resistance to crusting, etc.), its chemical behavior (Operating CEC, availability of phosphorus, bioavailability of trace elements and microelements, etc.) and its biological behavior (humification and mineralization of organic matter).

The total limestone rate varied between 5.20% and 34.80% and the active between 1.88% and 2.87%. The presence of limestone gives soil the specific characteristics in terms of physical and chemical behavior, and even affect its biological activity. The calcium content is related to the nature of the rock and explains the scrubland of installation in our study area resulting from the degradation of forest formations (Benabdeli 1983).

The soil, in almost all stations, was rich in organic matter. This organic matter plays an important role in physical, chemical, and biological functioning of soil by improving the cohesion of the structural elements, promoting useful water retention, participating in the reversible storage of nutritional elements, and increasing soil aeration. It should be noticed that the quantity of the organic matter varies according to the diversity and species richness of vegetation cover, climatic conditions, and the nature of the substrate (Duchaufour 2001).

Aristolochia baetica can grow on any soil at an altitude which can range from 0 to 1800 m, in well-watered areas or semi-arid (Maire 1961). This species, observed in woodlands, bushes or rocks *(*Maire 1961), can be encountered in Africa as in Iberia, and in the southwestern tip of Europe. All these characteristics are fairly extensive and represent a broad spectrum.

According to our results, it appears that *A. baetica* grows on light soils, essentially silty-sandy texture, with a high limestone content that allows us to classify the species as calcicole, rich in organic matter, and slightly alkaline pH.

Relationship between the floristics characteristics and soil parameters

The CFA, performed on all the floristic characteristics and soil parameters of the sampling stations, is shown in Figure 6. In this analysis, plane F1 / F2, that has been retained, provides the most information on the correlations between the studied species, floristic composition, and soil parameters on which it develops. Axis F1 providing the most important statistical information in the CFA, with 30.65% of inertia ratio, shows the existence of three groups: (i) Group 1 (Gr 1): represented by St1, St2, St3, St4, and St6 which dominated *A. baetica* (Ar ba) with its floristic cortege consisting essentially of: *Asparagus acutifolius* (As ac), *Asparagus albus* (As al), *Asphodelus microcarpus* (As mi), *Ballota hirsuta* (Ba hi), *Calycotome spinosa* (Ca sp), C*hamaeros humilis* L. (Ch hu), *Daphne gnidium* (Da gn), *Urginea maritima* (Ur ma). This species group is linked to a type of limestone substrate (Lt). The raised stations are represented by scrubland, in the south, with a highly calcareous soil, which promotes the installation of these stands reflecting a deterioration whose origin is the anthropogenic effect exerted by man and his flock on the one hand and the climate hostility on the other hand (Cherifi et al. 2011, 2014); (ii) Group 2 (Gr 2): represented by the matorals based on a *Quercus ilex* in St5 and St8 dominated with the following species: *Ampelodesmos mauritanicus* (Am ma), *Anagallis collina* (An co), *Olea europea* (Ol eu), *Salvia officinalis* (Sa of), correlated with the altitude factor (Alt), and a substrate rich in organic matter (OM), reflecting a high density of these plant formations; (iii) Group 3 (Gr 3): represented by a forest (St7) based on a *Pinus halepensis* (Pi ha) with domination of the following species: *Ammi visnaga* (Am vi), *Papaver rhoeas* (Pa rh), *Plantago albicans* (Pl al), *Rosmarinus officinalis* (Ro of), correlated with the altitude factor and a substrate rich in organic matter to slightly alkaline pH, and a high calcium levels with a steep slope.

HAC confirmed the three groups of stations identified by CFA (Figure 7). The HAC resulted in the categorization of the sampled stations into three main groups. This classification shows a great heterogeneity in the structuring of our study site, reflecting its diversity, which is essentially due to the different ecological conditions: soil, vegetation type, and stationary parameters.

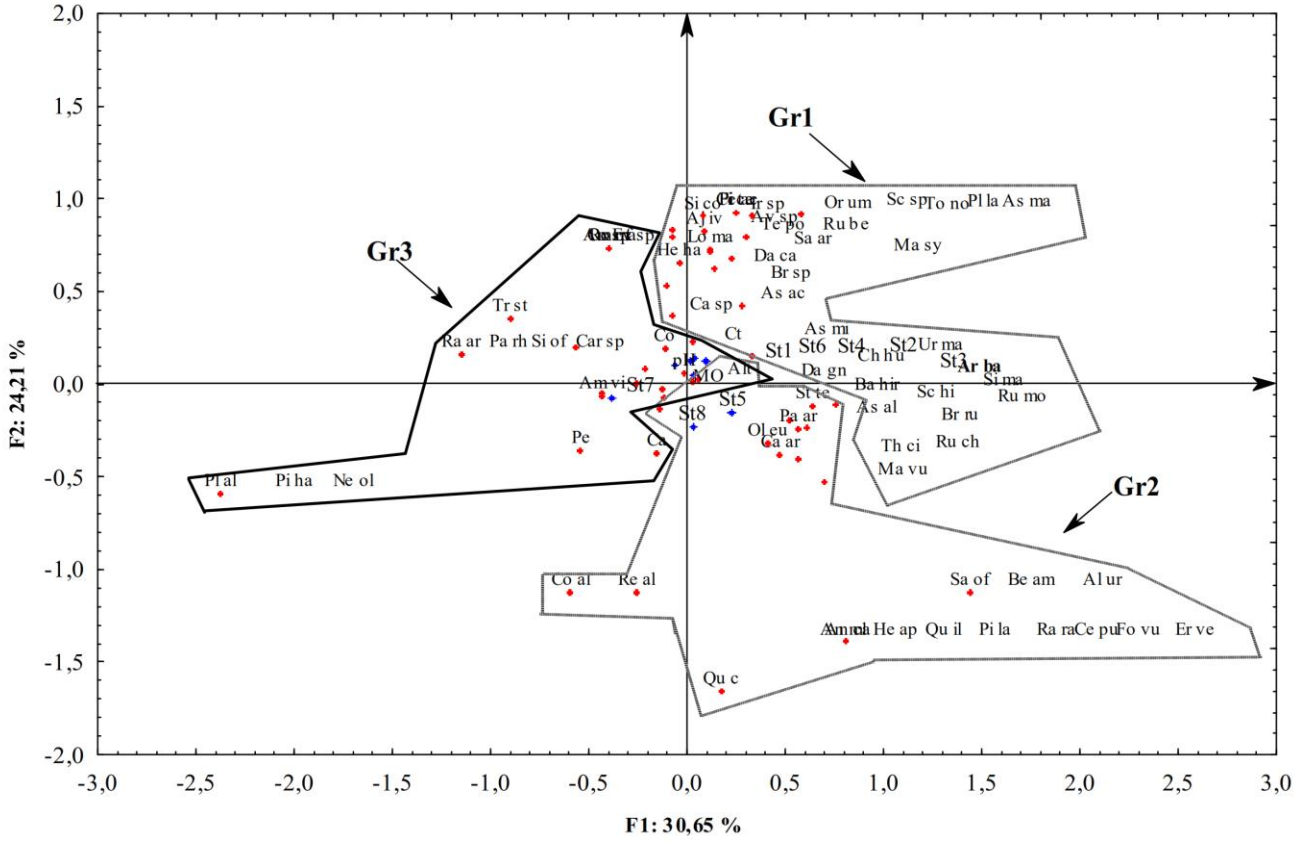


Figure 6. Representation of the correspondence factor analysis

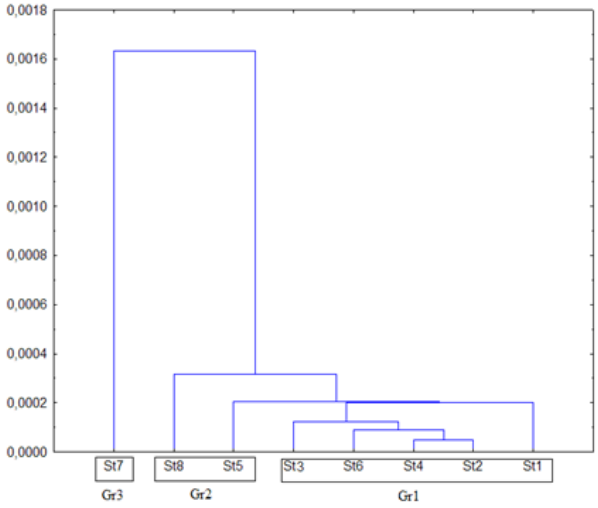


Figure 7. Representation of the hierarchical ascending classification

In conclusion, our findings permitted to characterize *A. baetica* in Tessala Mount (western Algeria) taking into account the physical and chemical factors of the soil at the level of the studied sites and it's floristic. The floristic inventory was represented by 68 species distributed in 31 families with dominance of Asteraceae and Lamiaceae, 6 biological types with dominance of therophytes, and 19 biogeographical types with dominance of Mediterranean element. The floristic data analysis and physicochemical parameters of soil performed through CFA and HAC, revealed that *A. baetica* is associated essentially with the degraded scrubland species, as: *Asphodelus microcarpus*, *Calycotome spinosa*, *Chamaerops humilis*, and *Urginea maritima*. Regarding edaphic parameters, *A. baetica* grows on calcareous soils, with essentially silty-sandy texture, unsalted with slightly alkaline pH. Some limitations of the study should be noticed. Further investigations are recommended by increasing the number of study stations. For that purpose, we plan for additional sampling in space and time to set other environmental parameters governing the habitat of *A. baetica*.

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Phenotypic variation and genetic alteration of *Spathoglottis plicata* resulted from in vitro cultured seed irradiated with X-Ray

SUYITNO ALOYSIUS[1,♥], AZIZ PURWANTORO[2], KUMALA DEWI[3], ENDANG SEMIARTI[3]

[1]Department of Biology Education, Faculty of Mathematics and Natural Science, Universitas Negeri Yogyakarta, Karangmalang, Yogyakarta.
[2]Faculty of Agriculture, Universitas Gadjah Mada, Jl. Flora Bulaksumur, Yogyakarta 55281, Indonesia. Phone/Fax: +62- (274) 551228.
[3]Graduate Program in Biology, Faculty of Biology, Universitas Gadjah Mada. Jl. Teknika Selatan, Sekip Utara, Yogyakarta 55281, Indonesia, Tel./fax.: +62-274-580839. ♥email: suyitno_al@uny.ac.id

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Abstract. *Aloysius S, Purwantoro A, Dewi K, Semiarti E.. 2018. Phenotypic variation and genetic alteration of* Spathoglottis plicata *resulted from in vitro cultured seed irradiated with X-Ray. Biodiversitas 19: 1642-1648.* A terrestrial orchid species among genus Spathoglottis as widely cultivated is S. plicata. Variability development of the species through mutation induction has been carried out, but its morphological variations and genetic changes have not been investigated. The purpose of this study is to identify the phenotypic variation and genetic alteration of S. plicata resulted from in vitro cultured seed irradiated with X-ray. Radiation was given at the doses of 0; 6; 12; 18 and 24 rad. The samples were surviving plants resulted from irradiated seeds. Phenotypic variations observed were the number, length and width of the leaf, number of tiller, and flower characteristics. Genetic alteration was detected from DNA homologous POH1, a key gene determining of shoot morphogenesis. Results show that there are variations of leaf color, length and width of the leaf, and the number of the tiller. Plants start to flower at the age of 30 months. The plants flowering reach 64.7% (WT), 50.0% (6 rad), 33.3% (12 rad), 33.3% (18 rad), and 40% (24 rad). Flower color is ranged from white, white slightly purple, purplish white, light purple, reddish purple and purple, found both in mutants and wild-type groups. The alignment result of POH1 homologous DNA obtained from PCR cDNA shows the nucleotide differences at some points between mutants and wild-type that indicate the occurrence of DNA alteration. X-ray induces the changes of POH1 homologous DNA, but it has no obvious relationship to the flower variation.

Keywords: Genetic, phenotypic, POH1 homologous gene, *Spathoglottis plicata*, X-ray

INTRODUCTION

The genus *Spathoglottis* (Orchidaceae) is still unknown status based on the IUCN Red List, but some experts suggest that some species within the genus are vulnerable (Dockrill 1992) or endangered (Murthy et al. 2012). A terrestrial orchid species among *Spathoglottis* as widely cultivated is *S. plicata.* The development of the species through mutation induction was important since the morphological diversity of *S. plicata* was still low (Romeida 2012). The conventional way of cross-breeding produces only limited variation. Mutation induction by radiation with random effects could potentially produce greater variations.

A technique of mutation induction by ionizing rays (X-rays and gamma-rays) was the easiest, useful, and secured way, and it has been widely applied in horticultural crops (Piri et al. 2011), such as *Hordeum* (barley) and *Triticum* (wheat). There were 3278 mutant varieties registered, 561 varieties resulted from X-ray induction, and only one mutant variety in orchids produced but it by another mutagent, those were *Cymbidium* (FAO/IAEA 2018). Seed is the most sensitive stage thus it is appropriately used as target irradiation to obtain new characters (Redei 1992). X-rays irradiation was proven capable of inducing the changes in the structure and color of *Zinnia elegans* flowers (Gultom and Gultom 2015) and many varieties of horticultural crops (van Harten 1998). X-ray irradiation is also expected to be capable of triggering phenotypic variations and its related genes on *S. plicata*. Based on the mutant variety database (FAO/IAEA 2018), there is no mutant variety of orchids produced by X-ray induction. In addition, studies related to the effects of X-ray irradiation on orchid morphogenesis genes have not been reported.

X-rays irradiation has a great ability to penetrate tissues and causes various changes or damages in cells. Ionizing irradiation (X-rays and gamma) triggers excitation and ionization in the material or living cells and causes rupture of chemical bonds in biological systems (Jan et al. 2012). The level of damage by ionizing rays was affected by the type or quality of light, irradiation dose and genotypic factor or sensitivity level of the organism (Hameed et al. 2008; Al-Enezi and Al-Khoyri 2012). The sensitivity of the organism was affected by age, type, genotype, level of activity or physiological conditions and complexity of the tissues (De Micco et al. 2010). Mutations have no necessarily effect directly to the morphological changes in the first generation plants (M1) (van Harten 1998). Mutations may start to appear in one or several offspring generations later on. Mutations might lead to the changes in prominent morphological characters, vague or do not affect morphological changes at all. Based on leaf lobe formation in mutants, mutations are distinguished into mild mutation, moderate mutation and severe mutation (Howell 1998).

The plants might be significantly affected (susceptible), moderately affected or tolerant (unsusceptible) by ionizing radiation. Most of *S. plicata* resulted from seed irradiation have morphological characters similar to wild-type plants (wildtype-like = WT-L). To detect the presence of mutations in a plant that was similar to its wild-type, DNA or RNA analysis was required.

Molecular characterization has been based on gene structure identification, genetic material at the genome (DNA) level, transcription (RNA) or its protein results (de Micco 2010). The POH1 or its homologous genes were members of Knox gene clusters (knotted-like orchid homeobox) which was a key gene in the shoot morphogenesis. From the results of PCR-RAPD, *S. plicata* seedlings experiencing prominent morphological changes show an increase in DNA polymorphism (Aloysius et al. 2017). Whether the orchids *S. plicata* surviving in experimental gardens also undergo genetic changes especially in homologous gene POH1, is an interesting thing to be studied. based on this reason, the purposes of this study were to identify phenotypic variation of *S. plicata* resulted from in vitro cultured seed irradiated by x-rays, and to detect genetic changes in homologous gene POH1 of mutants *S. plicata*.

MATERIALS AND METHODS

Materials

The material used in this study was *S. plicata* orchid plants resulted from acclimatization of plants produced by in vitro cultured seeds irradiated with X-rays. The number of plant samples were given as follows: 18 plants (0 rad); 8 plants (6 rad); 7 plants (12 rad); 6 plants (18 rad), and 10 plants (24 rad).

Orchid planting in the experimental garden

Cultured plants at the age of 8-10 months were acclimatized in stages given as follows: seedlings or plantlets were removed from the bottle, cleanly washed, and then soaked in fungicide (1g /L) for about 5-10 minutes, and then removed to organic fertilizer liquid (2-3 mL/L) for 5-10 minutes. The plants were then put into bottles that have been given cotton moistened with the same fertilizer solution. Furthermore, the bottles were closed using plastic lids with holes to keep connecting with the outside air and finally left them for 1-2 days. The plantlets were then transferred into plastic pots filled with moss which has been soaked in organic fertilizer solution (2-3 mL/L) for 5-10 minutes. The pots were then placed into a plastic lid (1 x 2 m^2) in the experimental garden with 50-75% light penetration. Plants were maintained by spraying water in twice a day. Plants were also sprayed with foliar fertilizer (1-2 mL/L) once in 3 weeks. The surviving plants were then transferred to the soil-manure medium (2 : 1) in plastic pots (8 x10 cm^2). Plants were then moved into a larger plastic pot (12 x 9 cm^2) with the same planting medium. Finally, at the age of 32 months, the plants were moved into a larger pot (23 x 20 cm^2).

Data collecting and processing

Morphological data

Morphological observation of plants includes plant height, number and length of leaf, leaf color, leaf sheet, and clump size (number of tillers). Flower morphology observation includes color and flower diameter, flowering number, length of the flower stalk, and the age of first flowering.

Analysis of POH1 homologous DNA transcript

Detection of genetic changes was focused on the analysis of POH1 homologous DNA transcript of wild-type plants (WT) and irradiated plants. PCR with Reverse Transcript (RT-PCR) was used for the POH1 RNA analysis (gene expression) of WT and mutants resulted from irradiated seeds. Total RNA was extracted and isolated from 0.1 g pieces of shoot bulb (rods) using RNA mini kit (Plant) GeneAid and conducted according to the procedures from the company. Total RNA (1 ug) was used to synthesize cDNA using Transcriptor One-Step RT-PCR kit. Synthesis of cDNA was conducted with PCR via Touch Down using the following condition stages. Phase I: 50^o C for 30′ (reverse transcription), 94° C for 7' (initial denaturation), 94° C for 10" (denaturation), 50° for C 30" (annealing), 68^o C for 1' (elongation), with 9 time cycles. Phase II: 94° C for 10" (denaturation), 55° C for 30" (annealing), 68^o C for 1' (elongation), with 11 time cycles. Phase III: 94° C for 10" (denaturation), 50° C for 30" (annealing), 68^o C for 1' (elongation), with 11 time cycles. Finally, 68^o C for 7' (final elongation) and 12^o C for 7' (hold). DNA complement (cDNA) was used for POH1 homologous DNA amplification.

Amplification of POH1 homologous cDNA uses master mix GoTaqGreen (GTG) (Promega), with POH1 homolog _F1 primer (5'-TAC TTC TAA CAA ATG GTG GGA-3') and' POH1 homolog'_R1 (5'-AAT GCG ATA AGA TAT TGT AGT -3'). The composition of the PCR mix for final volume of 40 µL was 20 µL GTG, 2 µL POH1 homolog_F1 primer and 2 µL POH1 homolog_R1 (10 pg/µL), 1.5 µL DNA template, and 14.5 µL nuclease-free water. PCR was performed in the following stages: pre-denaturation (95^o C for 2'), 40 time cycles with stages: denaturation (95° C for 30"), annealing (57^o C for 30"), elongation (72° C for 1'.30").Then, the extension phase (72° C for 5') and hold (12^o C for 5'). DNA amplicons were checked using electrophoresis on 1% agarose gel which was given 2 ul DNA staining of Florosafe brand, run on 100 V for 55 minutes. DNA ladder of Vivantis brand which provides DNA fragment sizes of 100 - 3000 bp was used as DNA markers. The electrophoresis results were observed under UV transilluminator and photographed.

Moreover, DNA amplicon was sequenced. The alignment was conducted using MEGA7 program. The alignment results were then utilized to identify the differences in nucleotide composition (nucleotide polymorphism) between mutant and WT.

Data analysis

Qualitative data of plant morphology were analyzed using qualitative descriptive analysis. Quantitative data

related to morphological parameters were analyzed using one-way analysis of variance followed by Duncan's multiple range tests with a significance level of 95%. Results of POH1 homologous DNA transcript alignment were identified to find the genetic changes (deletion, insertion or substitution of nucleotide bases).

RESULTS AND DISCUSSION

Identification of morphological variation

Spathoglottis plicata which were successfully acclimatized and survived have no prominent morphological character differences. Plants have enough strong habitus and fresh green linear (lanceolate) leaves. In general, the appearance of the plant habitus (Figure 1) was similar to WT-L plants.

Variations also appear on the characteristics of leaf size, the number of tillers, flower stem length, inflorescence number, and size of flower (Table 1), leaf color, lamina structure and number of tillers or cluster size (Table 2). These results show different influence or response of the plants. As stated by Borzoeui et al. (2010), Jan et al. (2012), and Minisi et al. (2013) that irradiation produces radiation stress as a result of free radicals (ROS) production which was highly toxic and mutagenic and also affect differently towards the morphology, anatomy, biochemistry, and physiology of plants depending on the dose given.

There were variations in the morphological traits, but not specific to a group of irradiated plants and also found in the WT plants group. Plants with yellow-green variegate leaf chimera were found in the WT group (5.5%), 18 rad irradiated plants (16.7%), and 24 rad irradiated plants (10%) and most of the other leaves were green or dark green. Variegated yellow-green leaf chimera was also found in *S. plicata* plants as a result of gamma irradiation (doses of 30-40 Gy) on the plantlets (Romeida 2012). Variations in leaf color were determined by the content or leaf pigment production, especially chlorophyll and xanthophyll. Mueller et al. (2012) stated that the xanthan or chlorine leaf mutants of barley (*Hordeum vulgare*) occur because of the decrease in the production of chlorophyll-a and chlorophyll-b.

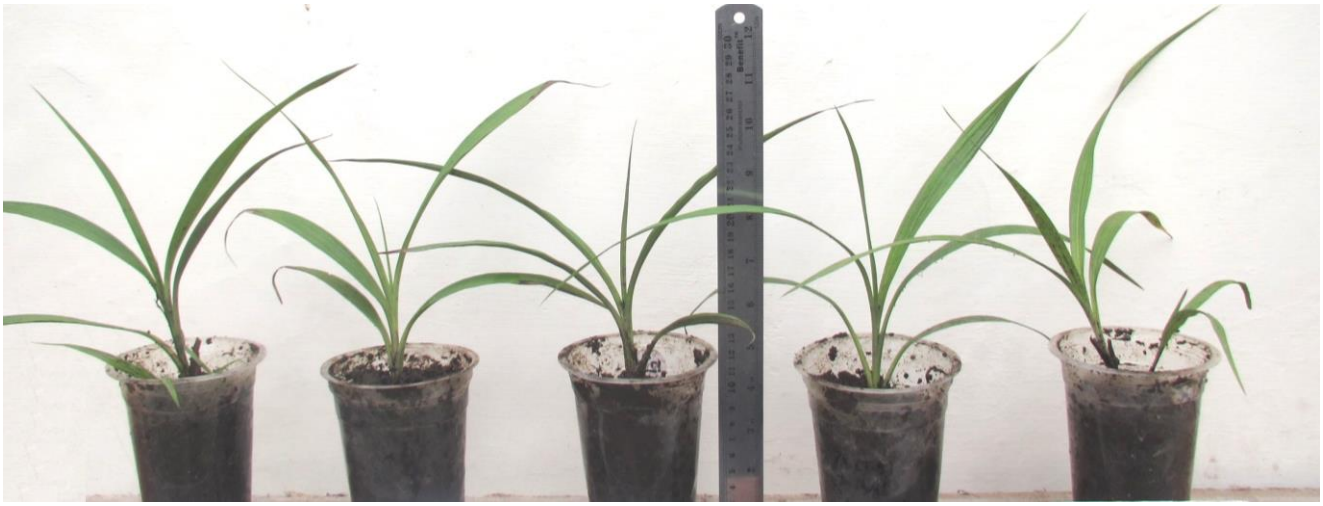

Figure 1. Samples of *Spathoglottis plicata* orchid at the age of 24 months resulted from in vitro culture of irradiated seeds at the doses of 0 (WT), 6, 12, 18, and 24 rad

Table 1. Morphological variation of *S. plicata* orchid resulted from seeds irradiation with X-rays

Doses (rad)	Length of flower stalk*	Flower size (l x w)*	Number of flower bud*	Number of inflorescence ns	Age at the first flowering (months)*	Number of tillers ns	Leaf length (cm)*	Leaf width (cm)ns
0	44.39±7.3b	20.58±2.9 ab	21.8±5.4ab	2.3±0.7 a	33.9±3.9ab	11.5±4.8a	73.3±7.5 a	3.5±0.7a
6	29.44±6.9a	16.35±3.4 a	18.7±10.2ab	3.7±3.6 a	41.5±6.5 b	9.0±3.1 a	58.9±8.4 a	3.6±1.1a
12	37.5±3.4bc	18.5 ±6.1a	15.2±6.6 a	3.3±0.9 a	39.7±1.7ab	13.3±2.0a	68.1±8.3 a	4.6±1.1a
18	49.9±10.9c	24.4±3.0b	17.0±3.3a	2.7±1.7a	46.0±5.1 b	12.0±3.7a	88.3±5.9 b	4.4±0.9a
24	45.7±8.4bc	20.8±3.1ab	28.1±2.3b	3.5±1.5a	31.2±7.3 a	7.5±5.0a	69.4±8.1 a	4.4±1.0a

NB. The same letter beside the average value of each variable shows no significant difference ($p > 0.05$) from the analysis of Duncan's Multiple Range Test (DMRT). * : significantly different

Table 2. Percentage of each morphological character of Spathoglottis plicata resulted from irradiated seeds

Morphological characteristic			Irradiated groups					Average
			0 rad	6 rad	12 rad	18 rad	24 rad	
Leaf color		Yellow-green variegata	5.5	0.0	0.0	16.7	10.0	2.19
		Green	94.5	100	100	83.3	90.0	97.81
Lamina		Open	88.9	100	100	83.3	100	95.92
		Roll/ twist	11.1	0.0	0.0	16.7	0.0	4.06
Clump: many (> 11); few (< 10)		Many tillers	77.78	57.14	100	83.3	60.0	75.51
		Few tillers	22.22	42.86	0.0	16.7	40.0	24.49

Moreover, variations in the structure of lamina (longitudinal roll or open), as well as the size of clusters (many or few tillers), were also found. Morphology variations which were not specified in the treatment groups show that these variations were not a result of irradiation. Yellow striped leaves on some plants may be a chimera. According to Leyser and Day (2003), plants can form two or more tissues that were genetically distinct and display different colors called chimera. For many plants, including orchids group, the difference in genome number between tissues often occurs because of endopolyploid (endoreduplication) events, but morphology differences did not accompany it. In *S. plicata* endopolyploid was found in various organs, except sepal, petal, and ovary (Yang and Loh 2004).

The variations concerning inflorescence and floral, no specific traits in plants irradiated groups were revealed (Table 1). Characteristic variations were found in stem length, flower diameter, number of buds produced, inflorescence number, and age of first flowering. This result indicates that the morphology variations of flowers occurring were also not associated with the given irradiation doses.

Prominent morphological variations were found in the color of flower, but this result was also not specific traits in irradiated plants groups. Flowers were generated in several levels of gradation (Table 3), including : (i) white on the entire piece of flowers, (ii) white with labellum part white purplish, (iii) purplish white, (iv) pink or light purple, (v) reddish purple, and (vi) dark purple. In general, the most emerging flower color was purple (48%), and the fewest was the white flower (7.4%). The same variations also occur in flower's labellum, both of the WT group and irradiated plants.

A similar study with gamma irradiation (20-300 Gy) was conducted by Romeida (2012) on *S. plicata* of Bengkulu accession. The study produces normal *S. plicata* plants with different flower colors from their parents. This study found that there were six color gradations of flowers and the phenotypic variation of flower colors might be formed not only as a result of X-ray irradiation but also because of free segregation during the formation of gametes. The discovery of six flower color gradations in WT plants indicates that multiple genes determined the inheritance of flower color in *S. plicata*.

Table 3. Number (%) of *S. plicata* orchid flowers resulted from irradiated plants

Flower color		Irradiated groups					Average (%)
		0 rad	6 rad	12 rad	18 rad	24 rad	
White		1 (7.69)	0	1 (33.3)	0	0	(7.40)
White, side lobe a bit of purple		1 (7.69)	1 (25.0)	0	0	1 (25.0)	(11.11)
Purplish white		3 (23.07)	0	0	0	0	(11,11)
Light purple		1 (7.69)	0	2 (66.7)	0	0	(11.11)
Red-purple		2 (15.38)	0	0	1 (33.3)	0	(11,11)
Purple		5 (38.46)	3 (75.0)	0	2 (66.7)	3 (75.0)	(48.1)
Total of flowered plant (%)		**13** (48.1)	**4** (14.81)	**3** (11.11)	**3** (11.11)	**4** (14.81)	(100)

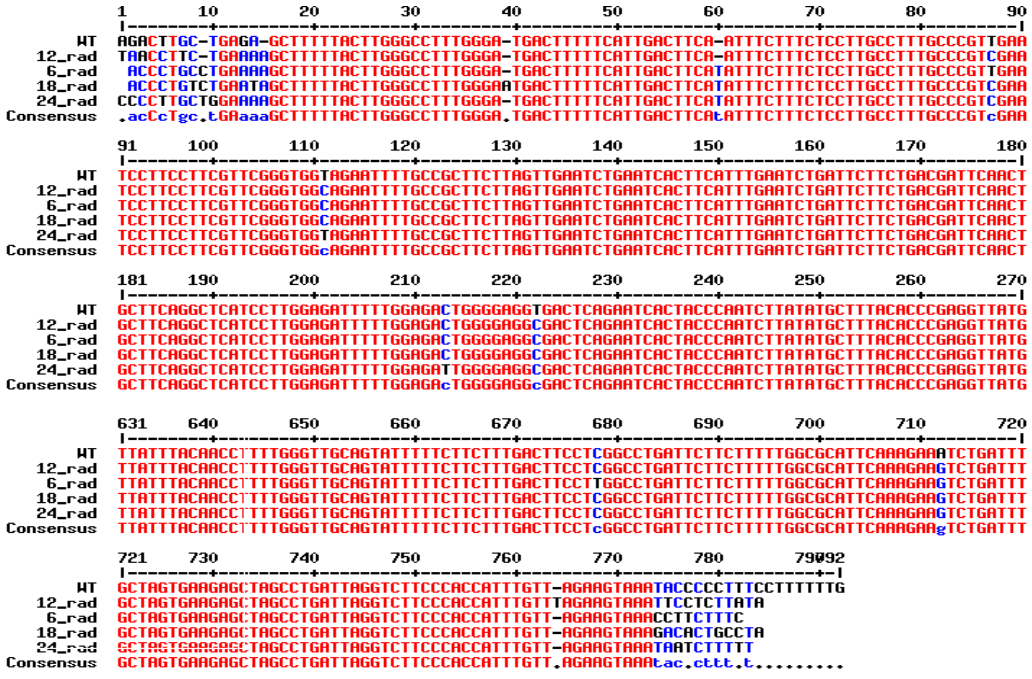

Figure 4. Alignment result of POH1 homologous DNA sequence of WT and irradiated plants

Table 4. Nucleotide changes in complementary DNA (cDNA) of homologous POH1 in irradiated plants.

Doses (rad)	Position of single nucleotide															
	5	**7**	**9**	**10**	**13**	**14**	**15**	**39**	**60**	**87**	**111**	**213**	**222**	**678**	**712**	**764**
WT	T	G	-	T	G	A	-	-	-	T	T	C	T	C	A	-
6	C	G	C	T	A	A	A	-	T	T	C	C	C	T	G	-
12	C	T	-	T	A	A	A	-	-	C	C	C	C	C	G	T
18	C	G	C	T	A	T	A	A	T	C	C	C	C	C	G	-
24	T	G	T	G	A	A	A	-	T	C	T	T	C	C	G	-

Flower color was determined by three main pigments including betalain, carotenoid, and anthocyanin (Grotewold 2006) *in* Lee et al. (2008). According to Lee et al. (2008), the primary pigment determining flower colors of orange, red, purple, dark purple, and purplish blue was anthocyanin. Irradiation of seeds with gamma rays also cause variations in structure and flower color on *Zinnia elegans* (Gultom and Gultom 2015) and *Chrysanthemum* (Lee et al. 2008), changes in leaves and roots morphology of *Dendrobium* (Sulistianingsih 2013), changes in flower color on *S plicata* (Romeida 2012) as well as an increased level of anthocyanin on *Moluccella laevis* (Minisi et al. 2013).

Analysis of POH1 homologous DNA Transcript

Results of POH1 homologous cDNA PCR on WT and irradiated plants at all dose levels show the DNA with a size of about 750 bp (Figure 3). This result suggests that X-rays irradiation does not affect the size of POH1 homologous DNA, let alone chromosome aberration. The sequence of POH1 homologous cDNA and its alignment result (Figure 4) indicates the presence of nucleotide changes in several points (gene mutation, point mutation).

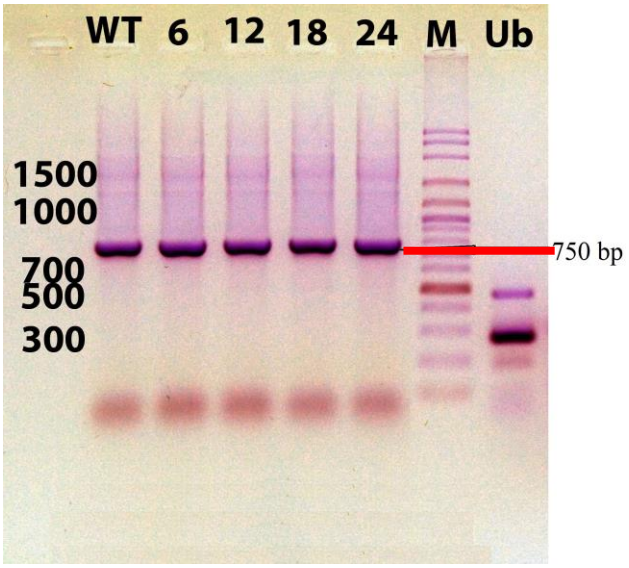

Figure 3. Electropherogram homologous DNA POH1 resulted from PCR cDNA of WT and irradiated plants (6-24 rad) *Spathoglottis plicata*. WT: wild-type, M: DNA Marker, bp: base pair, Ub: Ubiquitin.

Five samples for all level of irradiation dose were morphologically similar, but there were some differences (changes) in the structure of POH1 homologous DNA nucleotides. In POH1 homologous cDNA of irradiated plants, nucleotide substitutions occur in nine points and insertions happen in four points (Table 4). This result suggests that the nucleotide changes are not associated with morphological variations. Nucleotide changes allegedly occur on codon position that has no effect to the amino acid changes or protein produced. According to van Harten (1998), the mutation of nucleotides does not affect gene expression, depending on the changes of codon. Nucleotide changes on the 3rd base of codon do not affect to the changes in amino acid or protein produced.

Results of *S. plicata* from in vitro cultured seeds which were irradiated with X-rays shows phenotypic variations in color, length and structure of leaf lamina, number of tillers, and color of flowers. Some plants have yellow striped leaves, curled leaf, tall plants with few tillers and start flowering at various ages from the age of 30 months. Flower petal colors were graded from white, slightly purple white, light purple, reddish purple, and purple. Based on the gradation of flower color of *S. plicata* was allegedly determined by multiple genes. Based on sequence and alignment results of POH1 homologous DNA transcript, changes in nucleotide composition are found in the form of substitutions and insertions in some points (point mutation) although do not affect clearly on phenotypic changes of *S. plicata*.

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Diversity and abundance of termites along altitudinal gradient and slopes in Mount Slamet, Central Java, Indonesia

HERY PRATIKNYO[1,♥], INTAN AHMAD[2], BAMBANG HERU BUDIANTO[1]

[1]Faculty of Biology, Universitas Jenderal Soedirman. Jl. Dr. Soeparno No. 63, Purwokerto, Banyumas 53122, Central Java, Indonesia.
Tel./fax.: +62-281-638792, ♥email: hery.pratiknyo17@gmail.com

[2]School of Biological Sciences and Technology of Bandung Institute of Technology. Jl. Ganesa No. 10, Bandung 40132, West Java, Indonesia

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Abstract. *Pratiknyo H, Ahmad I, Bambang Heru Budianto BH. 2018. Diversity and abundance of termites along altitudinal gradient and slopes in Mount Slamet, Central Java, Indonesia. Biodiversitas 19: 1649-1658.* A study on diversity and abundance of termites along an altitudinal gradient and the different slope was done in plantation forest of Mount Slamet. This research aimed to define the species composition along the altitudinal gradient and slope and to define the environmental factors affecting it. The sampling of termites was carried out following standardized belt transects (100 m x 2 m) laid vertically on the altitude of 700 up to 1300 m asl on four slopes. Each 100 m length of the belt transects was divided into 20 sections (5 m x 2 m), and termites were taken in each section from the trees, branches, barks and the ground. Data of termites composition were analyzed for diversity index (Shanon-Wiener, H') and domination index (Simson, D); the correlation among environmental factors with the family were analyzed by multivariate correlation, and then a Canonical Correspondence Analysis (CCA) was used to define the most associated environmental factor to the diversity and abundance of termites. A total of 7349 individuals belonging to 11 species in nine genera, five subfamilies, and two families were recorded. Four wood feeder species were *Schedorinotermes javanicus, Macrotermes gilvus, Odontotermes javanicus* and *Microtermes insperatus*, while humus feeder was *Capritermes samarangi, Procapritermes stiger*, *Nasutitermes matangensis*, *N. matangensiformis*, *Bulbitermes* spp., and the soil feeders were *PeriCapritermes javanicus and P. dolichocephalus*. Based on the Shanon-Wiener index, the Western Slope was the highest in diversity with five main environmental factors (altitudes, maximal rainfall, N content, manure application and light intensity) the most influencing while the CCA ordination showed that the canopy closure and light intensity were the most associated factor to the diversity and abundance of termites. In conclusion, the slopes caused the species richness differently with the most associated environmental factors was the canopy closure and light intensity.

Keywords: Altitude, plantation, Mount Slamet, slope

INTRODUCTION

Termites are an integral part of tropical rainforest ecosystems (Ackermans at al.2009). Termites activity, such as mound-building, tunneling, and soil-feeding improves soil structure and quality (Lavelle et al. 2007), therefore, information of the species composition of termites be important to understand how the role of termites in their habitat (Jones and Prasetyo (2002).

Species composition, to most organisms, may decrease as air temperature decrease and is correlated to the increase of altitudinal gradient (Willig et al. 2003). Termite species composition and abundance generally decline with the increasing altitudinal gradient (Kayani et al.1979; Gao et al. 1981; Akhtar et al. 1992; Gathorne-Hardy et al. 2001), unlike wild bees pollinator that showed increases in mid-elevation (Widhiono and Sudiana 2016). Air temperature is considered a primary factor of the termite's species composition in the tropical area of South East Asia (Inoue et al. 2006). That species composition pattern declines due to increasing altitudes is monotic (Gathorne-Hardy 2001) as a research result in the Leuser Ecosystem of Aceh Province, Indonesia. To this phenomenon, Gathorne-Hardy et al. (2001) stated that termites species composition and abundance decrease is explained by the reduction of temperature, linked to the metabolic rate of termites as ectothermic fauna, where air temperatures drop by 1°C in every increase of 100 m in altitude. However, Stevens (1992) argued that any other factor caused the termites' species composition declines monotically. The monotonic decrease of termites species richness with altitudinal mirrors is often attributed to productivity and area, besides the reduction in temperature (Colwell and Lees 2000). According to this species composition pattern, Lomolino (2001) stated that there are other patterns of increasing of altitudinal gradient effect on declining of termites species composition, where the species composition initially increased in the lower to mid elevation, then decreased gradually on the higher elevation. This phenomenon is called the mid-domain elevation effect. Another species richness pattern, widely observed to closely associated with increasing altitude, is the mid-elevation peak (or hump-shaped curve) (Lomolino 2001); a pattern often explained by the presence of optimal climatic at mid-elevations, by the ecotone effect and/or by the mid-domain effect. The majority of species richness studies on tropical invertebrates show a mid-elevation peak pattern. This mid-elevation peaks in the termite species composition have been reported (Donovan et al. 2002; Inoue et al. 2006), which have been attributed to factors such as anthropogenic

disturbance and the limited altitudinal range of the study.

Termites were also found to have a smaller elevation range on smaller mountains as compared to the large ones. This is attributed to the Massenerhebung effect, a phenomenon in which cloud formation occurs at lower altitudes on smaller mountains, which in turn is reflecting sunlight and reducing daytime temperatures at lower elevations (Gathorne-Hardy et al. 2001). Mount Slamet is the most prominent mountain and the second highest mountain of 3428 m asl in Java Island (Daldjoeni 1982). Initially, the natural forest of Mount Slamet was a spot of tropical rainforest of Southeast Asia (Withmore 1998) but since colonial Deutch in 1917, the natural forest of Mount Slamet being converted to dammar plantation then in 1943 to pinus plantation (Richard 1979). Such conversion may cause to species composition of termites (Okwakol 2000; Jones et al. 2003; Turner and Foster 2008). Unfortunately, data on Massenerhebung effect in plantation forest of Mount Slamet has not been reported yet.

Although air temperature is often considered the critical factor influencing termite species composition, other non-mutually exclusive factors that may explain changes in termite species composition with altitude include slope, rainfall and the size of the regional species pool. Mount Slamet has four slopes with different microclimate. The slope is an important ecological gradient factor for species diversity and abundance. The western and eastern slope of Mount Slamet may receive higher sunlight radiation and more light intensity than other slopes. The slopes affect the nutrient cycling (Davies et al. 2003) although the distance is only a few hundred meters. The microclimate condition of slopes of Mount Slamet varies with the mountain altitude, where the **s**outhern and western slopes have a higher annual rainfall (4500-6500) mm than the Eastern and Northern slopes (3500-4800) mm (KPH Banyumas Timur 2015). Rainfall can have an adverse effect on termite species richness and abundance in tropical rainforest systems, as very high moisture can lead to inundated microhabitats and colony death (Dibog et al. 1999; Eggleton et al. 1997). Furthermore, termite species composition and abundance are correlated with human activity in farming by manure application which enables the increase of organic content of soil. Since 1987, State Forest Management (Perum Perhutani) Unit II Central Java (Semarang) has been allowing the local farmers to plant cereals under main trees without logging, and the last factor that may affect the species composition of termite is canopy loss (Davies et al. 2003).

As ectothermic fauna, the body temperature of termites depends on environmental temperature even though they respond it differently. The feeding group of soil feeders, for example, is generally more negatively affected. This is likely because soil feeder termites depend on lower energy food substrates than feeding group of wood feeders, providing them with less colony-wide energy to overcome the physiological costs of living at lower temperatures (Jones et al. 2000; Davies et al. 2003).

At the continental, level of termite species composition is anomalous; with South East Asia is the smallest species pool as compared to South America and Africa (Eggleton et al. 1996; Davies et al. 2003). Research on termites richness, relationship and bioindicator in various land use types in Mount Slamet had been done (Arthadi 1982; Pribadi et al. 2011); however, there is no information on species composition and abundance of termites in correlation with slope and altitudinal gradient. This research aimed to define how is the species composition and abundance of termites along an altitudinal gradient with the different slope, and what is the environmental factor affecting it in plantation forest of Mount Slamet, Central Java, Indonesia.

MATERIALS AND METHODS

Study site

The study was conducted in the plantation forest of Mount Slamet, Central Java, Indonesia (Figure 1), located in (S: 07° 18' 26.2"-E: 109° 14'26") up to (S: 07^0 16' 27,3"-E: 109^0 14' 25) and (S: 07° 16' 03.2"-E: 109° 18' 26.7") up to (S: 07^0 16' 32,8''-E: 109^0 04' 57), on the altitude of 700-1300 m above sea level (asl).

Sampling procedure

Termites collection

Termites were sampled followed a standardized transect method (Jones and Eggleton 2000). Plantation Forest of Mount Slamet was divided into four slopes (southern, eastern, northern and western) and each slope was split into six altitudes (700-800, 801-900, 901-1000, 1001-1100, 1101-1200 and 1201-1300) m asl. A belt transect (100 m x 2 m) was laid in each range of altitude vertically, and each of 100 m belt transect was divided into 20 sections (5 m x 2 m). On each section, one man searched termites for one hour on surface soil or humus, trees, roots, branches, and stumps, arboreal nests up to a height of 2 m above ground. Mainly the soldier and worker castes were collected. The collected specimen was stored in 80% alcohol for identification. The number of encountered termites (hits) along a transect was used as an indicator of the relative abundance of species within that plot. An encounter was defined as the presence of a species in one section (5 m x 2m). Thus, the relative abundance of a species per transect maximally was 20 individuals (Inoue et al. 2006; Vaessen, at al 2011)). Termites were divided into four putative feeding groups based on the site of discovery, the color of the abdomen and known dietary requirements of the workers. Feeding group ranged from groups 1 to 4 in which the diet of workers followed an increased humification gradient (Eggleton and Tayashu 2001). Nesting guilds included hypogeal nesters that build the subterranean nest, epigeal nesters that build aboveground mounds, hypogeal nesters that have partially subterranean and partially above ground nests, arboreal nesters that nested on living or dead trees, and wood nesters that nested in dead wood.

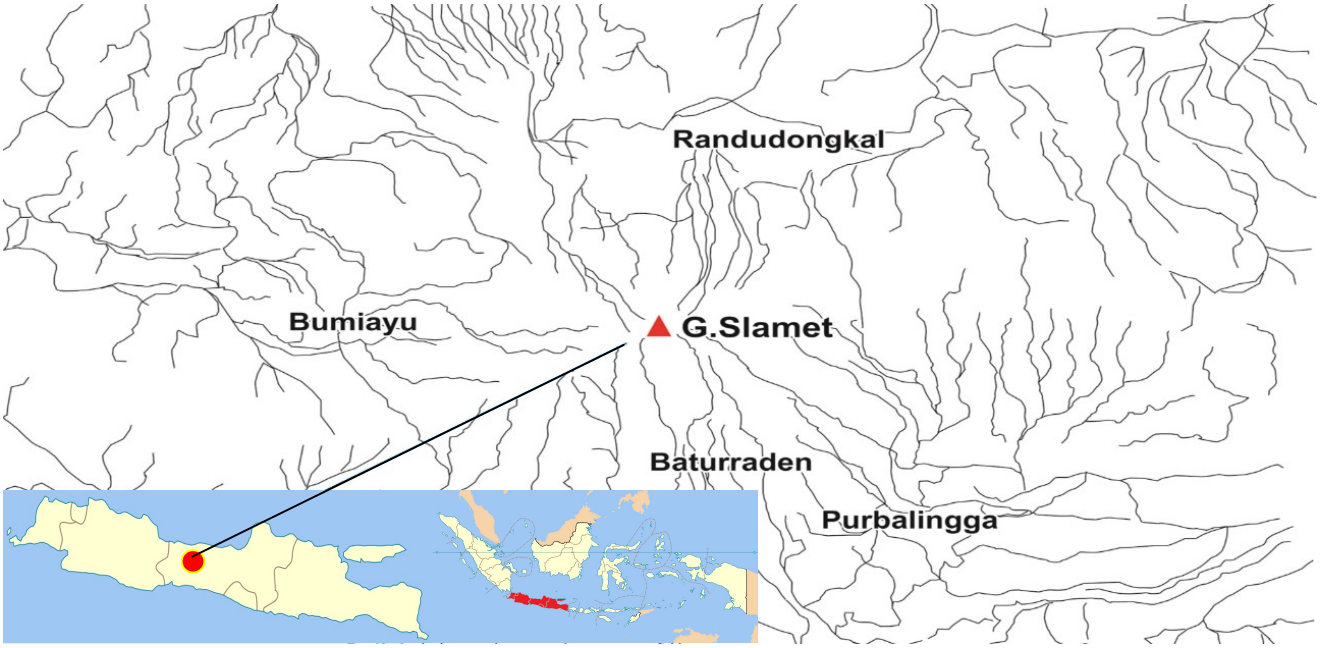

Figure 1. Sampling areas of termites in plantation forest of Mount Slamet, Java Island, Indonesia

Termites identification

The pictures of the specimen were taken under a Nikon Microscopes with 400x enlargement. Termites sampled were determined on basis of species identity, using the information given by Ahmad (1954), Thapa (1981) and relevant references. Recorded termites were placed into a feeding group based on their identity using the classification of Donovan et al. (1999), also certified by Research Center for Biology, Indonesian Institute of Science (LIPI), Bogor, Indonesia.

Data analysis

Species richness of each slope was analyzed for diversity (Shanon-Wiener) index and Domination (Sorensen) index by using PAST software. The differences in species composition and abundance of termites among slope and altitude was analyzed using ANOVA performed using SPP.S 11.0 software, and the correlation of environmental factor with species composition and abundance of each family was subjected to multivariate correlation analysis, and the most associated environmental factors of total termites was ordinated by Canonical Correspondence Analysis (CCA).

RESULTS AND DISCUSSION

Termites diversity

During sampling time we found two families, they were Rhinotermitidae and Termitidae. Familia Rhinotermitidae consisted of one subfamily Schedorhinotermitinae with only one genus, and the species found was *Shedorhinotermes javanicus.* Meanwhile, Family Termitidae comprised of four Subfamily, i.e., Macrotermitinae, Microtermitinae, Termitinae, and Nasutitermitinae. Subfamily Macrotermitinae consisted of two genera, i.e., *Odontotermes* and *Macrotermes*, each has one species (*Odontotermes javanicus* and *Macrotermes javanicus*). Sub-family Microtermitinae has one genus Microtomes with one species (*Microtermes insperatus).* The Termitinae Subfamily has three genera, i.e., *Procapritermes*, *Capritermes*, and *PeriCapritermes*. The Genus *Procapritermes* consisted of one species (*Procapritermes stiger*), Genus *Capritermes* of one species (*Capritermes semarangi*). Genus *Pericapritermes* has two species (*P. dolichocephalus* and *P. javanicus).* *Nasutitermitinae* genera (Nasutitermes and *Bulbitermes*), where the genus *Nasutitermes* comprises species (*Nasutitermes matangensis* and *N. matangensiformis*) and the genus *Bulbitermes*, has one species (*B. constrictoides*) (Table S1). The diversity and dominance indices of termites in all the slopes are presented in Table 1. Meanwhile, the termite's species dominance in each slope is shown in Table 2.

Table 1. List of Shanon-Wienner indices ($H^{'}$) and Dominance (Sorensen) indices (D) in each slope

Indices	Slopes			
	South	East	West	North
$H^{'}$	1.389	1.381	2.298	2.237
D	0.75	0.76	0.79	0.81

Table 2. Termite species dominance in each slope of Mount Slamet, Central Java, Indonesia

Species	Slopes			
	South	East	West	North
S. javanicus	(52.5%)	-	(51.9%)	-
C. semarangi	-	(27.8 %)	-	
O. javanicus	-	-	-	(19.7%)

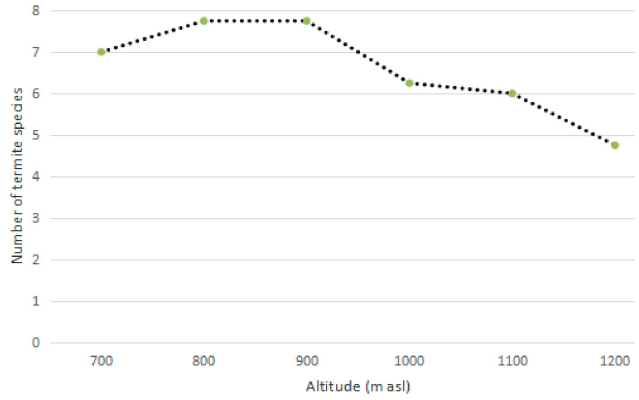

Figure 2. Correlation between the number of termites species and the altitude

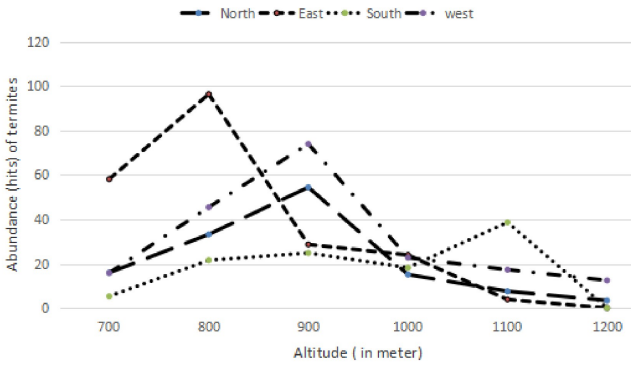

Figure 3. The pattern of correlation between altitudinal gradient with abundance (hits)

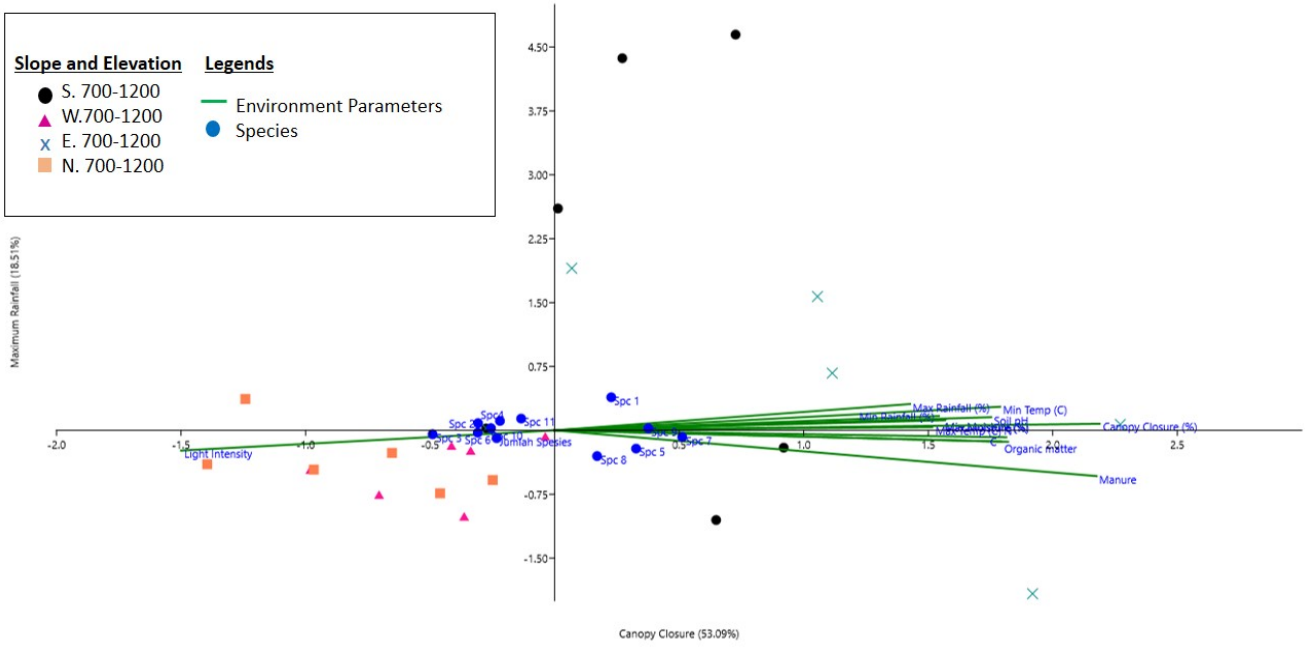

Figure 4. Visualization of ordination for 12 environmental factors on diversity and abundance of termites

The effect of slopes on the diversity of termites was significant ($P<0.05$), as well, the effect of slope on abundance (hits) except on altitudinal range of 1201-1300 m asl. The effect of altitudinal gradient on the diversity of termites was not significant ($P>0.05$) for all altitudinal range (700-1300 m asl) as well on the abundance of termites, except South Slope.

The pattern of correlation between altitudinal gradient and number species is shown in Figure 2. The pattern of correlation between altitudinal gradient with termite's abundance is shown in Figure 3. The effect of 12 environmental factors (Table S2) on diversity and abundance of termites is shown in Canonical Correspondence Analysis (Figure 4).

The most associated environmental factors on the abundance of Families Rhinotermitidae and Termitidae in Mount Slamet were altitude (not for Termitidae), N content (not for Rhinotermitidae), maximum rainfall, canopy closure and manure application.

Discussion

Environmental variables

Air temperature and soil temperature decreased with the increase of altitude. Air temperature dropped from 31.5 °C in the lowland plantation forest (700 m asl) to 28.5 °C at the highest sampling area (1300 m asl), except South Slope at the foggy condition, the temperature reached 15 °C. Soil temperature decrease from 24.5-22.5 °C. Canopy closure in Southern/Northern slopes (50% - semi-opened forest) were significantly different from that of Eastern/Western Slope (0%), especially at the mid-elevation site (800-1100 m asl). Annual rainfall peaked at Southern/Western Slopes (4500-6500 mm) in all elevation (700-1300 m asl), which were higher than Eastern/Northern Slopes (3800-4500 mm). The light intensity in Southern/Northern Slope was higher than that of Western/Eastern Slopes (Table S2).

Termites and slopes

ANOVAs results showed that number of termites species among the slopes differed significantly ($p<0.05$). The Western Slope had the highest species richness (11 species), followed by Northern Slope (10 species), Southern Slope (7 species), and the lowest was Eastern Slope with only six species (Table 1). The difference in termite species number in each slope of Mount Slamet was also shown by Shanon-Wiener index (Table 2). Based on Shanon-Wienner index, we could state that the Western Slope is the highest in species diversity. It was supposed that western slope has most appropriate microclimate and abiotic environmental factors for termites. Daldjoeni (1982) stated that Southern and Western Slopes of Mount Slamet has an Af microclimate type, where the microclimate is wet. This microclimate occurred along Serayu River, the big river with upstream from an area in Eastern Slope, flowing to the Indian Ocean. Consequently, the microclimate factor in the Southern and Western Slopes are wet, with the highest annual rainfall (4500-6500 mm), air temperature (23-26°C), and air humidity (95%). On the contrary, Northern and Eastern slopes of Mount Slamet are the area with an Am microclimate type, where the microclimate is drier than Af, with lower annual rainfall of 3800-4500 mm, hotter air temperature of 24-28°C and lower humidity of 90%. This microclimate occurs along Northern Coast of Java Island.

Even though there is a sharp difference of microclimate between Western and Northern Slope, they have an equal average number of species (10.66 and 10.55 species) as well, between Southern and Eastern Slope was 2.33 and 3.88 species, respectively. So, this result likely implies that the rainfall is not the single factor causing the high or low diversity of termites in Mount Slamet. We presumed initially that the average of number species would be divided into two group (Southern and Western in one group, then Northern and Eastern Slope in another group) based on the intensity of annual rainfall. But, rainfall factor likely has a close correlation to the species domination, where the species *O.javanicus* belonging to Subfamily Macrotermitinae was dominant in Northern Slope (higher air temperature and lower annual rainfall). This finding is in line with Collins (1980); Davies (1997); and Gathorne-Hardy at al. (2001) that the Subfamily Macrotermitinae is at a competitive advantage over other subfamilies in the area of high air temperature and low rainfall. Thus, in wet microclimate, the Subfamily Macrotermitinae appears to be replaced by wood-feeding from other subfamilies such as Nasutitermitinae and Termitinae. That is the reason why the Subfamily Macrotermitinae was more dominant at the Northern Slope. On the other hand, *S. javanicus* (Familia Rhinotermitidae) was dominant on two slopes with higher annually rainfall, i.e., the Western and Southern Slopes (Table 2). This fact was in accordance with statement by Nandika et al. (2006) that *S. javanicus* is very adaptive so that this species can be found at an altitude of more than 1000 m asl. This species is wood feeder termite, living in the xylem of the wood, adaptable to the high rainfall. Eventhough Dibog et al. (1998) and Bignell and Eggleton (2000) stated that rains could have an adverse effect on termite species richness in tropical rainforest systems, as very high levels of rain can lead to inundated microhabitats and colony death. The species *S. javanicus* has a good strategy to overcome extreme environmental factors such as low air temperature and high rainfalls by using the xylem of wood as a buffer.

The effect of Slope on abundance was significant ($F<0.05$) except in altitude of 1201-1300 m asl. The differences of microclimate such as rainfall, air temperature, air humidity, canopy closure and sunlight intensity are the likely causes of the observed variation in the termite abundance, but the most likely outstanding factors were air temperature and humidity. The altitude at 1201-1300 m asl have more extreme air temperature and humidity, the deep chasms preserves the fog. The elevation of 1201-1300 m asl is a permanent cloudy area at night and a temporal cloudy area in rainy season. The air temperature in the foggy condition of the altitude was very low (under 4 °C), which caused the decline in the termite survival, and hence, the poorest termites diversity. Furthermore, the fog reduces light intensity and light daytimes, which may also have affected the termite survival and abundance. The highly varied microclimate conditions affected organisms at all levels (Auslander et al. 2003). Davies et al. (2003) stated that lower light intensity causes the temperature becomes lower as well and affect termites inhabited in that habitat. Maintaining the environmental air temperature in a constant state is vital for the survival of termite colonies (Korb and Linsenmair 1998). Unstable air temperature cause colony death (Bong 2012). Therefore, only certain species can survive at both hotter and cooler air temperatures. This fact is closely related with the domination of species (Table 2), where *S. javanicus (*Family Rhinotermitidae) was dominant in the Southern Slope while *O. javanicus* (Family Termitidae) was dominant in the Northern Slope.

In the Southern Slope, a half of 120 sections of termites sampling are semi-opened area (appendix 2). This semi-opened forest enables affect soil, and humus feeder termites than wood feeder termites (De Souza and Brown 1994) and soil be drier (Vaessen 2011). In Southern Slope, the local farmers are forbidden by the State Forest Management Unit II to plant any cereal under the main

plantation. Consequently, organic content in soil of the slope was the lowest. The most critical factor to the termites species is availability of food (FAO 2000). This condition tends as supporting factor of changing as pest. On the contrary, in other slopes, the local farmers were planting cereals under main plantation trees, and along altitudinal gradient 700-1200 m asl, they applied manure periodically per year, which enabled the feeding group of soil termites became abundant, especially in the Western and Eastern Slope. Likely, manure application support food availability of soil termites. This finding is in line to the state of Ngugi et al. (2011) that soil feeder termites prefer to use simple component of sugar in peptide form as nutrient.

Termites and altitudinal gradient

The effect of altitudinal gradient on termite species number was not significant ($p>0.05$) except for the Southern Slope. Figure 1 shows that species number initially increased in altitude 800-900 m asl then finally decreased. This phenomenon is called *mid-elevation effect* (Lomolino 2001); a pattern often explained by the presence of optimal climatic condition at mid-elevations, by the ecotone effect and/or by the mid-domain effect (Lomolino 2001). The majority of species richness studies on tropical invertebrates showed a mid-elevation peak pattern. This mid-elevation peaks in the termite species composition have been documented (Donovan et al. 2002; Inoue et al. 2006), which have been attributed to factors such as anthropogenic disturbance and the limited altitudinal range of the study. This result was different with that of research by Gathorny-Hardy (2001) in the Leuser Ecosystem, where the pattern of declining diversity by increasing altitudinal gradient was monotically.

The effect of altitudinal gradient on the abundance of termites on all the slopes was not significant ($P>0.05$), except for the Southern Slope. Similar with the effect of altitudinal gradient on the species number, it is supposed that these altitudes (700 up to 1300 m asl) have identical range of air temperature (23-28°C), which enable all inhabitant species to develop well, but in the Southern Slope, the air temperature sometimes remarkably dropped under 4°C at night with air humidity reached up to 95%, which is detrimental for termites (Korb and Lismaier 1998). This phenomenon is called the *Messernerhebung* effect, caused by lack of sunlight covered by the thin cloud rising in specific elevation. The Southern slope has a deep chasm laying to the bottom of the Mount, which keeps the cloudy air along the altitude of 700-1300 m asl.

Distribution and effect of the environmental factor

The termite species identified from this research were the wood feeder consisted of *S. Javanicus, O javanicus, M gilvus Microtermes. insperatus,* while the humus feeders termites consisted of *N. matangensis, N. matangensiformis Bulbitermes constrictoides* and *Procapritermes stiger.* Furthermore, the soil feeder consisted of *P. dolichocephalus, P javanicus.* and *C. semarangi*. These results were new record findings at the plantation forest of Mount Slamet. It is likely that these results were caused by the belt transect sampling methods used in the study. This method is different with the method used in the previous research carried out in Plantation forest of Mount Slamet, namely *direct* sampling or *biting sampling* methods. The two last methods are appropriate and relevant to the wood feeder species. It means that the change of the sampling method to the belt transect sampling method resulted in more reliable species discovered, consist of the wood, humus and soil feeder.

Data on the distribution and composition of termites among four slopes showed that *P. stiger* occurred only in the southern and western slope while *B. constrictoides* occurred in the western slope, both were specifically inhabiting humus habitat under Pinus trees. The Pinus leaf waste layer was up to 10 cm thickness with humus acidity 6. Gathorne-Hardi et al. (2001) stated that *Bulbitermes* is one of the genera having broad environmental tolerance. In this research, *Bulbitermes* was found at the base of hill with mid-elevation of 900 m-1000 m asl with a high slope of 60-70°. Such steep slope with water runoff enables nutrient to accumulate (Hemachandra et al. 2010; Rao 2012). *Bulbitermes* spp was firstly found in plantation forest in the eastern part of Mount Slamet (Pribadi at al 2011).

Certain species were found to dominate in the Plantation Forest of Mount Slamet. Calculated Dominance (Simson) index showed the dominance index was more than 75%. The highest dominance index was shown by the Northern slope (0.81) suggesting that the Northern slope was the most preferred ecosystem for certain termites. The most dominant species in Southern and Western slope was *S. javanicus*, and the most dominant in Northern slope was *O. javanicus*, while that in the Eastern slope was *C. semarangi.*

Based on feeding habit, the most abundant termites in the Northern Slope with a hotter air temperature hotter (24-26°C) and the maximal annual rainfall lower (3500-4500 mm), and N content of 0.7%, was the wood eater *O. javanicus.* On the contrary, the Sothern and Western slope with lower air temperature (23-25°C) and maximal annual rainfall (6000-6500 mm) and N content of 0.6%, was dominated by low-level wood feeder termite *Schedorhinotermes javanicus.* Based on this fact, likely rainfall factor affects the preference of termites species in choose of slope. That finding is in line with the state of De Blauwe et al. (2008) that scale spatial potential affects diversity of Termites.

Analysis of 12 environmental factors showed that only five environmental factors have significant ($F<0.05$) relationship with termite's abundance. The five environmental factors were the percentage of canopy closure, maximal rainfall, altitudes, N content, and manure application. These five ecological factors have a close correlation with the termite's abundance ($R=62\%$). Furthermore, the ordination of Canonical Correspondence Analysis (CCA) shows that canopy closure (eigenvalue 53.09%) and maximal rainfall (eigenvalue 18.51%) were the most associated environmental factors to the species number and abundance of termites in Mount Slamet Plantation. In accordance with to this result, Davies et al. (2003) stated that the local factor such as canopy closure is

more attributable effect on termites species number and abundance. There are coexistence relationships between termites composition and canopy closure of vegetation; in a denser canopy, the termites are more potential to inhabit and develop than in opened area. Canopy closure decreases diversity and abundance of termites (Carrijo et al. 2009). Also, canopy closure factor keeps soil humidity in optimal condition for the development of termites diversity. (Davies et al. 2003).

Ordination of CCA also showed that almost all of the environmental factors were positively associated with species number and abundance of termites species 1 (*S. javanicus*), species 5 (*P. delicocephalus*), species 7 (*C. semarangi*), species 8 (*P. stiger.*), and species 9 (*N. matangensiformis*) in Southern and Eastern slope while light intensity factor was associated with number species and abundance of species 2 (*O. javanicus*), species 3 (*M. gilvus*), species 4 (*M. insperatus*) and species 6 (*P.javanicus*) in the northern and western slopes.

In conclusion, Mount Slamet is relatively low in diversity with only two families (Rhinotermitidae an Termitidae) comprised of five subfamilies (Rhinotermitinae, Macrotermitinae, Microtermitinae Termitinae and Nasutitermitinae), nine genera (*Schedorhinotermes, Macrotermes, Odontotermes, Microtermes, Pericapritermes, Procapritermes, Capritermes, Nasutiermes,* and *Bulbitermes*) and eleven species. There was domination of individual species in each slope, i.e., *S. javanicus* was dominant on the Western and Southern slopes*, O. javanicus* was dominant on the Northern slope, and *C. semarangi* was dominant on the Eastern slope. However, Western Slope was the wealthiest slope. The environmental factors mostly affected diversity and abundance of termites in the plantation forest of Mount Slamet were light intensity, maximal rainfall, altitudes, N content, canopy closure and manure application.

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Table S1. Termites that were sampled during research in Mount Slamet, Central Java, Indonesia

		Slope	SS							WS							ES							NS						
		Altitude (100 m)	7- 8	8-9	9-10	10-11	11-12	12-13	∑	7- 8	8-9	9-10	10-11	11-12	12-13	∑	7-8	8-9	9-10	10-11	11-12	12-13	∑	7- 8	8-9	9-10	10-11	11-12	12-13	∑
F	SF	Species																												
Rhi	Rhino termitinae	*S. javanicus*	50	65	377	195	137	5	829	12	147	67	18	53	5	22	235	243	98	50	42	5	673	15	42	124	33	26	10	250
Ter	Macrotermitinae	*O. javanicus*	8	9	39	7	48	0	111	8	14	5	13	9	4	3	29	31	0	0	0	0	60	59	126	170	12	8	6	381
		M. gilvus	2	6	10	5	0	0	23	12	34	7	26	17	1	87	0	0	0	0	0	0	0	46	118	158	21	7	0	350
	Microtermitinae	*M. insperatus*	4	7	10	6	3	0	30	3	12	4	0	0	0	9	0	0	0	0	0	0	0	14	23	23	1	7	4	72
	Termitinae	*P. dolichocephalus*	2	5	14	9	0	0	30	9	45	0	31	21	8	94	100	108	98	69	0	0	375	15	23	45	24	11	2	120
		P. javanicus	2	9	20	6	0	0	37	23	31	47	15	18	6	280	0	0	0	0	0	0	0	8	16	34	27	13	4	102
		C. semarangi	3	8	18	7	2	0	38	50	67	0	56	23	2	288	54	200	30	23	0	0	307	7	11	28	21	5	4	76
		P. stiger	0	149	0	0	0	0	149	37	82	1	45	19	9	29	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Nasutitermitinae	*N. matangensi formis*	31	40	83	38	28	0	220	11	15	3	13	7	5	4	21	133	50	11	0	0	215	6	8	8	3	3	5	33
		B. constrictoides	0	0	0	0	0	0	0	7	48	3	26	19	3	96	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		N. matangesis	20	39	70	28	21	0	178	2	5	7	7	4	4	9	200	341	39	11	0	0	591	5	9	9	6	3	2	34
		Taxa_S	9	10	9	9	6	1	1645	11	11	11	10	10	10	2065	6	6	5	5	1	1	2221	9	9	9	9	9	8	1418
		Individuals	122	337	641	301	239	5		174	500	814	250	190	137		639	1056	315	164	42	5		175	376	599	148	83	37	
		Dominance_D	0.27	0.26	0.38	0.44	0.39	1		0.16	0.15	0.13	0.13	0.14	00.2		0.27	0.22	0.24	0.29	1	1		0.20	0.23	0.20	0.15	0.17	0.16	
		Shannon_H	1.59	1.67	1.41	1.26	1.2	0		2.04	2.06	2.14	2.13	2.10	10.9		1.48	1.61	1.50	1.36	0	0		1.82	1.72	1.78	1.94	1.97	1.95	
		Simpson_1-D	0.73	0.73	0.62	0.55	0.61	0		0.83	.841	0.86	0.86	0.85	00.8		0.73	0.77	0.75	0.70	0	0		0.79	0.76	0.79	0.84	0.83	0.84	
		Evenness_e^H/S	0.55	0.53	0.46	0.39	0.55	1		0.70	0.71	0.77	0.84	0.81	00.7		0.73	0.83	0.89	0.78	1	1		0.69	0.62	0.66	0.77	0.80	0.88	

Note: F: Family, SF: Subfamily, Rhi: Rhinotermitidae, Ter: Termitidae

Table S2. Environmental factors conditions of Mount Slamet, Central Java, Indonesia during research

Slopes and Altitudes (m)	Organic content	N content	C content	T soil (°C)	T air (°C)	R Min (%)	RFMax (%)	RF Min (mm)	RF Max (mm)	Manure (time/y)	Soil pH	Light Intensity (candela)	Canopy closure (%)
Southern Slope													
S. 700	17.88	0.71	11.19	25	32	80	95	4500	6500	0	4.50	69802	100
S. 800	17.60	0.70	11.09	24	31	80	95	4500	6500	0	4.50	69800	50
S. 900	16.81	0.70	10.20	23	31	80	95	4500	6500	0	4.50	69800	50
S.1000	16.78	0.70	10.02	23	30	85	97	4500	6500	0	4.50	69801	50
S.1100	15.60	0.70	09.19	22	29	85	97	4500	6500	0	4.50	69802	100
S.1200	15.50	0.69	09.10	22	29	85	97	4500	6500	0	4.50	69803	100
Western Slope													
W. 700	22.98	0.91	13.39	24	31	80	95	4500	6500	1	4.50	56200	100
W 800	22.70	0.90	13.19	23	30	80	95	4500	6500	1	4.50	56200	100
W.900	21.90	0.90	12.90	22	29	80	95	4500	6500	1	4.50	56200	100
W.1000	21.78	0.90	12.80	21	28	85	97	4500	6500	1	4.50	56200	100
W.1100	20.91	0.90	11.39	20	27	85	97	4500	6500	1	4.50	56200	100
W.1200	20.68	0.89	11.30	20	27	85	96	4500	6500	1	4.50	56200	100
Eastern Slope													
E. 700	22.90	0.91	13.39	24	31	80	90	3800	4500	1	4.40	56220	100
E. 800	22.78	0.90	13.00	24	30	80	90	3800	4500	1	4.40	56202	100
E. 900	21.80	0.90	12.19	24	29	80	90	3800	4500	1	4.40	56204	100
E 1000	21.18	0.90	12.00	24	29	85	95	3800	4500	1	4.40	56202	100
E.1100	20.91	0.90	11.13	24	28	85	95	3800	4500	1	4.40	56204	100
E.1200	20.79	0.70	11.00	24	28	85	97	3800	4500	1	4.40	56201	100
Northern Slopes													
N. 700	17.68	0.71	10.69	25	32	80	90	3800	4500	0	4.00	69805	100
N. 800	17.18	0.70	10.50	24	32	80	90	3800	4500	0	4.00	56203	100
N. 900	16.80	0.70	09.69	23	32	80	90	3800	4500	0	4.00	56204	50
N.1000	16.28	0.70	09.50	22	32	85	95	3800	4500	0	4.00	69803	50
N.1100	15.68	0.70	09.50	22	32	85	97	3800	4500	0	4.00	69805	50
N.1200	15.28	0.69	09.45	21	32	85	97	3800	4500	0	4.00	69804	100

Notes: T= air temperature, R= Air humidity, RF= Rainfall, S= South, E=East, W=West, N=North, LS = Light Intensity

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Biodiversity and distribution of termite nests in West Papua, Indonesia

NIKEN SUBEKTI[1,♥], ISMA NURVAIZAH[1], JAN HENDRIEK NUNAKI[2], HENGKY LUKAS WAMBRAU[2], RO'IYYATUL MAR'AH[1]

[1] Department of Biology, Faculty of Mathematics and Natural Sciences, Semarang State University. Jl. Raya Sekaran, Gunungpati, Semarang, Indonesia. ♥email: nikensubekti@mail.unnes.ac.id; ismanurvaizah08@gmail.com; roiyyatul@gmail.com

[2]Department of Biology Education, Faculty of Teacher Training and Education, University of Papua. Jl. Gunung Salju, Amban Manokwari, Papua Barat, Indonesia. ♥email: yan_bol2000@yahoo.com; hengky_lukas72@yahoo.co.id

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Abstract. *Subekti N, Nurvaizah I, Nunaki JN, Wambrau HK, Mar'ah R. 2018. Biodiversity and distribution of termite nests in West Papua, Indonesia. Biodiversitas 19: 1659-1664.* Termites play an important role in plant nutritive cycles by contributing to the disintegration and decomposition of organic matter. However, termites also cause damage to wood in nature as well as in buildings. Therefore, termites are potential pests and need to be controlled. Effective control of termites requires knowledge about their species prevalence and distribution. The current study aimed to identify the termite species and nest distributions in West Papua. A survey to determine the distribution of termite nests used the transect line method with intervals of 50 m in width and length. The results showed that there were 35 termite nests on 10 host trees species, namely *Calophyllum inophyllum* (Bintanggur), *Mastixiodendron pachyclados* (Lancat), *Intsia bijuga* (Kayu besi), *Inocarpus fagifer* (Gayang), *Canarium hirsutum* (Kenari), *Horsfieldia parviflora* (Pala hutan), *Diospyros papuana* (Black wood), *Aleurites moluccana* (Kemiri), *Pometia coreacea* (Matoa), and *Vatica rassak* (Resak). These nests harbored three termite genera, including *Microcerotermes*, *Longipeditermes*, and *Bulbitermes*. *Microcerotermes* were the most commonly found and had a wide distribution across almost all points of observation.

Keywords: Biodiversity, distribution, Papua, termites nest

INTRODUCTION

Termites play an important role in the recycling of plant nutrients through the disintegration and decomposition of organic materials found in wood and plant litter. The insects' main food sources are wood, cellulose materials, and fungi. However, termites frequently destroy wood and other cellulose materials in built structures and attack living trees and plants and are thus considered pests (Subekti 2016). The total annual economic losses associated with termite infestation of buildings and preventive treatments worldwide were estimated to be US$40 billion in 2012 (Ghaly and Edwards 2011).

Termites have a high species diversity, with 2500 species having been successfully identified. Termite species are divided into seven families, 15 subfamilies, and 200 genera, which occur in various countries around the world (Nandika et al. 2015). In Indonesia, 200 species of termites within three families (Kalotermitidae, Rhinotermitidae, and Termitidae) have been identified. Termites have a high diversity in tropical forests because these areas have diverse ecosystems (Indrawan et al. 2007). The main environmental factors that affect the distribution of termite nests include the temperature and humidity, while other factors are precipitation and vegetation structure (Cookson and Trajstman 2002). Each of these factors varies, which has driven the ability of termites to adapt and survive and to develop colonies under a broad range of conditions.

Climatic and soil conditions in Indonesia strongly support termite survival (Indrayani et al. 2017). In almost all tropical and subtropical areas, termites (Ordo: Isoptera) have become pests that pose a large damage threat to various crops and forest products (Subekti 2016). Based on observations in West Papua, Indonesia wood-feeding termites can attack a living tree and build a nest in it, which eventually kills the tree. Manokwari, the capital of the province of West Papua, Indonesia, is ecologically suitable for breeding termites. Termite colonies can be easily found in the city, especially in areas of vegetation.

Manokwari (0.015′-3.025′ S, 132.035′-134.045′ E) has flora and fauna that is very different from the other major islands of the country. Research on the types and distribution of termites in West Papua has never been done. However, West Papua is a natural laboratory that contains a large biodiversity of flora and fauna, even in the heart of the city of Manokwari.

Observationally, termite nests often occur in several tree species in West Papua. Since some trees are grown for harvest, termites have the potential to cause economic harm in the region. However, detailed information about termites in West Papua is not yet available, which hinders the development of effective control measures.

MATERIALS AND METHODS

Termite sampling was conducted at the Gunung Meja Nature Tourism Park, Manokwari, West Papua, Indonesia.

The identification of host plants was done in the Biology Laboratory of the University of Papua, Manokwari, Indonesia, while termite identification and data analysis were conducted at the Laboratory of Biology, State University of Semarang, Central Java, Indonesia.

Soldier caste termites were collected from the Gunung Meja Nature Tourism Park, Manokwari and placed in 70% alcohol. A global positioning system (GPS) was used to pinpoint geographical locations, and a lux meter was used for measuring the intensity of light. Additional equipment included a thermohygrometer to measure air temperature, a soil tester for measuring soil moisture and pH, a compass, a machete, plastic containers, tweezers, petri dishes, brushes, sample bottles, raffia, plastic straps, stationery, a digital camera, a microscope, markers, paper labels, measuring tape to determine the height and diameter of nests, tally sheets, and identification books.

A survey to determine the distribution of termite nests was done using the transect line method (Turner 2000; Lee et al. 2003). This method is often used to collect data on species and the number of termite nests. The observation path was systematically specified for the entire forest, with intervals of 50 m in width and length. When, a nest of termites was found researchers recorded the location using GPS. The starting point for each line of observation was marked with the direction in which observations were made, using the compass. The data collected included the position of termite nests according to the GPS, the height and the size of the nest, and the species of tree in which it was found. Termite nests were classified according to size, namely, small (nest height ≤ 0.49 m), medium (0.5-0.99 m), and large (≥1 m) (Subekti et al. 2008).

Soldier caste termites, up to 25 from each site, were collected using tweezers or paint brushes and placed in sample bottles containing 70% alcohol. Each sample bottle was with a number, the number of nests (to assign an identifying number to each nest) and the nest location.

Termite identification was based on soldier caste termites. The sample insects were examined with a binocular microscope to observe the morphological characteristics, including the length of the mandibles, the length of the head and length of antennae. After photomicrographs were taken, the insects were stored in specimen containers. Termite identification was done to the level of the species based on Sornuwat et al. (2004) and Tho (1992).

The identification of host plant species was based on Womersley (1978) and Lekitoo et al. (2008). Determination of the distribution of the termites used the points of observation of nests in the field using GPS, with further processing with the software ArcView 10. The results are presented as a map of termite species in forested areas.

RESULTS AND DISCUSSION

Termite species diversity

The results of the identification of the termite species according to Sornuwat et al. (2004) and Tho (1992) indicated that three termite species occur in the Gunung Meja Nature Tourism Park, Manokwari. These species are from the family Termitidae and belong to three genera (*Microcerotermes*, *Longipeditermes*, and *Bulbitermes*) from two subfamilies (Amitermitinae and Nasutitermitinae).

Termites are polymorphic social insects that live in colonies. A caste system exists in each colony, and each caste has a different body morphology. In this study, termite identification was based on soldier caste termites because insects in this caste have a distinct mandible shape that differs by species, permitting easy identification (Haneda and Firmansyah 2012).

Microcerotermes spp. have small soldiers that are similar in size to their workers. Typical morphological characteristics of soldier caste termites of *Microcerotermes* spp. were a rectangular head capsule and curved, serrated mandibles (Figure 1.A). The length of the head in *Microcerotermes* spp. was half of the body length, and the insects had paired antennae with 13 segments. According to Sornuwat et al. (2004), this genus has a rectangular shaped head with curved mandibles and antennae with 13-14 segments. Based on the results of the study, *Microcerotermes* spp. were identified as nesting in trees on living and dead wood. In addition, this species of *Microcerotermes* spp. made nests from cardboard.

Microcerotermes spp. generally nest in trees, but close to the ground. The termites cause damage to the trees in which they nest because they eat wood of living or dead trees. *Microcerotermes spp.* usually nest on the main stem of a tree. Nest material is a mixture of chewed wood and dirt (Nandika et al. 2015).

A B C

Figure 1. Morphology of termites found in Gunung Meja Nature Tourism Park Manokwari West Papua: A. *Microcerotermes*, B. *Longipeditermes*, and C. *Bulbitermes;* 40 × 10 magnification

The soldier caste *Longipeditermes* sp. termites had a dark brown to blackish head capsule. The length of the rostrum can exceed the length of the head by more than half, and the antennae and legs were tinted light brown. Antennae had 14 segments. The third segment was three times as long as the second segment and less than twice the length of the fourth segment (Figure 1.B). These traits are similar to those described by Sornuwat et al. (2004).

Longipeditermes termites are often found on the tropical forest floor. These termites do not require burrows to move and do other works. Their dark coloring and rapid movements help termites of this species avoid predators. Activities outside the colony are often done in the morning and afternoon to reduce the risk of predation. Because of their color and rapid movement in the forest litter, individual termites are not easy to find and collect (Syaukani 2011).

Soldier caste termites of *Bulbitermes* spp. were found to have morphological characteristics including brown coloring, the head is triangular, and antenna with 13 segments. The average body length was 3.75 mm, and the head length with the mandible was 0.98 mm. The insects were found burrowing in living trees (Figure 1.C). According to Husni and Syaukani (2012), *Bulbitermes* spp. have triangular-shaped heads and antennae with 12-14 segments. The length of the head up to the nasus is 1.24-1.45 mm, the length of the head with the mandible is 0.98-1.12 mm, the length of the rostrum is 0.32-0.37 mm, and the length of the pronotum 0.26-0.18 mm.

The morphological features are similar to those of *Nasutitermes* spp., but the two species can be distinguished by the shape of the head. *Bulbitermes* spp. are also characterized by having a monomorphic soldier caste of soldiers and living in burrows (non-free-ranging species). The upper teeth (left mandible) are generally the same length or shorter than the first teeth, and the notch located at the tip of the right mandible is not well developed. The important characteristics used to identify the genus are based on the worker caste. Some morphological characters have been tested for consistency with molecular characteristics (Syaukani and Thompson 2011).

The *Bulbitermes* nests are round or oval-shaped, depending on the burrows. The main nest materials are small fragments of decayed or rotten wood, dried foliage, and soil that is attached with saliva. The nest lining is composed of two layers. The outer layer is relatively thin and soft, and it is instrumental in protecting the nest from rain. The inner layer is relatively hard and stiff, and it is primarily composed of rotted wood and soil.

Distribution of termite nests

Gunung Meja Nature Tourism Park Manokwari has an area 460.25 ha with varies topography from sloping, slightly wavy, and light to heavy, with the highest peak (Bonay peak) ± 177 m asl. The area with a sloping topography is found on a ridge that is relatively flat and resembles as table, light wavy topography is on the hillside to a height of 70 m asl, whereas the heavy wavy topography is on the north and the south ridge. We found 35 termite nests that were evenly spread evenly along the Gunung Meja Nature Tourism Park, Manokwari and followed the distribution pattern of primer plant in the area (Figure 2). Termites tend to build nests near river by utilizing moist soil to be inserted in the nests in order to keep the humidity and water flow inside the nests (Gautam & Henderson 2014). The 35 termite nests occurred in 10 species of host trees, namely, *Calophyllum inophyllum* (Bintanggur), *Mastixiodendron pachyclados* (Lancat), *Intsia bijuga* (Kayu besi), *Inocarpus fagifer* (Gayang), *Canarium hirsutum* (Kenari), *Horsfieldia parviflora* (Pala hutan), *Diospyros papuana* (Black wood), *Aleurites moluccana* (Kemiri), *Pometia coreacea* (Matoa), and *Vatica rassak* (Resak).

According to Lekitoo et al (2008), there are two groups of flora in the Gunung Meja Nature Tourism Park, which are woody plant (woody vegetation) and non-woody plant (non-woody vegetation). The dominant two of woody plant species in tree level are *P. coreacea* dan *I. bijuga,* whereas in the stake level is dominated by *Horsfieldia sp.* In accordance with Agriculture Department of Directorate General of Forestry, Indonesia, *Pometia sp.* has a hardy wood but is not resistant against termites and moss attack, while *C. inophyllum*, *M. pachyclados, H. parviflora*, *A. moluccana* and *Canarium sp.* have a slightly hardy and heavy wood, so that is easy to be attacked by termites. Nakayama & Osbrink (2010) reported that *A. moluccana* oil can not act as toxic agent for termites and only can be the feeding deterrent at more than 27% concentration. This is the reason why termites utilize *A. moluccana* as their host tree in the area. There is no relationship between specific plant communities and termite nests, but the occurrence of the nests in a certain area can induce the increasing of woody and forbs plant diversity (Gbeffe et al. 2017).

Based on the results of this study, the dominant termite species was *Microcerotermes* spp. As many as 33 termite nests were built by a *Microcerotermes* sp., which included eight large nests (height ≥ 1m), 12 medium nests (height 0.5-0.99 m), and 13 small nests (height ≤ 0.49 m). The nests were located at an altitude of 124-223 m asl. Only one nest each was found for *Longipeditermes* spp. and *Bulbitermes* spp., specifically, nests number 5 and number 13 (Figure 3). These nests were medium size (0.52 m and 0.72 m) and located at an elevation of 149 m asl and 161 m asl. The spread of termites in natural forests at varying elevations shows their adaptability to diverse habitat conditions. High termite colony distribution found along elevation were truly influenced by climate changes and vegetation around there (Nunes et al 2017).

Cheng et al. (2008) stated that land with a mineral soil type will be dominated by members of the Termitidae. It may be for that reason that only species of Termitidae were found in Gunung Meja Nature Tourism Park. The land in this forest area is a bit acidic to neutral, the availability of C-organic was very low to high, with N, P_2O_5, Ca, Mg, K, and Na (Lekitoo et al. 2008). Chemical elements content such as K, P, Ca, Mg, C-organic (Kaschuk et al. 2006), NH_4^+ and NO_3^- (Muvengwi et al. 2016) in the termite nest soil is higher than in the surrounding soils.

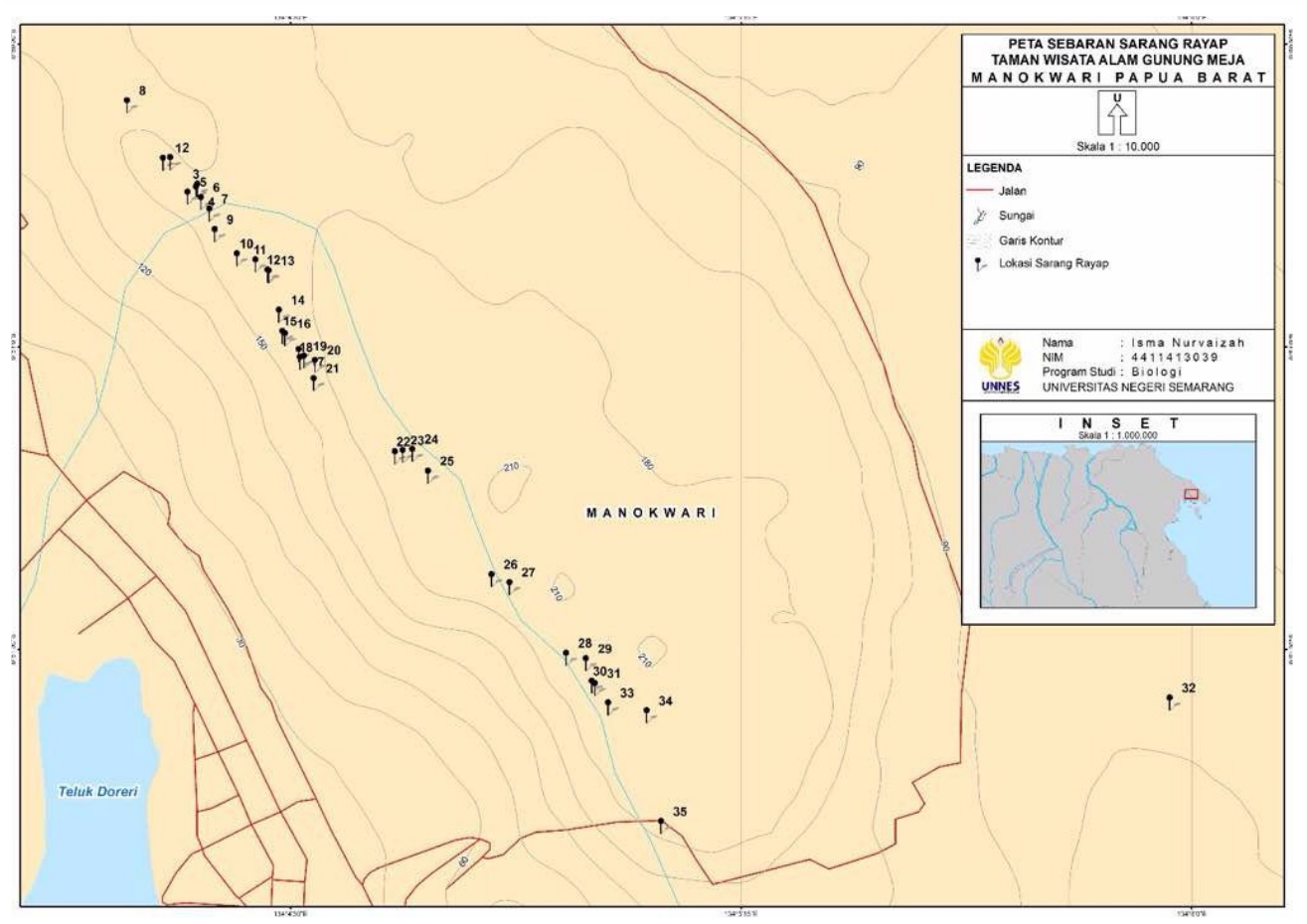
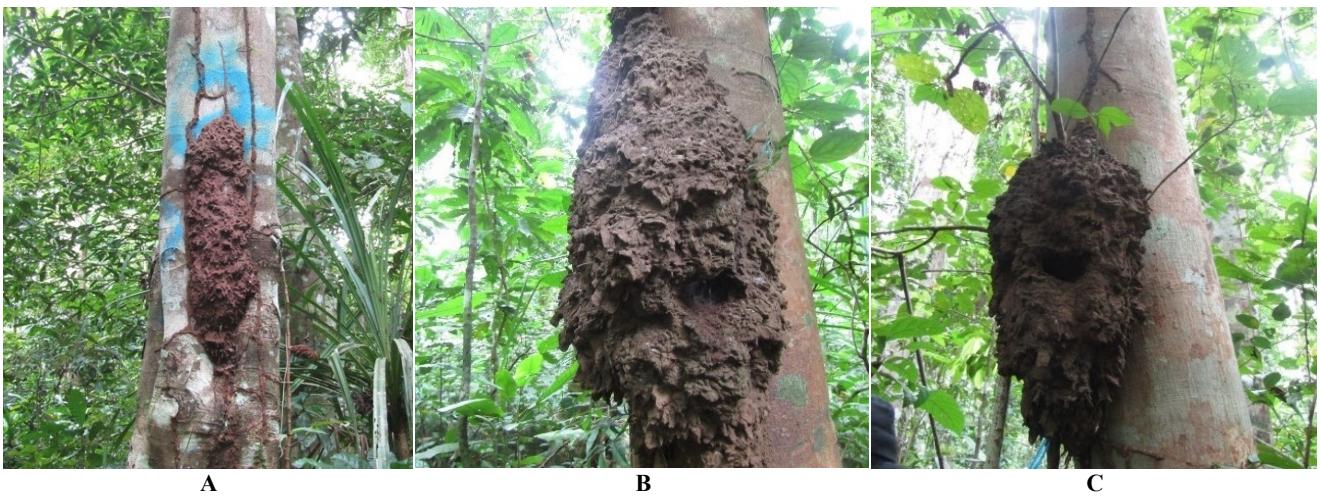


Figure 2. Map of termite nest distribution in Gunung Meja Nature Tourism Park, Manokwari, West Papua, Indonesia



A **B** **C**

Figure 3. Nests of three termite species found in Gunung Meja Nature Tourism Park, Manokwari, West Papua, Indonesia. (A) *Microcerotermes* spp., (B) *Longipeditermes* spp., and (C) *Bulbitermes* spp.

Termite nests/mounds characteristic depends on the surrounding soil properties. Soil in Gunung Meja Nature Tourism Park greatly varies and generally has very thin topsoil (<30 cm). The Soil variety consists of clay, calcareous soil, rocky soil and coral soil. This variation caused the differences of vegetation in the forest area. Sample soil analysis conducted by Soil Research Association Bogor confirmed that soil texture in Gunung

Meja Nature Tourism Park consists of loamy clay, dusty loam, dusty loamy clay and loam. de Lima et al. (2018) reported that soil with termite mounds performed higher clay content, acidity, and Al^{3+} content. Moreover explained by Mujinya et al. (2013) that clay content in termite nests/mounds can be twice higher than in the surrounding soil. This proves that soil in Gunung Meja Nature Tourism Park which tends to clay, can be the preferred place for termite to build nests by utilizing mineral clay contents. So that termites play an important role as weathering agents of clay minerals (Jouquet et al. 2002). Abe & Wakatsuki (2010) found that termites of Termitidae preferred to collect clay particles from argillic horizon (illuvial) because of the existence of phyllosilicates and crystalline sesquioxides minerals. Mica group is one of phyllosilicates contained in the nest. Crystalline sesquioxides in the termite nest are different in content, for example, Mn oxides (Mn_2O_3) in the nest was relatively greater than Fe oxides (Fe_2O_3). The poorly Fe oxides in the nest caused the higher degree of clay dispersibility than in the surrounding soil (Mujinya et al. 2013). The other Sesquioxides such as Al oxides (Al_2O_3) was used as the main aggregating agent especially as water-stable aggregates (Barthès et al. 2008).

Microcerotermes spp. are included among termites feeding on wood and litter, and they may potentially be pests in natural forest areas. These findings accord with previous research (Cheng et al. 2008; Vaessen et al. 2011; Bong et al. 2012; Kon et al. 2012). Wood-feeding termites are the type of termites that are most likely to be pests (Hanis et al. 2014). The species are present in abundant quantities in the forest area because of the presence of plant residues containing cellulose being abundant.

Nasutitermitinae is found in secondary forests that have highly diverse flora. They can be bioindicators of forest health because they are a soil-feeding group and they include wood eaters who inhabit relatively undisturbed forests (Syaukani 2013). *Longipeditermes* spp. and *Bulbitermes* spp. belong to the Termitidae family, and they eat soil with a high organic content (Faszly et al. 2005). *Longipeditermes* spp. and *Bulbitermes* spp. can be difficult to find because these termites have a specific habitat that is rarely to be found in this area.

Generally, the nest architecture of *Microcerotermes* spp., *Longipeditermes* spp., and *Bulbitermes* spp. in the Gunung Meja Nature Tourism Park Manokwari did not differ by species. Termite nests are among the most complex and sophisticated structures built by insects (Himmi et al. 2015). The selection of certain microhabitats for nest building is presumed to be associated with reducing the risk of predation by the ants, birds, lizards, bears, and orangutans. Some colonies build nests that are round- or oval-shaped, depending on the host tree. The main nest materials consist of small fractions of decayed or rotten wood, dried foliage, and soil that is attached with saliva. Lining nest is composed of two parts: the outer layer is relatively thin and soft is more instrumental in preventing the nest when the rain, while the inner layer is relatively hard, stiff, and there are many kinds of wood rotted material and soil.

Nest architecture features connected rooms, with hallways always guarded by soldier caste termites. If soldier caste termites are harassed, they immediately go from the nest and confront the attacked. Young soldiers preferred to be the royal guard and the older soldiers were in charge to encounter the more hazardous task (Yanagihara et al. 2018). Meanwhile, the worker caste termites hide in the nest and return to their normal activity if the conditions are secure. Du et al. (2016) found that most of young workers performed the grooming in the central nest, whereas the older workers maintained the nest and sanitation, especially looking after for the royal pair and the royal chamber. The room of the king and queen (royal chamber) is not easy to find. This structure is undoubtedly built under pheromone regulation, especially cement pheromone emitting by queen. This pheromone can enhance not only the shape of royal chamber but also the dome foundation (Nakanishi et al. 2017). The characteristics of the royal chamber for termites of all species do not differ from the conditions of the rooms of other castes.

Architecture of termite nest is likely influenced by soil properties utilized to build the nest, and this certainly depends on the ecological needs of termite in controlling temperature and humidity inside the nest. Jouquet et al. (2015) confirmed that soil properties can affect the physicochemical characteristics of *Odontotermes obesus* (Termitidae) nest material and also impress their nest shape. Nest termite architecture is also equipped by solar-powered ventilation in order to adapt with environmental changes, and it seems like external lung system in human (Ocko et al. 2017).

Shelter-tube architecture of termite colony can be different between each species, even between one and another colony from the same species. Mizumoto & Matsuura (2013) demonstrated that each termite colony builds a specific architecture model referring to its shelter-tube construction system. Shelter-tube architecture built by groups of individuals from the same colony presented similar construction pattern, whereas groups of individuals from the different colony performed a different construction pattern. This is associated to those foraging strategy differences of termites, and also related to the distribution of food resources in their environment.

Based on the research in this study, three genera of termites are present in West Papua, including *Microcerotermes Longipeditermes*, and *Bulbitermes*. The termites were found in 35 different nests. *Microcerotermes* were the most commonly found and had a wide distribution, being present at almost all points of observation. *Longipeditermes* and *Bulbitermes* were less common, with only within one point observation each.

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Dracorhodin: A potential marker compound for detecting the presence of dragon's blood resin from *Daemonorops* originated from Indonesia

TOTOK KARTONO WALUYO♥, SANTIYO WIBOWO♥♥
Forest Product Research and Development Center. Jl. Gunung Batu No. 5, Bogor 16610, West Java, Indonesia.
Tel.: +62-251-8633378, Fax.: +62-251-8633413, ♥email: waluyo60@yahoo.com, ♥♥santiyowibowo1973@yahoo.co.id

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Abstract. *Waluyo TK, Wibowo S. 2018. Dracorhodin: A potential marker compound for detecting the presence of dragon's blood resin from* Daemonorops *originated from Indonesia. Biodiversitas 19: 1665-1671.* Dragon's blood typifies as red-colored resin, which is presented by several plant genus, i.e., Dracaena, Daemonorops, Croton, and Pterocarpus. In Indonesia, dragon's blood is originated from Daemonorops which grows scattered in Sumatera and Kalimantan islands. Relevantly, this study was conducted to identify the specific compounds of dragon's blood originated from Indonesia's Daemonorops. Dragon's blood test samples were originated from 16 different towns in Indonesia, which are known as the center of dragon's blood-production. The samples were in powder and solid formation. The compounds of samples were analyzed using GC-MS instrument. Results revealed that dragon's blood in powder and solid formation were inherently similar. Dragon's blood powder was obtained from wet extraction, while dragon's blood solid from dry extraction. Results of chemical analysis on 16 dragon's blood samples disclosed that three compounds were frequently detected associated with dragon's blood presence. Dracorhodin compound was detected in 16 dragon's blood samples i.e 3,4-dihydro-5-methoxy-6-methyl-2-phenyl-2H-1-benzopyran-7-ol in 13 samples; and trendione in 9 samples. Accordingly, dracorhodin could serve as the most compound containing in dragon's blood originated from Indonesia's Daemonorops, which could be observed from 16 tested Daemonorops dragon's blood samples from several regions (towns) in Indonesia.

Keywords: Dragon's blood, *Daemonorops*, GC-MS, marker compound, dracorhodin

INTRODUCTION

Jernang (Dragon's blood) typifies as a red-colored resin consecutively belonging to genus *Dracaena* (Dracaenaceae), *Daeomorops* (Aracaceae), *Croton* (Euphorbiaceae) and *Pterocarpus* (Fabaceae) (Pearson and Prendergast 2001). Dragon's blood resin has been widely used as a coloring agent for varnishes, ceramics, marbles, stone-made tools, woods, rattans, paintings, etc. Besides, dragon's blood resin could also be used as drug ingredients, among others for antidiarrheal (Gupta et al. 2008), antimicrobials (Edward et al. 2001; Waluyo and Pasaribu 2015), antivirus (Gupta et al. 2008; Waluyo and Pasaribu 2015), anticancer (Gupta et al. 2008; Alonso-Castro et al. 2012), antiplatelet (Yi 2011), antiinflammation (Gupta et al. 2008; Lopes et al. 2014), antioxidant (Gupta et al. 2008; Lopes et al. 2014) and wound healing (Gupta et al. 2008; Waluyo and Pasaribu 2015; Namjoyan et al. 2015).

The adopted techniques to obtain dragon's blood resin vary depending on the species of their host trees. For example, dragon's blood resin living in the host trees, i.e., *Dracaena cinnabari*, *Croton*, and *Pterocarpus* can be obtained by performing the tapping technique on the stem part of those tree species (Pearson and Prendergast 2001). Meanwhile, for the tree species of *Dracaena cinnabari* Balf.f., dragon's blood resin is acquired using tapping technique as well on the stem part of the tree. Meanwhile, for the species of *Dracaena cochinchinensis* (Lour.) S.C. and *Dracaena cambodiana* Pierre ex Gagnep. both originated from China, it is obtained by inducing *Fusarium proliferatum* fungi at the tree stem and leave parts of those species. Therefore, the infected plant organ will produce dragon's blood resin (Fan et al. 2008; Wang et al. 2010; Ou et al. 2013).

Dragon's blood resin originated from rattan species is the species that belong to the genus *Daemonorops*. The resin results from the secretion of the rattan fruits, adhering to the outer part of fruit skins. Dragon's blood of this plant only exists in Indonesia and Malay Peninsula (Yi et al. 2011). Several rattan species producing dragon's blood resin are among others *Daemonorops draco* BL.; *D. maculata*.; *D. mattanensis* Becc.; *D. micrantus* Becc.; *D. propinquess* Becc.; *D. rubber* BL.; *D. sabut* Becc.; *D. micracanthus* Becc.; *D. didymophylla* Becc.; *D. melanochaetes* Blume.; *D. longipes* Mart.; *D. draconcellus* Becc.; *D. motleyi* Becc., etc (Heyne 1987; Dransfield and Manokaran 1994; Januminro 2000; Waluyo 2013). One of the several simple techniques to obtain dragon's blood resin from rattan species commonly performed by the tribe community residing in Jambi by pounding fresh rattan fruits so that the resin that adheres to the outer fruit skins fall apart or become loose from those skins (Waluyo 2008).

Dragon's blood resin produced from fruits various rattan plant species (*Daemonorops*) grows widely in Nangro Aceh Darussalam province until Lampung province in Sumatera and several regions in Kalimantan. Accordingly, the relevant research was conducted to know the reliable information about the particular chemical

compounds and to obtain the compound entity itself that could have functioned as a convincing marker to detect the presence of dragon's blood particularly from the genus *Daemonorops* originated from Indonesia. Expectedly, the result of the research could be beneficial and use as a reliable reference to distinguish whether the dragon's blood resin is originated rattan or other plant species from Indonesia.

MATERIALS AND METHODS

Materials and equipment

The materials of this research consisted of dragon's blood resin both in powder and in solid/block formation (Figure 2), derived from genus *Daemonorops* seeds (Figure 3), collected from several regions in Indonesia (Figure 1). The solid-shaped dragon's blood resin stuff were collected from the regions in Jambi, West Sumatera, and Kalimantan provinces. The location of 9 samples collection is presented in Table 1. Meanwhile, the powder-shaped of dragon's blood sample was originated from the regions in Aceh (Meulaboh/ML, Lhokseumawe/LS), Sumatera Utara (Medan/MD, Sipirok/SP), and Lampung (Liwa/LW) provinces. In relevant, 7 samples of powder-shaped dragon's blood were collected from 7 (towns) particular sites (towns) in those three regions (Table 1). The chemical used for compound analysis was mainly acetone, while the equipment consisted of consecutively soxhlet extraction apparatus, rotary vacuum evaporator, and GC-MS (Gas Chromatography-Mass Spectrometry) instrument.

The extraction techniques for rattan fruits in the field

Dragon's blood resin was extracted from rattan fruit using dry and wet extraction techniques by the community who reside in three regencies that consisted of Sarolangun/SR, Lhoksemawe/LS, and Meulaboh/ML. The procedure of the extraction as follows:

The wet extraction technique using conventional method

The wet extraction technique was performed by the local community in Lhokseumawe/LS using water as media (Januminro 2000). The rattan fruits were dried under the sun until dry; and were then ponded to easily separate rattan fruit skin from fruit seed. The separated rattan fruit skin was then put into the container filled with water, and stirred or squeezed vigorously so that the resin portion enabled to dissolve in water. Furthermore, the water solution was sieved/filtered using a screen made of sacks or woven plastics. The water filtrate was saved and then placed inside the container; and let them for some duration, until the dragon's blood resin was precipitated or settled down perfectly. It was then dried under the sun (Figure 3).

Table 1. Location origin and shapes of dragon's blood

Origin of location	Province	Forms/ shapes	Code
Meulaboh	Aceh	Powder	ML
Lhokseumawe	Aceh	Powder	LS
Tapaktuan	North Sumatra	Powder	TT
Medan	North Sumatra	Powder	MD
Sipirok	North Sumatra	Powder	SP
Solok	West Sumatra	Solid	SL
Sungaidareh	West Sumatra	Solid	SD
Sarolangun	Jambi	Solid	SR
Muarabungo	Jambi	Solid	MB
Jambi	Jambi	Solid	JB
Palembang	South Sumatra	Powder	PL
Liwa	Lampung	Powder	LW
Pontianak	West Kalimantan	Solid	PT
Sanggau	West Kalimantan	Solid	SG
Putussibau	West Kalimantan	Solid	PS
Murateweh	Central Kalimantan	Solid	MT

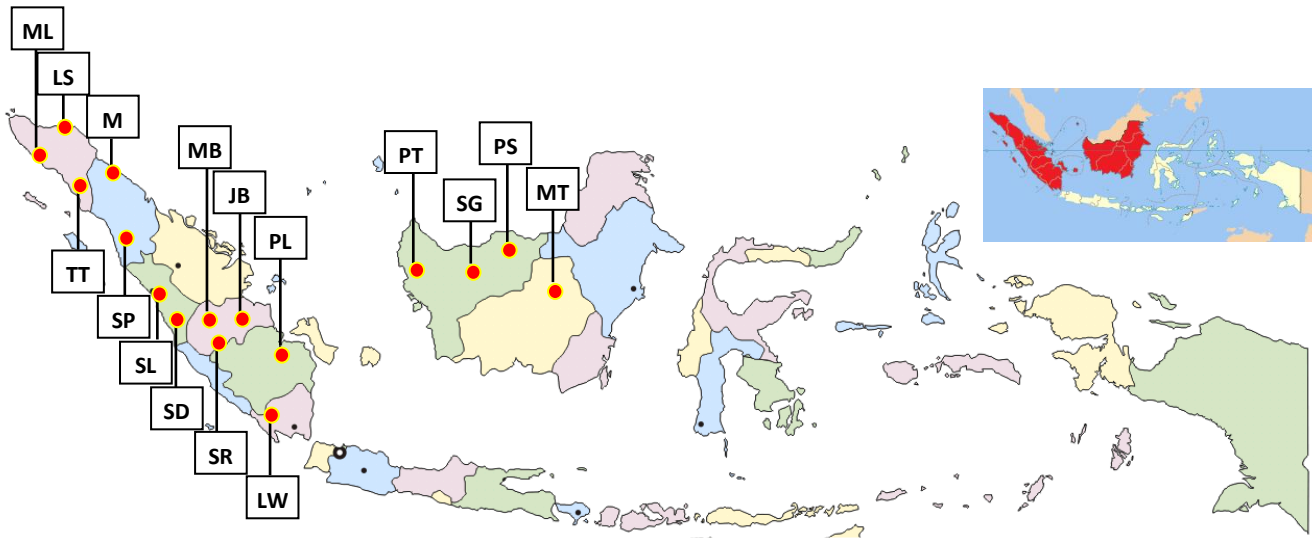


Figure 1. A map featuring the origin for location of dragon's blood resin. Abbreviation of the cities refer to Table 1

A B

Figure 2. Dragon's blood resin stuffs. A. Solid shape; B. Powder shape

A B

Figure 3. Rattan fruits from the genus *Daemonorops*. A. Rattan fruits that still contained dragon's blood resin at their outer skin; B. Rattan fruit that no longer contained dragon's blood resin at their outer skin (after being extracted)

Wet extraction using machineThe extraction technique conducted in Meulaboh/ML could also be categorized as wet extraction, which was only slightly different from the technique performed by the community in Lhokseumawe/LS. Rattan fruits were put into a cylinder-shaped container or tube already filled with water (Figure 4). Afterward, the cylinder-shaped tube was revolved vigorously until the fruit resin dissolved completely in water. Furthermore, the water portion was separated through the sieving; and the obtained filtrate was let stand for some duration for resin precipitation. Afterward, the resin was separated from the water, which was then dried under the sun.

Dry extraction technique

Dry extraction technique was conducted by pounding the fresh rattan fruits (Figure 5) to powder shape. Modern mechanical equipment can be used to speed up this process, as presented in Figure 6 (Waluyo 2008). Furthermore, the resulting dragon's blood powder was kept inside plastic containers/bags; and not long afterward, the powder would be hardened/solidified. The chemical used for compound analysis was mainly acetone, while the equipment consisted of consecutively soxhlet extraction apparatus, rotary vacuum evaporator, and GC-MS (Gas Chromatography-Mass Spectrometry) instrument.

Determination of resin content

The resin content determination followed the standard procedure of ASTM D297-9318. 5 g dragon's blood sample was extracted with acetone using soxhlet apparatus. The process of extraction was for 6 hours or until the solution was clear. The extracts were then concentrated using rotary vacuum evaporator. The percentage of acetone extract (resin content) was calculated as follows:

$$\text{Acetone extract (resin content), \%} = (A/B) \times 100$$

Where:
A: Grams of extract
B: Grams of sample used

Identification of compounds in dragon's blood resin

The concentrated resin solution was further analyzed using GC-MS instrument, adopted from electron-attacking ionization method at the gas chromatograph of GC-17A (SHIMADZU) type, which was set up tandemly with mass spectrometry device of MS QP 5050A type, using capillary column, DB-5 ms (J&W) (silica 30 m x 250 µm x 0,25 µm), with column temperature operated at 50 °C (zero minute) until 290 °C with 15 °C/minute rate, involving helium carrier gas at fixed pressure (7.6411 psi) and using Wiley 7N, year 2008 as database.

RESULTS AND DISCUSSION

Resin contents

Acquiring the values of resin content was necessary to assess the purity of dragon's blood resin. Results of analysis on resin content were disclosed in Table 2. The resin content in solid-shaped dragon's blood (SL, SD, SR, MB, JB, PT, SG, PS, and MT) varied about 83.79-93.62%. Meanwhile, resin content in powder-shaped dragon's blood (ML, LS, TT, MD, PL, and LW) ranged about 94.90-97.44%; which was relatively higher than the resin content in solid-shaped dragon's blood. The lower resin content of the solid-shaped dragon's blood material were strongly attributed to the presence of debris and other contaminants such as rattan fruit skin (Waluyo 2008).

The resin contents of dragon's blood materials from all the tested samples were quite high, reaching above 80% (Table 2). Based on Indonesia's National Standard (BSN 2010), those entire of dragon's blood samples belonged to the super quality category with the content minimally reaching about 80%.

Figure 4. Wet extraction technique. A. Extraction using water media, B. Filtering process, C. Drying process under the sun

Figure 5. Technique for dry extraction of dragon's blood

Figure 6. Equipment for the extraction of dragon's blood resin

Identification of Dragon's Blood compounds

Results of GC-MS analysis of all (16) dragon's blood resin stuff samples (Tables 1 and 2**),** showed that 42 chemical compound had been identified (detected). Those compounds almost had similarities in chemical formula and chemical structure corresponding to 42 types of standard reference's compounds with 80% of similarity index. (Table 3). Out of those 42 chemical compounds, 3 (three) compounds were mostly detected or abundantly present in the dragon's blood samples. Those three compounds

consisted of dracorhodin followed by 3,4-dihydro-5-methoxy-6-methyl-2-phenyl-2H-1-benzopyran-7-ol and trendione (Table 3). Dracorhodin was detected in all of samples of dragon's blood stuff; while 3,4-dihydro-5-methoxy-6-methyl-2-phenyl-2H-1-benzopyran-7-ol was found in 13 samples; followed by, trendione was found in 9 samples (Figure 7).

Dracorhodin turned out to be the only one of 42 compounds which were detected in all 16 samples of dragon's blood stuff (Table 3). Accordingly, dracorhodin could be found as a major compound of dragon's blood resin in *Daemonorops* (rattan genus). This result was different with dragon's blood resin originated from China (genus *Dracaena*), which contained loureirin as major active compound, so that could be used as a marker compound for the presence of the dragon's blood resin (Gupta et al. 2008; Jia et al. 2014).

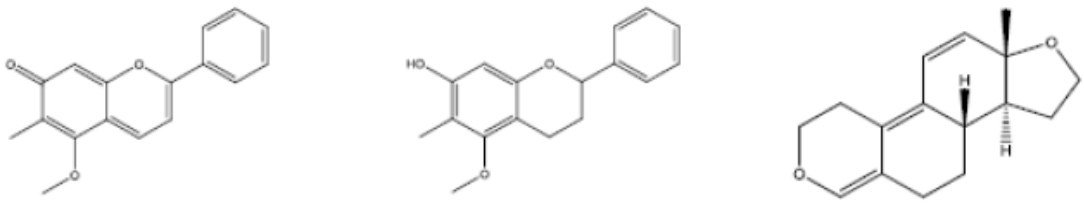
Until now dracorhodin was identified as an active compound in the species of *Daemonorops draco* BL. (Gupta et al. 2008, Baumer and Dietermenn 2010), whereas many species of the genus *Daemonorops* are grown in Indonesia. *Daemonorops acehensis* in Aceh province (ML, LS, TT), *Daemonorops uschdraweitiana* in North Sumatra (MD, SP), *Daemonorops brachystacliys* and *Daemonorops draco* in Jambi and West Sumatera (SR, MB, JB, SD, SL), *Daemonorops siberutensis* in South Sumatra and Lampung (PL, LW), *Daemonorops micracantha* and *Daemonorops didymophylla* are mostly found in Kalimantan (PT, SG, PS, MT) (Rustiami et al. 2004; Purwanto et al. 2005). Thus, it is suspected that all of the genera of *Daemonorops* contain dracorhodin compounds.

Dracorhodin typified as a derivative of anthocyanin's flavonoid compounds, which rendered the dragon's blood resin stuffs to exhibit their specific colors (Melo et al. 2007; Shi et al. 2009). Those specific-colored dragon's blood resin stuff were utilized as coloring agent for art items of the 15th century (Baumer and Dieterman 2010). The outstanding color of dracorhodin was due to the presence of double or triple bond system inside its molecules, which were intricately conjugated and further, in general, afford antioxidant actions. These compounds were obtained from research of methanol extract as well as ethyl acetate extract of dragon's blood resin exhibited antioxidant activities (Waluyo and Pasaribu 2013). Furthermore, the particular compounds belonged to anthocyanin group tended to have anticancer activities. The free radical as one of factors causing cancer diseases were able to be caught by the system of conjugated double or triple bonds in anthocyanin (Amin and Mousa 2010). Other benefits of anthocyanin's flavonoid compounds were as antimicrobials, antivirus, and antitumor agents; and able to perform cytotoxic activities (Gupta et al. 2008; Edward et al. 2001; Alonso-Castro et al. 2012; Waluyo and Pasaribu 2015).

In addition, dracorhodin was also apparently efficacious to cure lung cancer diseases. This was strongly indicated that the use of darcorhodin perchlorate (inherently the synthetic dracorhodin) could overcome lung cancer diseases (Zhang et al. 2015). In chemical structure, 3,4-dihydro-5-methoxy-6-methyl-2-phenyl-2H-1-benzopyran-7-ol compound resembled a lot those of dracorhodin. Accordingly, it could also serve as an effective marker compound, besides dracorhodin. Meanwhile, tendione is typified as a prohormone compound belonging to the steroid groups, which were utilized a lot by sports fan person, particularly bodybuilders (Parker et al. 2012).

Table 2. Resin contents of dragon's blood

Origin of location	Forms/ shapes	Resin contents (%) (Mean ± SD, n = 3)
Meulaboh (ML)	Powder	95.50 ± 1.70
Lhokseumawe (LS)	Powder	94.90 ± 0.92
Tapaktuan (TT)	Powder	97.44 ± 0.82
Medan (MD)	Powder	97.08 ± 1.39
Sipirok (SP)	Powder	95.73 ± 1.51
Solok (SL)	Solid	93.62 ± 1.22
Sungaidareh (SD)	Solid	87.45 ± 1.84
Sarolangun (SR)	Solid	84.29 ± 1.93
Muarabungo (MB)	Solid	86.91 ± 1.51
Jambi (JB)	Solid	92.33 ± 2.09
Palembang (PL)	Powder	95.84 ± 2.94
Liwa (LW)	Powder	95.38 ± 1.49
Pontianak (PT)	Solid	86.14 ± 2.22
Sanggau (SG)	Solid	87.69 ± 2.23
Putussibau (PS)	Solid	83.79± 2.64
Murateweh (MT)	Solid	92.00 ± 3.09



Dracorhodin

3,4-dihydro-5-methoxy-6-methyil-2-phenyl-2H-1-benzopyran-7-ol

trendione

Figure 7. Structure of dracorhodin and 3,4-dihydro-5-methoxy-6-methyl-2-phenyl-2H-1-benzopyran (Shi et al. 2009); trendione (Parker et al. 2012).

Table 3. Compounds which were found and identified in 16 samples of dragon's blood resin stuff

Chemical compounds	Retention time (minute)	Samples of dragon's blood stuff (each corresponded to their location origin)																No. identified chemical compounds
		ML (%)	LS (%)	SL (%)	TT (%)	MD (%)	SP (%)	SD (%)	MB (%)	SR (%)	JB (%)	PL (%)	LW (%)	PT (%)	SG (%)	PS (%)	MT (%)	
ρ Vinilguaiacol	5.794	-	-	-	0.23	-	-	-	-	-	-	1.58	1.25	0.30	-	-	0.83	5
α Cubebene	5.995	-	-	-	-	0.02	0.04	-	-	-	-	-	-	-	-	-	-	2
4-Vinil-2-methoxy-phenol	6.038	-	-	-	0.09	-	-	-	-	-	-	-	-	0.07	-	-	0.10	3
α Copaene	6.225	-	-	-	-	0.07	0.05	-	-	-	-	-	-	-	-	-	-	2
Isopiperitenone	7.053	-	-	-	0.03	-	-	-	-	-	-	-	-	0.05	-	-	0.12	3
α Amorfene	7.170	-	-	-	-	0.02	0.06	-	-	-	-	-	0.06	-	-	-	-	3
Vianole	7.313	0.01	-	-	-	-	-	-	-	-	-	0.02	0.12	-	-	0.03	-	4
δ Cadinene	7.480	-	-	-	-	0.01	0.01	-	-	-	-	-	-	-	-	-	-	2
Diethyl phthalate	8.034	-	-	0.10	-	-	-	-	0.04	-	0.10	0.10	0.24	-	0.22	0.47	-	7
(+)-Spatulenole	8.235	-	-	-	-	0.05	0.08	-	-	-	-	-	-	-	-	-	-	2
5-Methoxy-4-methyl-1,3-benzenediole	8.638	-	-	0.03	-	-	-	-	-	-	-	-	-	-	0.04	0.12	0.10	4
Koiganal	10.139	-	-	-	-	0.01	0.02	-	-	-	-	-	-	-	-	-	-	2
Methyl esters palmitic acid	10.332	0.05	0.03	-	-	0.02	0.07	-	-	-	-	-	-	-	-	-	-	4
Viridoflorene	10.415	-	-	-	-	-	-	-	0.01	-	0.01	0.02	0.04	-	-	-	-	4
N-Hexadecanoic acid	10.651	-	-	-	0.08	-	-	-	-	-	-	-	-	-	-	-	-	1
Aromadendrene	11.028	-	-	-	-	-	-	0.13	-	0.03	-	-	-	0.09	-	0.12	0.08	5
16-Octadecenoic methyl ester acid	11.465	-	0.06	-	-	0.13	-	-	-	-	-	-	-	-	-	-	-	2
2-Hydroxy cyclopentadecanoneone	11.473	-	-	-	0.09	-	-	-	-	-	-	-	-	0.05	-	-	0.07	3
9-Octadecenoic acid	11.742	-	-	-	-	0.77	1.01	-	-	-	-	-	-	-	-	-	-	2
(E)-9-Octadecenoic acid	11.767	0.10	-	-	1.87	-	-	-	-	-	-	-	-	1.77	-	-	0.94	4
Linoleic acid ethyl ester	11.784	0.70	0.80	0.05	-	-	-	-	-	-	-	-	-	-	0.12	0.35	-	5
Diepisedrene-1-oxide	12.128	-	-	-	-	-	-	-	-	-	-	0.01	-	-	-	-	-	1
Heptadecane- (8)-carbonate- (1)	12.245	0.07	-	-	-	-	-	-	-	-	0.01	-	-	-	-	-	-	2
4a,8-Dimethyl-2-isopropyl perhydronaphtalene	12.622	0.18	-	-	-	-	-	-	-	-	-	-	0.10	-	-	-	-	2
Olealdehyde	12.631	-	0.30	-	-	0.41	0.20	-	-	-	-	-	-	-	-	-	-	3
Dodecyl succinic anhydride	12.849	-	-	-	-	0.22	0.32	-	-	-	-	-	-	-	-	-	-	2
1,3 Diphenylisobenzofuran	12.916	-	-	0.54	-	-	-	-	-	-	-	-	-	-	0.35	1.08	-	3
7-Pentadiene	12.983	0.09	1.10	-	0.63	1.06	2.02	-	-	-	-	-	-	0.85	-	-	1.65	7
Linoleic acid	12.168	0.70	-	-	1.19	-	0.40	-	-	-	-	-	-	2.05	1.60	0.21	1.92	7
Triphenyl phosphate	13.269	-	-	-	-	-	-	-	-	-	-	0.36	0.50	-	-	-	-	2
2-Monooleine glycerol	13.369	-	-	-	-	-	-	1.05	1.10	-	-	-	-	-	-	-	-	2
3,4-Dihydro-5-methoxy-6-methyl-2-phenyl-2H-1-benzopyran-7-ol	13.520	5.30	5.19	11.26	-	3.71	0.40	4.57	2.84	5.10	-	7.19	5.20	2.92	3.06	1.12	-	13
(Z)-9,17-Octadecadienoate	13.626	-	-	-	1.50	1.15	0.25	-	-	-	6.53	-	-	-	-	-	-	4
1,8-Dihydroxy-3-methoxy-6-methyl-anthraquinone	13.855	-	-	-	-	-	-	1.13	1.43	1.07	-	-	-	0.27	-	-	1.19	5
4- (4-Ethylcyclohexyl-1-penthyl-cyclohexene	14.099	0.58	0.24	-	1.26	1.50	2.86	-	-	-	-	2.01	1.37	-	-	-	-	7
Trendione	14.267	5.23	4.28	-	-	-	-	3.30	3.10	4.50	1.75	-	1.25	1.15	-	-	1.07	9
9,12-Octadecadiene-1-ol	14.787	-	-	-	3.78	-	-	-	-	-	-	-	-	-	-	-	-	1
9.10 Dideutero octadecanoic acid	14.904	1.56	1.83	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
4-Hydroxy-3,3,4-tri methoxy stilbene	14.913	-	-	-	3.16	-	-	-	-	-	-	-	-	-	-	-	-	1
2,6,10,14-Tetramethyl-pentadecane	15.424	-	-	-	-	-	-	1.58	1.03	1.20	-	3.14	2.23	0.05	-	-	0.34	7
Dracorhodin	15.785	6.29	5.87	5.93	4.56	3.05	4.07	4.06	2.47	4.24	1.36	0.28	2.65	0.67	2.21	1.26	0.91	16
4-Methoxy-6-methyl-2- (3',5'-dimethoxy benzyl)benzoic acid	17.236	-	-	-	0.84	-	-	-	-	-	-	-	-	0.08	0.56	-	0.16	4

Remarks: ML: Meulaboh, LS: Lhokseumawe, TT: Tapaktuan, MD= Medan, SP: Sipirok, SL: Solok, SD: Sungaidareh, SR: Sarolangun, MB: Muarabungo, JB: Jambi, PL: Palembang, LW: Liwa, PT: Pontianak, SG: Sanggau, PS: Putussibau, MT= Muarateweh

In conclusion, dragon's blood resin stuff is obtained from the extraction process of rattan fruits originated from Indonesia, which belongs to the genus *Daemonorops*. Chemically, there were 42 active compounds found in dragon's blood resin samples in both solid and powder formation. Three out of those 42 compounds were mostly detected and abundantly present in resin contents, of which dracorhodin compound was the highest rank, followed by 3,4-dihydro-5-methoxy-6- methyl-2-phenyl-2H-1-benzo-pyran-7-ol, and trendione. Therefore, dracorhodin may be applied as a most convincing marker compound for the presence of dragon's blood resin stuffs in *Daemonorops* (rattan genus) originated from several regions in Indonesia. It was also presumed that dracorhodin is a major compound, which relates to the widespread uses of dragon's blood resin stuff. These prospective results may confirm dracorhodin as a contained in the dragon's blood resin in rattan species originated from Indonesia. Although, the further research on other compounds (e.g. trendione, etc), which can potentially be used as effective compounds to distinguish the dragon's blood resin stuffs from other countries or other plant species should be done.

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Short Communication: Macropropagation – An important tool for conservation of North Sumatran endangered tree species, *Dryobalanops aromatica*

ARIDA SUSILOWATI[1,♥], CUT RIZLANI KHOLIBRINA[2], HENTI HENDALASTUTI RACHMAT[3], DENI ELFIATI[1], ASWANDI[2], ILLA MASYITAH RAENI[1]

[1]Faculty of Forestry, Universitas Sumatera Utara. Jl. Tridharma Ujung No.1 Kampus USU Medan, 20155. ♥Email: arida_iswanto@yahoo.co.id
[2]Aek Nauli Forest Research Agency. Jl. Raya Parapat Km 10,5. Parapat Sumatera Utara
[3]Forest Research, Development and Inovation, Ministry of Environment and Forestry. Jl. Raya Gunung Batu, Bogor-West Java

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Abstract. *Susilowati A, Kholibrina CR, Rachmat HH, Elfiati D, Aswandi, Raeni IM. 2018. Short Communication: Macropropagation - An important tool for conservation of North Sumatran endangered tree species, Dryobalanops aromatica. Biodiversitas 19: 1672-1675. Dryobalanops aromatica* or locally known as *kapur* is a tree producing borneol used for pharmaceutical purposes. Due to illegal harvesting for wood and borneol, depauperate population with low and rare reproductive mother trees and land conversion to oil palm plantation, its population decreases every year. *Ex situ* and *in situ* conservation efforts are needed to prevent this species from extinction. One of the *ex situ* conservation efforts that can be done is propagating this species vegetatively with shoot cuttings. Shoot cutting is technically simple and inexpensive method to produce new planting stock for further purposes such as production and conservation. This method has also been successfully used for the propagation of endangered and highly economically valuable species. Despite many applications of shoot cutting for clonal forestry, there was still lack of information about the successfulness of this method for *Dryobalanops aromatica*. Therefore the objective of this research was to get data about the successfulness of *kapur* cuttings using different media and growth regulator treatment. A factorial experiment using a Completely Randomized Design with two factors was conducted for this research. The first factor was cutting media, consisting of three types, namely (i) sand, (ii) combination of sand and soil, and 3) combination of sand, soil and rice husk. The second factor was plant growth regulator (PGR), consisting of two levels, namely (i) without PGR addition and (ii) with PGR addition. The parameters observed were survival percentage, rooting percentage, number of primary and secondary roots, length of primary and secondary roots and adventitious root formation. Results showed that rooting percentage of cutting using this technique varied from 30 to 60% and thus this technique was prospective to be developed as a tool for propagating *kapur* trees. Adventitious roots originated from the wounded area near the cambium which was later followed by the formation of callus and root primordia.

Keywords: adventitious roots, camphor, cuttings, medium, PGR, rooting

INTRODUCTION

Kapur, *Borneo camphor*, *camphor tree*, or *Sumatran camphor* is an emergent canopy tree species with hermaphroditic bee-pollinated flowers. This species is distributed in the lowland dipterocarp forests of Malaya, Sumatra, including Riau archipelago, and Borneo (Kitamura et al. 1994). *Dryobalanops aromatica* C.F.Gaertn. (Syn. *Dryobalanops sumatrensis* (J.F. Gmelin) A.J.G.H. Kostermans) or locally known as *kapur* is commonly found in mixed Dipterocarpaceae forests up to 300 m in altitude on hillsides with sandy soils. Its distribution includes Peninsular of Malay, Sumatra and Borneo. In Sumatra, it is distributed in the western part of Singkil, Natal River, between Sibolga and Padang Sidempuan to Airbangis, the south of Rokan River to the north of Batanghari. In the east part of Sumatra, it can be found in the Riau Island, in the west on Mursala Island, but not in Simalur, Nias, or Batu islands (Heyne 1987; Subiakto and Rachmat 2015; Rachmat et al. 2018). The timber has been logged by local people as it is known to have high quality of wood for construction and it is also extracted for resin production. The timber is categorized as Class I for its strength and durability, meaning that it can be used as major timber for construction and building.

Its resin is also well known in commercial market and has potential use in a wide range of medicinal purposes. *Kapur* crystal resin is present in the axial parenchyma tissues of the stem (Yamada and Suzuki 2004), and it is often harvested by cutting and splitting the stems. The resin can also appear on the bark of broken or injured stems. These white crystals contain borneol compounds, a form of terpene alcohols ($C_{10}H_{18}O$), which are widely used in the manufacture of fragrances, antiseptic and others (Huo 1995). In China, it is known as *Bing pian's* that serves as anti-inflammatory medicine and analgesic. Currently, it is also popular to be used in bio-panty which reduces pain during menstruation, reduces muscle and joint pains, helps cleanse the blood clots, and prevent the proliferation of germs (Duke 2005). Borneol originated from *Dryobalanops* is one of the most effective medicines for blood clots or blockage of blood vessels in human heart and brain (Dharmananda 2003).

The high utilization of *kapur* both for its wood and resin has not been followed by intensive conservation efforts including habitat protection. Habitat alteration due to conversion into palm oil plantations, settlements and agricultural purposes caused the decrease of its population both in numbers of trees and also the size of the trunks. The very apparent evidence of extreme condition for this species was described by Rachmat et al. (2018) in Mursala Island where big mature trees almost disappeared. Fruiting period that usually occurs once in 4-year interval may contribute further to its higher risk of extinction.

Conservation status for this species based on IUCN listing is Vulnerable (VU) (Barstow and Randi 2018). Therefore, a rapid effort is needed in order to conserve this species, and one of the approaches is by creating a breeding shortcut through vegetative propagation.

Vegetative propagation techniques are the first step in tree species domestication and offer opportunity of avoiding the problem of recalcitrant seeds. Vegetative propagation also facilitates the transfer of genetic potential as well as the non-additive variance of the parent to the new plant and offers availability of superior individuals in a short period of time for large-scale commercial plantation. One of promising vegetative propagation techniques is macrocutting. Compared to other techniques (e.g., microcutting, grafting) macrocutting is easier, cheaper, faster and economically more beneficial.

Root formation is a critical phase in determining the success rate of rooted cuttings. The root formation of cutting is a complex mechanism and influenced by physiological, environmental and genetic factors. Physiologically, the cutting becomes stressed after severance from the stock plant, and if there is little water or nutrient uptake, the cutting usually reduces stomatal conductance, until the root formation has been developed (Druege and Kadner 2008, Pop et al. 2011). Environmental factors, especially temperature, light, and relative humidity also affect the root formation (Sakai and Subiakto 2007). When environmental factors do not support the cutting, it will be unable to promote root formation. From the genetic aspect, individual cutting has its own ability to form the adventitious root because this phenomenon is related to gene activation after cutting process (Tiberia et al. 2011). Therefore it is necessary to find combination of some factors that will support root cutting formation (Susilowati et al. 2017) and produce new seedlings with high-quality root system (De Klerk et al. 1997).

MATERIALS AND METHODS

The cutting materials were originated from orthotropic shoot of 1-year-old naturally regenerated seedlings available at Aek Nauli Forest Research Agency, Pematang Siantar. The cutting media were sand, topsoil and carbonized rice husk (1: 0: 0; 1: 1: 0, 1: 1;1 v/v in ratio). Commercial auxin powder growth regulator was used for promoting root formation. Propagator box, shading net, pot tray and cutting scissors were also used as tools for this research.

Cutting experiment was carried out based on KOFFCO technique (Subiakto and Subiakto 2007) with minor modification. The cutting materials were taken from orthotropic branches and cut about 7-10 cm and immediately stored in container water. The cuttings were then repeatedly washed using sterilized water. The cuttings were planted in pot-tray containing sterilized medium and placed in propagation boxes according to the treatment. The propagation boxes were stored in a greenhouse with light intensity reduced to about 50 %. Watering was done twice daily, once in the morning before 10.00 a.m. and once in the late afternoon after 4.00 p.m to ensure the seedlings received enough water during the initial growth stage.

The factorial complete block design with two factors was used in this cutting experiment. The first factor was cutting media (I), consisting of I_1 (sand), I_2 (sand: topsoil) and I_3 (sand: topsoil: carbonized rice husk). The second factor was plant growth regulator treatment (A), consisting of A1 (without PGR addition) and A2 (PGR addition). Each combination was replicated into 10 pot-trays. Observation was done at 20 weeks after planting, parameters observed were cutting survival percentage, rooted cutting percentage, primary and secondary root length, and primary and secondary root number. Monthly assessments of cuttings were carried out for five months, i.e. until no more new roots were formed. At the end of each month, the number of rooted cuttings, number of roots and length of roots were recorded.

RESULTS AND DISCUSSION

The successfulness of cutting propagation varied with the plant species, some of which were easy to propagate, and others were difficult. The results of medium and auxin treatment on *kapur* cuttings after 20 weeks are presented in Table 1. At the end of observation period (20 weeks), all cuttings were healthy as indicated by their green leaves. Survival rate varied from 50 to 80%.

The high survival percentage of *kapur* cuttings at the end of observation was probably caused by the juvenility of cutting source materials. Similar research conducted by Maura-costa and Lundoh (1994) found the higher survival ability (80%) by using juvenile materials of *D. lanceolata*. In *Diploknema butyracea,* Zargar and Kumar (2018) found the decrease in survival percentage from 92.2% to 37.20% by using mature donor plants. According to Mitchelia et al. (2004), maturity stage of donor plants has negative influence on the performance of rooted cuttings. Hartman and Kester (1983) also stated that the juvenility of the stock plant can also be an overriding factor in root formation especially for plants which are difficult to root. The effect of juvenility on rooting ability may be related with low levels of rooting inhibitors as well as high levels of photosynthates, but as the plant grows older, the inhibitor levels increase (Kontoh 2016). A general decline in rooting ability, root quality, and survival has been associated with donor plants having reached a stage of reproductive or ontogenetic maturity (Hacket 1985).

The rooting ability of *kapur* varied from 30-60%. Lower rooting ability of *kapur* cutting may be caused by terpenoid present in cutting materials. The highest rooting percentage occurred in sand media and PGR treatment (60%) and the lowest rooting percentage occurred in sand: rice husk (1: 1) with no auxin addition (30%). The variance analysis (Table 2) showed that media and interaction between media and auxin did not significantly affect all observed parameters of cuttings, while the auxin addition only affected the length of primary roots. The same results were also found in *D. oblongifolia*. Brodie (2003) found that interaction between media and exogenous auxin treatment did not influence the rooting ability of cuttings and suggested other factors (e.g. individual condition of stock plant) were more important for rooting in *D. oblongifolia*.

Auxin addition significantly affected the length of primary roots in this research. Auxins play a critical role in the formation of adventitious roots by increasing initiation of the root primordium and growth via cell division (Fogaca and Fett Neto 2005). Auxins promote starch hydrolysis and mobilize sugars and nutrients to the cutting base (Das et al. 1997). During cell division and auxin transport, auxins act primarily through selective proteolysis and cell wall loosening with receptor protein transporting inhibitor response 1 and auxin-binding protein 1 (Da Costa et al. 2013). In this research, we used commercial auxin powder containing a mixture of auxins (IBA and NAA). Davies and Haissig (1990) stated that mixtures of root-promoting substances are sometimes more effective than either compound alone, and adding a small percentage of certain *phenoxy* compounds to either IBA or NAA increased rooting and produced root systems better than using *phenyl* compounds alone.

Adventitious root formation was also observed in this study. The results on *kapur* morphological observation of roots are described in Figure 1. The formation of adventitious roots is a high energy requiring process, which involves cell division, in which predetermined cells switch from their morphogenetic path to act as mother cells for the root primordia; hence more reserve food material is needed for root initiation (Aeschabacher et al. 1994). It is known that root formation is a critical phase that determines the success of propagation by cuttings. Physiologically the formation on cuttings depends on endogenous auxin in plant material and other synergistic components such as *diphenol*. The synergistic component promotes the synthesis of ribonucleic acid (RNA), which stimulates root initiation (Hartmann et al. 2002).

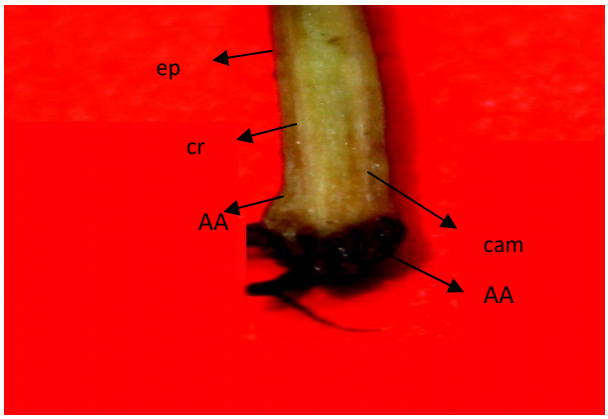

Figure 1. A root of *kapur* cutting (5x magnification). Epidermis cell (ep), cortex (cr), cambium (cam) and an adventitious root (AA)

Table 1. The average value of *kapur* cutting parameters (20 weeks after cutting)

Parameters	Treatment					
	I_1A_1	I_1A_2	I_2A_1	I_2A_2	I_3A_1	I_3A_2
Survival percentage (%)	70	80	50	60	60	70
Rooting percentage (%)	50	60	30	40	40	40
Number of primary roots	2	3	2	2	2	3
Number of secondary roots	24	29	16	28	20	27
Length of primary roots (cm)	1.76	2.81	2.45	4.50	2.81	8.42
Length of secondary roots (cm)	5.57	2.17	2.59	5.83	2.93	1.79

Table 2. The summary of variance analysis for cutting rooting ability

Variable	Treatment		
	Media	PGR	Media* PGR
Number of primary roots	0.88^{ns}	0.92^{ns}	0.86^{ns}
Number of secondary roots	0.90^{ns}	0.69^{ns}	0.64^{ns}
Lenght of primary roots	0.33^{ns}	0.05^{*}	0.79^{ns}
Lenght of secondary roots	0.67^{ns}	0.83^{ns}	0.34^{ns}

Note: ns = not significant at 5% level probability

The bud or leaf produces a complex compound different from auxin which stimulates the formation of roots. The compound found by Bouillenne and Went (1933) is called *rhizocaline,* a complex compound consisting of three components: (i) Specific factors translocated from leaf with chemical properties as ortho-dihydroxy phenol, (ii) Non-specific factors (auxin) transited and found in low biological concentrations, and (iii) The enzymatic factor residing within the cellular tissue, possibly polyphenols. The root initiation process occurs when *ortho-dihydroxy phenol* reacts with the addition of auxin and enzyme, resulting in acceleration of the respiratory process and cell mitotic division leading to cell and tissue differentiation.

Generally, adventitious root developments occur outside of the central portion of vascular tissue (Hartmann et al. 2002). The results of histological observations showed that root formation in *kapur* cuttings started from the outer bark outside the meristem on the cambium. Root formation began with the formation of callus, followed by root primordial formation and ended with the appearance of an adventitious root. Symptoms of rooting at *kapur* cuttings occurred at 18 -20 weeks of cuttings, indicated by the callus formation on the cuttings. In the conifer, Hartmann et al. (2002) found 4 stages of adventitious root formation process that begins with cell proliferation at the basal of cuttings, followed by differentiation of vascular tissue and periderm in the wounded area, differentiation of cutting areas around the cambium and phloem to further form prospective roots and end with meristem formation at the root. Furthermore, Tiberia et al. (2011) stated that physiologically, root formation was the role of auxin-responsive genes in controlling cutting response to auxin addition. This process is also affected by environmental factors and donor plant condition.

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Association between *XRCC1* exon 10 (*Arg399Gln*) gene polymorphism and micronucleus as a predictor of DNA damage among radiation workers

HARRY NUGROHO EKO SURNIYANTORO♥, YANTI LUSIYANTI, TUR RAHARDJO, SITI NURHAYATI, DEVITA TETRIANA

Center for Technology of Safety and Radiation Metrology, National Nuclear Energy Agency of Indonesia. Jl. Lebak Bulus Raya, Pasar Jum'at Jakarta Selatan 12070, Jakarta, Indonesia. Tel.: +62-21-7513906, Fax.: +62-21-7657950, ♥email: harry_nes@batan.go.id

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Abstract. *Surniyantoro HNE, Lusiyanti Y, Rahardjo T, Nurhayati S, Tetriana D. 2018. Association between* XRCC1 *exon 10 (*Arg399Gln*) gene polymorphism and micronucleus as a predictor of DNA damage among radiation workers. Biodiversitas 19: 1676-1682.* This study was aimed to examine the association between *XRCC1* exon 10 gene polymorphism and micronucleus frequencies in radiation workers and their relation to the confounding factors. This study involved 37 radiation workers and 37 controls from several hospitals in Indonesia. Genotyping of X-ray cross-complementing group 1 (*XRCC1*) exon 10 gene polymorphism and micronucleus assay were performed using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and Cytokinesis-Block Micronucleus assay (CBMN assay), respectively. The results indicated that MN frequencies were not significantly higher in the exposed workers than in controls (20.46±6.42 versus 16.89 ±9.72; P=0.07). The micronucleus frequencies of radiation workers with mutant genotype showed not significantly higher than controls in the same genotypes (22±6.64 versus 11.75 ± 8.13; P=0.11). The confounding factors, like age, years of employment and equivalent doses were significantly associated with micronucleus frequencies (P<0.05). The equivalent dose has a significantly positive correlation with micronucleus frequencies among radiation workers, increasing the MN frequencies by 16.3 per 1 mSv of equivalent dose (P=0.001). The genetic polymorphism of *XRCC1* gene exon 10 demonstrated no association with the extent of DNA damage in the hospital radiation workers. The MN frequencies were strongly associated with age, equivalent dose and years of employment.

Keywords: Gene polymorphism, *XRCC1* exon 10, micronucleus, ionizing radiation, radiation workers

Abbreviations: BER: Base Excision Repair; CBMN: cytokinesis-block micronucleus; DNA: deoxyribonucleic acid; MMR: Mismatch Repair; MN: micronucleus; NER: Nucleotide Excision Repair; PARP: poly-ADP-ribose polymerase; SNP: Single nucleotide polymorphisms; *XRCC1*: X-ray cross-complementing group 1

INTRODUCTION

Experts who work in radiobiology have studied the health risks of radiation workers exposed to low-dose ionizing radiation. The risk is not only in the form of the disease, but also the inherited mutations that indirectly increase the risk of a disease in offspring. Factors affecting health risks from low-dose radiation exposure include interactions between radiation with mutagen and carcinogens, varying repair mechanisms, cell sensitivity to radiation exposure and adaptive response variations that depend on antioxidants and radiation doses (Prasad et al. 2004). Individual survival requires genetic stability. Genetics stability maintaining requires an accurate mechanism of DNA replication and mechanisms to correct errors that occur continuously during the replication process. Less than one per thousand basic changes produce a permanent mutation, while the others will be fixed by DNA repair (Alberts et al. 2002).

Systems available in the human body, such as Mismatch Repair (MR), Base Excision Repair (BER), and Nucleotide Excision Repair (NER) can prevent the DNA damage. It can be repaired by enzymes through the BER pathway. X-ray cross-complementing group 1 (*XRCC1*) is one of the Poly (ADP-ribose) polymerase (PARP) family proteins that play a role in BER pathway by binding a single-strand of DNA and recruiting DNA repair protein (Wood et al. 2001). The *XRCC1* gene is located on chromosome 19q13.2-13.3, 33 kb in length, consisting of 17 exons, encoding a 2.2 kb transcript and 633 amino acids. Some studies (Norjmaa et al. 2016)) have shown that the *XRCC1* gene is related to single-strand breaks and base excision repair pathways. The *XRCC1* gene also plays an important role in DNA damage repair caused by ionizing radiation, X-rays, gamma rays, oxygen and alkylation agents. The *XRCC1* gene as the coder of *XRCC1* protein will produce three important functional enzymes, namely poly-ADP-ribose polymerase (PARP), DNA ligase III, and DNA polymerase β (Audebert et al. 2004).

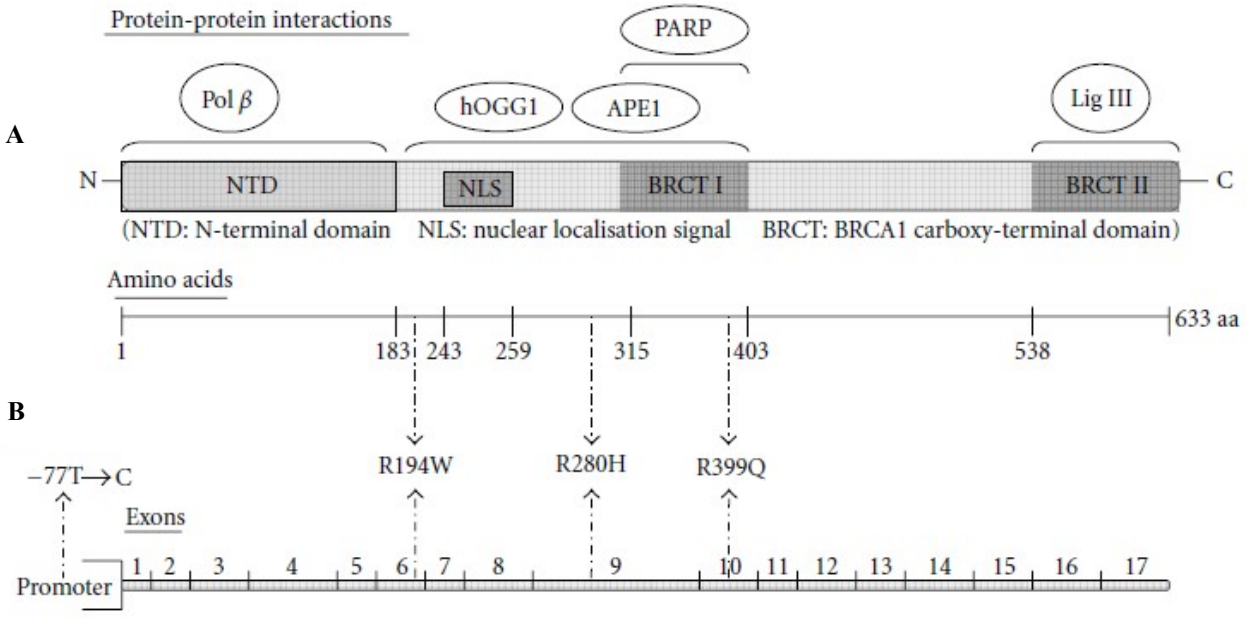

Figure 1.A. The domains of *XRCC1* gene exon 10 and it's interaction with other components of BER. B. The position of Single nucleotide polymorphisms of *XRCC1* gene (-77T>C, Arg194Trp, Arg280His and *Arg399Gln*) (Sterpone and Cozzi 2010)

Single nucleotide polymorphisms (SNPs) are the most common polymorphisms in humans with a frequency ≥ 1% in the population. SNPs that occur in DNA repair genes resulting in decreased DNA repair ability, increased mutation rate and cancer risk (Ochiai 2015). SNPs that occur in the *XRCC1* exon 10 gene change a nitrogen base G to A at codon 399 resulted in changes of amino acid arginine to glutamine (Norjmaa 2016). Some studies have shown an association between *XRCC1* gene polymorphism and DNA damage (Jiang et al. 2006; Zhao et al. 2006; Zhang et al. 2012; Saad-Hussein et al. 2017). One of DNA damage predictors is micronucleus, which is a biomarker of chromosomes damage or loss (Fenech 2007). Micronucleus is formed from fragments of chromosomes left in anaphase during the process of cell division. The cytokinesis-block micronucleus (CBMN), cytome assay method, is used to measure the frequency of micronucleus because it is more specific, accurate and the most widely used for measuring DNA damage in human populations (Heddle 1973; Fenech 2000).

Micronucleus occurrence is an important biomarker as a result of a response to the environment including diet and ionizing radiation exposure. Micronucleus measurements in radiation-working populations or people living in areas with high natural radiation exposure can be used to determine the impact of exposure to the DNA damage (Chang et al. 1997). This study was aimed to examine the correlation between *XRCC1* exon 10 gene polymorphism and micronucleus frequencies in radiation workers and their relation to gender, age, smoking status, years of employment, and an equivalent dose of ionizing radiation.

MATERIALS AND METHODS

Procedures

Study population

The study included 37 medical workers occupationally exposed to low doses of ionizing radiation in the Radiology and Radiotherapy Department at several hospitals in Indonesia and 37 controls who had never been occupationally exposed to ionizing radiation. Questionnaires were given to the subjects to find out complete information about gender, age, smoking status, years of employment, equivalent dose and history of disease ever suffered. Exclusion criteria included diagnostic X-rays, and radiotherapy underwent six months before sampling, which could have affected to the equivalent dose and/or DNA damage. The characteristics of the subjects are shown in Table 1.

There are five different working division among radiation workers: doctor, radiologists, radiotherapists, radiographers, and nurses. Each participant was briefed about the study protocol, with specific written information about the cytogenetic test, the aims of the study and signed informed consent. Blood samples (5 mL) was obtained from each subject (radiation workers and controls) and taken to the laboratory of Molecular Biology for further analysis.

Micronuclei assay

The CBMN-assay was performed as described by Fenech (2007) with some modifications. Lymphocyte cultures were incubated for 48 hours at 37°C, cytochalasin-

B (Sigma-Aldrich, St. Louis, MO) at a final concentration of 3 g/mL was added to cultures to block cytokinesis after 44 hours of incubation. The cultures were stopped at 72 hours, treated with a hypotonic solution for 4 minutes and fixed with two changes of methanol: acetic acid (3: 1, v/v). The fixed cells were spread onto clean glass slides and stained for 10 minutes with a 4% Giemsa solution. Microscopic analysis was performed under a light microscope with a 40 × 10 magnification and CBMN-assay parameters of micronucleus, verified under 1000× magnification. A score was obtained for slides from each subject. The frequency of binucleated cells containing one or more micronuclei was scored in 1000 cells per subject, to determine cytotoxicity following published CBMN-Cyt scoring-criteria refers to IAEA manual (Fenech 2007).

DNA isolation

DNA was isolated from lymphocytes extracted from whole blood using the QIAamp DNA Kit (Qiagen) according to the manufacturer's instructions. The obtained DNA was stored at -20°C.

Genotyping of XRCC1 exon 10

Genotyping of *XRCC1* exon 10 gene polymorphism was performed using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), as described previously by Andreassi et al. (2009) with the forward primer was 5'- AGTAGTCTGCTGGCTCTGG-3' and the reverse primer 5'-TCTCCCTTGGTCTCCAACCT-3'. The PCR reactions were carried out with a denaturation of 95°C for 3 minutes, followed by 35 cycles of 15 seconds at 95°C (denaturation), 15 seconds at 60°C (annealing) and 15 seconds at 72°C (extension) and finally 1 minute at 72°C (final extension). Following amplification, PCR products were digested using 10 U of restriction enzyme *Msp*I (BioLabs, Inc.) for 16 hours at 37 °C, and electrophoresed on a 3% agarose gel. The wild-type GG genotype for codon 399 was determined by the presence of two bands at 269 and 133 bp, the mutant heterozygous GA genotype was determined by the presence of three bands at 402, 269 and 133 bp, while the mutant homozygous AA genotype was determined by the presence of the uncut 402 bp band (indicative of the absence of the *Msp*I cutting site).

Data analysis

The data analysis was conducted with SPSS version 16.0 for Windows. All of the data were expressed as mean±standard deviation. Independent sample T-test was used to test micronucleus frequencies difference between exposed workers and controls and to test a significant relationship between micronucleus and various genotypes. Linear regression analysis was performed to assess the relationship between years of employment, equivalent dose, and micronucleus frequencies on exposed workers. Poisson regression analysis was applied to evaluate the influence of gender, age, smoking status, years of employment, and the equivalent dose of ionizing radiation at micronucleus frequencies in the whole population and both groups separately. The genotype and allele frequencies were showed on frequencies distribution table and were checked for consistency with Hardy-Weinberg equilibrium and compared between the radiation workers and controls group by $\chi 2$ tests. The level of significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

In this study, single nucleotide polymorphisms (SNPs) of *XRCC1* gene *Arg399Gln* was investigated. The genotype analysis of this SNPs of *XRCC1* gene, for medical radiation workers from several hospitals in Indonesia, was performed using PCR-RFLP method. The characteristic of subjects according to age and gender data was displayed at Table 1, and the genotyping results are shown in Figure 2. Based on Figure 2, GG genotype (wild-type) was showed with 269+133 bp fragment length, GA genotype with 402+269+133 bp, and AA genotype (mutant homozygous) with 402 bp.

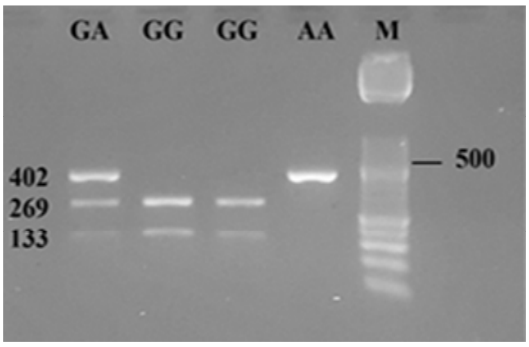

Figure 2. Results of genotyping for *Arg399Gln* polymorphism of *XRCC1* gene on 3% electrophorese gel. GG (wild-type), GA (mutant heterozygous), AA (mutant homozygous), M (DNA ladder 100 bp)

Table 1. Demographic characteristics of the study population

Parameters	Radiation workers	Controls	Total
Sample size (N)	37	37	74
Gender			
Females (%)	19 (51.35%)	18 (48.65%)	37 (50%)
Males (%)	18 (48.65%)	19 (51.35%)	37 (50%)
Age (years)			
Mean±SD	45±8.01	43.08±11.47	44.04±9.94
Range	29-59	20-58	20-59
Smoking status			
Never (%)	31 (83.78%)	30 (81.08%)	61 (82.43%)
Current (%)	6 (16.22%)	7 (18.92%)	13 (17.57%)
Years of employment (years)			
Mean±SD	20.78±7.47	-	-
Range	7-35		
Equivalent Dose (mSv)			
Mean±SD	0.21±0.2	-	-
Range	0.022-0.731		

This study was used totally 74 subjects which consisted of 37 radiation workers and 37 controls. Statistically, the characteristics of subjects, including gender, age, smoking status, years of employment and equivalent dose between radiation workers and controls did not show any differences among the subjects. The range of age was 29-59 years, the range of exposure duration (years of employment) to ionizing radiation for radiation workers was 7-35 years and the range of equivalent dose was 0.022-0.731 mSv (Table 1).

Micronucleus analysis

Micronucleus test results were reported as a total number of micronucleus per 1000 binucleated (BN) cells (Table 2). MN frequencies were not significantly higher in the hospital radiation workers compared to the controls (20.46 ± 6.42 versus 16.89 ± 9.72, P = 0.07). The frequency of MN in the never-smokers group was higher than in the smokers group, both in radiation workers (20.58 ± 6.42 versus 19.83 ± 6.57) and controls (17.77 ± 9.72 versus 13.14 ± 9.85), although the difference was not significant (P=0.19). The present study showed that the MN frequencies being higher in the AA genotypes group compared to the homozygous GG (wild-type) group (22±6.64 versus 21.39±6.42) in radiation workers. On the contrary, a decrease in MN frequency was observed in controls with AA genotype as compared to homozygous GG (wild-type) group (11.75 ± 8.13 versus 16.77 ± 9.84).

The years of employment for radiation workers were 7-35 years with an average working period was 20.78 ± 7.47 and the equivalent dose for radiation workers were 0.022-0.731 mSv with an average dose was 0.21 ± 0.2 (Table 1). Linear regression analysis was used to examine the effect of years of employment and equivalent dose to MN frequencies. There are a significant relationship between equivalent dose and MN frequency (β = 0.508, P = 0.001; Figure 3). On the contrary, no significant relationship between years of employment and MN frequency (β = 0.064, p = 0.706; Figure 4).

Poisson regression analysis results on gender, age, and smoking status are shown in Table 3. Gender has a significant effect on MN frequencies in control (P <0.0001), Age has a significant effect on MN frequencies in radiation workers (P=0.02) and controls (P <0.0001), smoking status has no significant impact on MN frequencies. Both of years of employment and equivalent doses have a significant effect on MN frequencies with P-value 0.044 and 0.001, respectively. Poisson regression analysis in the present study showed that the effect of gender on MN frequencies was higher in males than in females.

Correlation between equivalent dose and years of employment to MN frequencies

Linear regression analysis was used to examine the correlation between equivalent dose and years of exposure to MN frequencies. The result showed positive correlation between equivalent dose and MN frequencies among radiation workers. It means that MN frequency tended to rise with equivalent dose. This correlation have a statistically significant relationship (β = 0.508, P= 0.001) as shown in Figure 3. Correlation between years of employment and MN frequencies have no statistically significant (β = 0.064, P= 0.706) as shown in Figure 4.

Poisson regression analysis was also applied to evaluate the influence of age, gender, smoking status and dose equivalent of ionizing radiation on MN frequencies in the overall population and in both groups separately. The results showed that significant effects are indicated by age (P<0.0001), years of employment (P0.044) and equivalent dose (P=0.001) in the overall population, whereas in the controls group, the confounding factors influenced significantly to the MN frequencies are gender (P<0.0001) and age (P<0.0001), and in the radiation workers group is age (P=0.02), as shown in Table 3.

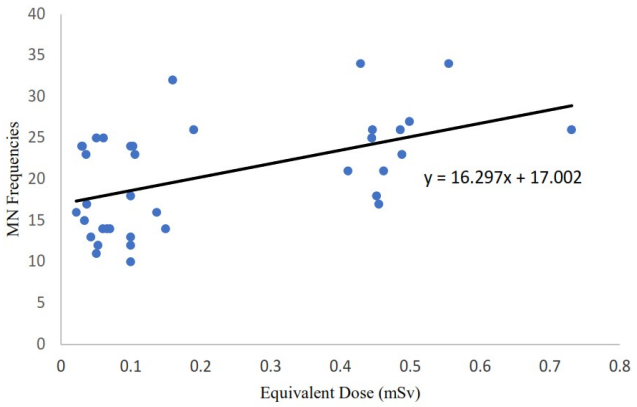

Figure 3. The relationship between DNA damage, assessed as MN frequencies in peripheral lymphocytes, and equivalent dose (mSv). The thick line is the result of linear regression analysis of the data. β = 0.508 , P = 0.001

Figure 4. The relationship between DNA damage, assessed as MN frequency in peripheral lymphocytes, and years of employment to ionizing radiation. The thick line is the result of linear regression analysis of the data. β = 0.064 , P = 0.706

Table 2. Micronucleus frequencies in the study population

	Radiation workers			Controls			P
	Subjects	**MN±SD**	**95% CI**	**Subjects**	**MN±SD**	**95 % CI**	
`All	37	20.46±6.42	18.39-22.53	37	16.89 ±9.72	13.76-20.02	0.07
Never smokers	31	20.58±6.42	18.32-22.84	30	17.77 ± 9.72	14.29-21.25	0.19
Current smokers	6	19.83±6.57	14.57-25.09	7	13.14 ± 9.85	5.84-20.44	0.19
XRCC1 exon 10							
GG	18	21.39±6.42	18.42-24.36	13	16.77 ± 9.84	11.42-22.12	0.14
GA	15	18.93±6.32	15.73-22.13	20	18 ± 9.85	13.68-22.32	0.73
AA	4	22±6.64	15.49-28.51	4	11.75 ± 8.13	3.78-19.72	0.11

Table 3. Poisson regression analysis of confounding factors on MN frequencies in peripheral lymphocytes of the study groups.

Confounding factors	**IRR**	***P***	**95% CI**
All			
Gender (1,2)	1.104	0.09	0.985-1.237
Age (years)	1.024	<0.0001	1.018-1.030
Smoking status (1,2)	1.087	0.296	0.929-1.273
Years of employment (years)	0.981	0.044	0.963-0.999
Equivalent dose (mSv)	1.978	0.001	1.345-2.910
Controls			
Gender (1,2)	1.455	<0.0001	1.213-1.745
Age (years)	1.034	<0.0001	1.026-1.043
Smoking status (1,2)	0.898	0.41	0.695-1.160
Radiation workers			
Gender (1,2)	1.050	0.56	0.891-1.237
Age (years)	1.021	0.02	1.003-1.039
Smoking status (1,2)	0.974	0.81	0.786-1.207

Note: IRR: Incidence Rate Ratio; [a]Gender: 1. Females; 2. Males; Smoking status: 1. Never; 2. Current

Table 4. Genotype and allele frequencies of *Arg399Gln* in the study population

	N (74)	**%**	**Radiation workers (n=37) (%)**	**Controls (n=37) (%)**	***P value***
Genotype codominant					
GG	31	41.89	18 (48.65)	13 (35.13)	
GA	35	47.29	15 (40.54)	20 (54.05)	
AA	8	10.82	4 (10.81)	4 (10.82)	0.39
Allele					
pG			0.69	0.62	
qA			0.31	0.38	

Genotype analysis

The genotype distribution in this study was consistent with the Hardy-Weinberg equilibrium for all the SNPs studied, both in radiation workers and controls, as shown in Table 4. The frequencies of genotypes for radiation workers were GG (48.65%), GA (40.54%) and AA (10.81%) with frequencies of G allele were (0.69) and A (0.31). The frequencies of genotypes for controls were GG (35.13%), GA (54.05%) and AA (10.82%), with frequencies of alleles, were G (0.62) and A (0.38). The results of the χ2-test showed no significant difference in the same genotype between radiation workers and controls (P=0.39).

Discussion

The strength and novelty of the present study is the investigation of biological markers of effect and susceptibility on the same population exposed to the chronic low level of ionizing radiation. To our best knowledge, this research has not been done in Indonesia. The present population study is the first in vivo study that combines genotype analysis in DNA repair genes with both exposure and micronucleus frequency in somatic cells of hospital workers, occupationally exposed to low levels of ionizing radiation, and controls. The use of micronuclei as one of the cytogenetic damage biomarkers has been done in previous research and proven to be a reliable biomarker of molecular DNA damage in the population exposed to ionizing radiation (Sakly et al. 2012). This approach offers the opportunity to complete cancer prevention programs for health surveillance of radiological workers, still based mainly on physical dosimetry.

The results of our study showed that MN frequencies were higher in the radiation workers compared to controls, although the difference was not significant (20.46 versus 16.89, P=0.07). The MN frequencies in current smokers-radiation workers were also higher than current smoker-controls (20.58 versus 17.77, P=0.19). The MN frequencies based on the division of the genotype group also showed the same results. The MN frequencies were higher in the radiation workers compared to controls, although not statistically significant. In the present study, MN frequencies were lower in the current-smoker subjects compared to never-smokers, both in the radiation workers and controls. This fact was in accordance with the previous study which states that nicotine can protect against reactive carcinogens contained in tobacco smoke (Nersesyan et al. 2011).

The subjects used in this study never received exposure exceeded the permitted dose limit, recommended by the

Nuclear Energy Regulatory Agency of Indonesia for radiation workers (20 mSv per year). The results of this study indicate a positive correlation between years of employment and an increase in MN frequency of 0.055 per 1 year, but it is not significant (P=0.706). On the other hand, the radiation workers group will be significantly increased in MN frequencies of 16.3 per 1 mSv of equivalent dose (P=0.001). This is similar to the study by Sakly et. al. (2012) which states an increase of MN frequency in hospital radiation workers in Tunisia, and the previous study which indicates any direct relation between MN frequencies in lymphocytes and radiation dose which can be used for purposes of biological dosimetry (Ozdal et al. 2016).

MN frequencies can indicate the confounding factors such as gender, age, and lifestyle inducing the chromosomal damage. Bonassi et al. (2011) stated that female subjects will experience higher MN frequencies than in male lymphocyte cells, but not significantly different in exfoliated buccal cells. In the other hand, Ferraz et al. (2016) stated that the gender factor did not affect the frequencies of MN, except for age, which is the most influential factor among all of the confounding factors. In our study, gender is significantly influenced only in the control group (P<0.0001), while in the radiation workers did not affect substantially (P=0.56), as well as in the whole population (P=0.09). The previous studies have shown that individuals at age range from 23-50 years will have an increase in MN frequencies, followed by a decline in age above 50 years (Orta & Gunebakan, 2012). The occurrence of decreased MN frequencies in individuals over 50 years is due to decrease cell proliferation ability with increasing age (Milosevic-Djordjevic et al. 2002). In our study, age is significantly influenced by the MN frequencies both in controls (P<0.0001), radiation workers (0.02) and the whole group (P<0.0001). The smoking habit also as a factor that affects the MN frequencies. Bonassi et al. (2003) stated that in most reports, the results are unexpectedly negative, and in many cases, smokers had lower MN frequencies compared to non-smokers subjects. This fact only occurs in smokers with less than 30 cigarettes per day, whereas for smokers with greater than 30 cigarettes or more per day will experience a significant increase in MN frequencies. The current-smokers subjects in this study spent cigarettes less than 16 cigarettes per day, so their MN frequencies were lower than never-smokers subjects. The smoking habit in our study is not significantly influenced to MN frequencies in all of the groups. The influence of these three factors showed the different results in many studies, so it is necessary to do a more in-depth study of these confounding factors.

Radiation workers with 7-35 years of employment and equivalent doses between 0.022-0.731 mSv were also studied in this study using Poisson regression analysis. The results show that years of employment and equivalent doses have a significant influence on MN frequencies with P value 0.044 and 0.001, respectively. This is due to the longer working period caused the increase in equivalent doses accumulation which received by radiation workers. A previous study by Qian et al. (2015) stated that radiation workers with >20 years of exposure time had higher MN frequencies compared to radiation workers with <20 years of exposure time. Syaifudin et al. (2017) also stated that the MN frequencies after irradiation of lymphocytes increased with the increased radiation dose, mainly for higher doses (>2 Gy). This is in accordance with the previous study which showed that the DNA damage is significantly affected by equivalent doses of ionizing radiation exposure and the length of the work period. Higher equivalent doses produced higher MN frequencies (Tucker, 2008).

Previous studies suggested that DNA repair (*XRCC1* and XRCC3) and folate-metabolism genes (MTHFR) also influence MN formation (Iarmarcovai et al., 2008). The genetic polymorphisms of DNA repair genes play an important role in the sensitivity of the individual genomes exposed to ionizing radiation (Damiola et al. 2014). The genotype analysis in this study showed no association between *XRCC1* gene polymorphism in all of genotypes groups and MN frequencies (Table 2). This result may be due to insufficient sample size thereby decreasing the strength of the research statistics. There are some limitations to this study. First, this retrospective study is characterized by some disadvantages including insufficient sample size, unavailable data, no randomization and limited accuracy of medical records. Second, the average of equivalent doses in this study was collected from the health records of workers exposed to ionizing radiation, and these data may be unstable since the data may be influenced by factors such as the population background. Finally, a population with 7-35 years of employment was selected, which may affect the precise measurements of the equivalent dose, consequently creating variances of the MN frequencies.

In conclusions, our study reported that radiation workers had higher MN frequencies compared than controls. The genetic polymorphism of *XRCC1* gene exon 10 demonstrated no association with the extent of DNA damage in the hospital radiation workers. The equivalent dose has a significantly positive correlation with micronucleus frequencies among radiation workers. Furthermore, it was found that the MN frequencies were strongly associated with age, equivalent dose and years of employment. In the subsequent studies, it is necessary to examine the DNA repair genes polymorphism in populations with controlled non-genetic factors, such as lifestyles, environments, and exercises that affect the MN frequency as a biomarker of DNA damage.

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Morphological characteristics and isozyme banding patterns of *Cucurbita moschata* at different altitudes

NUR RAHMAH HIDAYATI[1], SURANTO[1,2,♥], SAJIDAN[1]

[1] Bioscience Graduate Program, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57 126, Central Java, Indonesia. Tel./fax.: +62-271-662450
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57 126, Central Java, Indonesia. Tel./fax.: +62-271-663375, ♥email: surantoak@yahoo.com, nur.rahmah.hidayati@gmail.com

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Abstract. *Hidayati NR, Suranto, Sajidan. 2018. Morphological characteristics and isozyme banding patterns of* Cucurbita moschata *at different altitudes. Biodiversitas 19: 1683-1689.* Aims of this research were to investigate the morphological character and isozyme banding patterns of *Cucurbita moschata* plants grown at three different altitudes. Samples in this study consisted of leaf, stem, and flowers. The morphological characters were conducted by direct observation in the field and analyzed descriptively as well as statically by one way ANOVA. The isozyme bands appearance of esterase and peroxidase of leaf samples were conducted using polyacrylamide gel electrophoresis (PAGE). Qualitative approach was used to analyze the presence and the absence of isozyme bands, while Retardation factor (Rf) was used to analyze quantitatively. The results showed that most plants grown at middle altitude (351-750 m asl.) were well-developed in terms of length of leaves, stems and flowers. Accordingly, the isozyme banding pattern of peroxidase was also found varied in plants grown at middle altitudes from which the presence of very unique bands was detected. Conversely, the band detected in plants grown at the lower and the highest altitudes was similar in term of band's number but it was different in the quality of the bands. Meanwhile, esterase isozyme banding pattern of plants grown at the lower and higher altitude had more bands than the middle altitude. Based on this result it is obvious that the isozyme data could be used to support in understanding the diversity morphological characters of plants grown in three different altitudes. This early result suggests that altitudes as a crucial factor in contributing the expression of isozyme appearance, which is useful for further pumpkin characterizations.

Keywords: altitude, *Cucurbita moschata*, isozyme, morphology

INTRODUCTION

Cucurbita moschata D., which belonged to the family of Cucurbitaceae, have been recorded its ability to grow and develop in both the tropical area and the sub-tropic region with altitudes of 2200 m asl. (Paris 2010; Jacobo-Valenzuela et al. 2011). As perennial plants, pumpkin fruit is a valuable source of energy and nutrition due to contents of carbohydrate, lipids, protein, and minerals are high (OECD 2012; Suranto et al. 2015). Besides, its fruit contains sugar such as fructose, glucose, sucrose, myo-inositol and raffinose (Kami et al. 2011), this fruit also produces secondary metabolism, i.e., α and β carotenes acting as an antioxidant (Zaccari and Galetta 2015). This plant is easy to grow worldwide including in the area of Tegal District of Central Java-Indonesia. The altitudes of this district were ranging from lower land area or even 100 meters below sea level until more than 1200 meters above sea level (m asl.) (BPS 2017). These habitats enable plants to grow and adapt to the varied environmental conditions. The morphological variations of pumpkin plants could be interpreted as the active response of plant organs to varied environmental factors, such as soil, temperatures and weather conditions. These environmental factors could cause the morphological appearances of plant varied in between their populations, and this occurrence could be observed both during vegetative and generative periods. Many approaches have been employed in order to know the diverse morphological appearance was due to genetically induced variation, or environmentally induced variation. In recent years, the characterization of plants has been conducted not only based on morphological characters but also the isozyme banding pattern as reported by Premoli (2003) and Zolfghari et al. (2010). The use of plant isozyme in the characterization of plants have been conducted widely (Rejon et al. 2012; Houmani et al. 2016; Hartanti et al. 2017).

The electrophoretic isozyme approach was not only used to clarify the position of plant species in the correct taxon, but it could also be used to detect the presence of certain disease on plant organs (Suranto et al. 2017). As reported by Gautam et al. (2018) that isozyme banding pattern could be used to determine the effect of heavy metal in the soil on particular crop. Isozyme banding pattern has been widely used by researchers because of the electrophoretic plant proteins can be conducted very easily. And therefore esterase and peroxidase were chosen as the first consideration due to the fact that only small amount of samples used and only very limited times required as well as very low prices needed in running the experiment. Therefore in this study, morphological characters and isozyme banding pattern data were conducted in order to investigate the differences of *C. moschata* grown at three different altitudes.

MATERIALS AND METHODS

Environmental measurement

Prior to collecting the data for laboratory works, a number of environmental parameters were tested in the field. There was relative humidity (%), temperature **(°**C), soil pH and the quality of light intensity. All data were collected at three different level of altitudes.

Samples locations

Pumpkin plants used in this study was local rounded fruits type which is collected in every altitudes location of Tegal District, Central Java, Indonesia. The stratified sampling method was employed, and the only flowering plants were taken as a sample. There were three different altitudes used for sampling locations: I (1-350 m asl.), II (351-750 m asl.), and III (751-1050 m asl.). Those sample locations were classified as lower (I), middle (II), and high (III) altitudes, respectively. Observation in every single location of sampling was observed three times (Figure 1).

Morphological character

Stem

Thirty-six of stems from every single plant-resulted from every three different altitudes were used to characterize the stem morphology. The third internode from the top was chosen as the sample, which was then measured by its length, diameter, shape, surface, color, and hairness of the stem. The length and width were calculated quantitatively, while for the rest data were analyzed qualitatively.

Leaf

Using the same number as stem samples, leaves were measured in terms of length, width, and diameter of leaf, length, and diameter of petiole, petiole stripe, shape, dentateness, apex, basal, venatio, color, nervus lateralis, as well as hairness.

Flower

Employing thirty-six of flowers collected from every single plant at three different levels of altitudes. The flower characters such as color, number of petals, diameter and length of flowers, pedicellus, length and total number of calyxes.

Planting procedures

The transplanted plants from the natural habitat were collected and planted in the experiment field at Departement of Biology, UNS. Forty-five (45) plant samples from nine (9) locations of three (3) different altitudes were grown in 45 polybags containing compost soil. Watering was done every two days using tap water, and fertilizer was given once in three weeks times.

Electrophoresis and isozyme procedures

Total number of 45 leaves were collected from three different location (Figure 1). Procedures of running electrophoresis of isozymes which consisted of preparation of solution, acrylamide gel, extraction of sample, electrophoresis, and staining procedure were conducted according to Suranto (2001), with some modification.

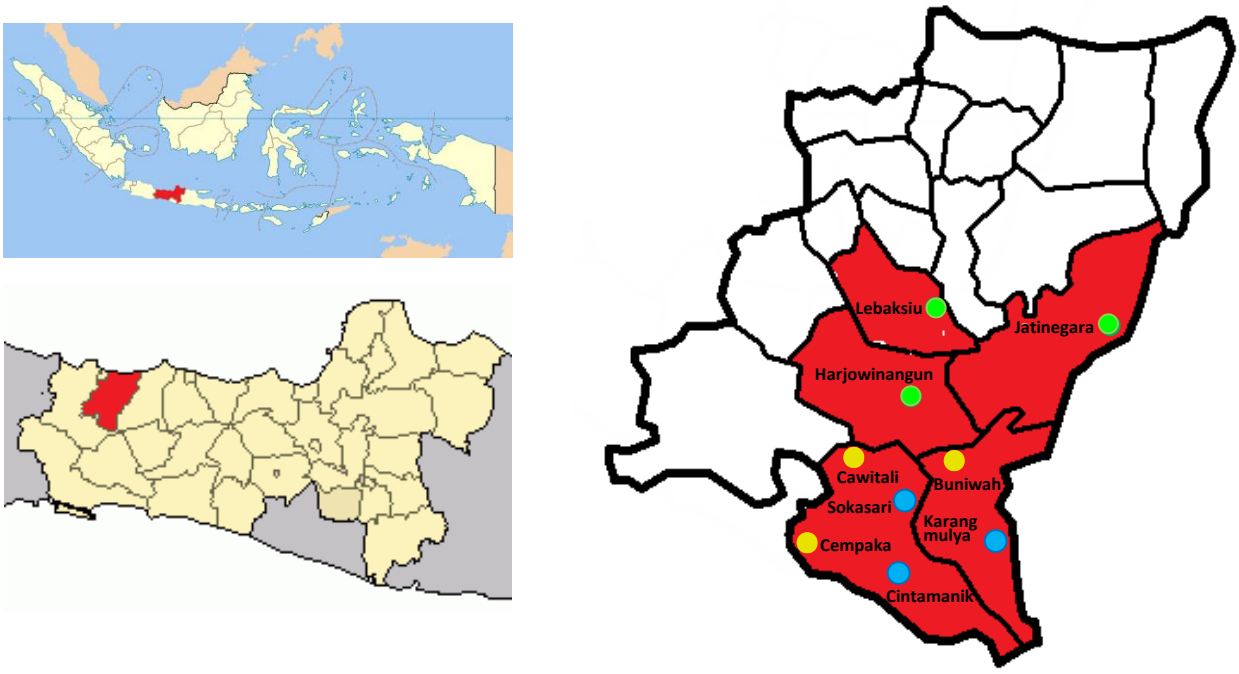

Figure 1. Map area of Tegal District, Central Java, Indonesia, where samples of *Cucurbita moschata* are collected. Note: ● = 1-350 m asl., ● =351-750 m asl., ● = 751-1050 m asl.

Buffer solution

Buffer solution consists of a tank buffer that is used during the electrophoresis process and extraction buffer which is used to extract fresh leaves. The buffer tank is made by dissolving 14.4 grams of boric acid and 31.5 grams of boric into distilled water until it reaches a volume of 2 liters. Extraction buffer is made by dissolving 0.018 grams of cysteine, 0.021 grams of ascorbic acid, and 5 grams of sucrose into 20 ml of pH 8.4 tank buffer.

Stock solution

Stock solutions consist of stock A made by dissolving 4.5 grams of TRIS (Hydroxymethyl) Methylamine (PURISS); 0.51 grams of citric acid, and 500 ml of distilled water and stock B by dissolving 30 grams of acrylamide; 0.80 gram N N'-methylene-Bis-Acrylamide; and 100 ml of distilled water. Stock A and stock B are prepared for making of acrylamide gel. *Preparation of acrylamide gel* Acrylamide gel is made by mixing 5 ml of stock A solution and 2 ml of stock B solution then being shaken until homogeneous. After homogeneous 5 µl TEMED is added and 7.5 µl 10% APS.

Leaf extraction A total of 0.5 g of fresh leaves were extracted using 0.5 ml of extraction buffer. The leaves are mashed using cold mortar to maintain the stability of the enzyme. Leaf samples were centrifuged at a speed of 4000 rpm for 3 minutes. The supernatant obtained is used for the electrophoresis process.

Electrophoresis

Peroxidase: as much as 2.5 µl of supernatant were inserted into the gel well and run at constant voltage (80 V) for 55 minutes. Esterase: as much as 3 µl of supernatant were put into a gel well and run at a constant voltage (80 V) for 55 minutes.

Enzyme staining

Peroxidase: the gel is soaked into the peroxidase enzyme dye made by dissolving 0.0125 grams of O-Dianisidine into 2.5 ml of acetone. Next, 20 ml of acetate buffer pH 4.5 was added and 2 drops of H_2O_2 were added. The gel was soaked for 5 minutes until the band pattern appeared and rinsed using distilled water.

Esterase: esterase enzyme staining was carried out by mixing 0.025 grams of 1-naphthyl acetate, 0.0125 grams of fast blue BB salt dissolved in 2.5 ml of acetone and 20 ml of phosphate buffer pH 6.5. Gel is left for 3 hours and rinsed with distilled water

Data analysis

For the morphological data have been analyzed descriptively, meanwhile, the morphometric data was analyzed using One way ANOVA, and then followed by Duncan Multiple Range Test (DMRT). Data of electrophoretic isozyme were analyzed both using qualitative and quantitative methods. For the presence and absence of the bands detected including the thickness. Qualitative approach was chosen, meanwhile, the movement of bands was calculated based on the Retardation factor (Rf) as explained by Lehmann et al. (1989).

RESULTS AND DISCUSSION

Environmental conditions

The condition of microclimate of each altitude is presented at Table 1. All parameters of environmental conditions varied except for the soil pH (7). In this study, air and soil temperatures decreased along with the increased altitudes. Meanwhile, contrary pictures were recorded for the relative humidity (RH), and no different data were obtained when soil pH from three altitudes was tested. The lowest light intensity was found at the highest altitude, while the highest light intensity recorded in the low altitude.

Morphological characters

Pumpkin plants grew very well at the second altitudes (Table 2). This could be proved by the result of the stem length (12.567±0.071 cm) and stem diameter (1.023±0.008 cm). For the rest of two altitudes showed quite similar in their length and diameter. In general, the shape of observed stem was similar (pentagonal). However for the color of stem and hairness were a bit varied. Dark green was detected for the plants which grew at second altitudes (351-750 m asl.), while for the other two altitudes were light green. Accordingly, the very dense, smooth, long hairness were detected at the highest altitudes, while for the lowest altitude occurred conversely. In all cases, the second altitudes of plants showed to have quite long dense hairs with the texture was quite rough.

For the leaf character examinations, there was significant difference on the length of leaf and petiole, but those in the first (I) and second (II) altitudes were not examined. It is recorded that the longest leaf was found at second altitude (12.966±0.055 cm). Conversely, the longest petiole was found at the first altitude (9.100±0.055 cm) and the smallest of petiole diameter (0.426±0.005 cm) was detected at the first altitude. This occurrence was also recorded for narrowest leaf (10.800±0.550 cm) which was found at the first altitude.

The morphological characters of leaves were examined for their shape, dentateness, basal leaf, hairness, apex, venatio, and nervus lateralis. Results showed that the morphological characters of leaves were indifferent in leaf, shape, dentateness, and basal (Table 5). However, the color of leaves, as well as the hairness, were quite varied. The light green was detected at the highest altitude.

Other morphological characters such as flower were also shown similarly. The only petiole and calyx length of *C. moschata* flowers were recorded varied, but the other characters were not (Table 6).

Table 1. Microclimate condition of each three different altitudes

Parameters ($\bar{X}$)	Altitudes (m asl.)		
	I (1-350)	II (351-750)	III (751-1050)
Air temperature (°C)	38.64	33.82	26.95
Relative humidity (%)	53.55	56.22	81.00
Soil temperature (°C)	31.06	28.41	25.33
Soil pH	7	7	7
Quality of light intensity	High	Normal	Low

Table 2. Result of morphological character tested of *Cucurbita moschata* a three different level of altitudes

Stem characters (cm)	Altitudes (m asl.) I (1-350)	II (351-750)	III (751-1050)
Length	9.800[a]±0.057	12.567[b]±0.071	11.733[c]±0.082
Diameter	0.690[a]±0.005	1.023[b]±0.008	0.786[c]±0.007

Note: Based on the Duncan Multiple Range Test (DMRT), the numbers followed by the different letters in same row are significantly different, P<0.05

Table 3. Result of morphological characters tested from *Cucurbita moschata* at three different level of altitudes

Stem characters	Altitudes (m asl.) I (1-350)	II (351-750)	III (751-1050)
Shape	Pentagonal	Pentagonal	Pentagonal
Colour	Light green	Dark green	Light green
Stripe surface	Clear	Very clear	Clear
Hairness	Rough, short, very seldom	Pretty rough, long, Pretty dense	Smooth, long, very dense

Table 4. Result of leaf character tested of *Cucurbita moschata* at three different level of altitudes

Leaf characters (cm)	Altitudes (m asl.) I (1-350)	II (351-750)	III (751-1050)
Length	11.833[a]±0.088	12.966[b]±0.055	11.867[c]±0.149
Width	10.800[a]±0.550	11.633[b] ±0.080	11.867[c]±0.054
Diameter	11.267[a]±0.083	12.200[b]±0.119	11.867[ab]±0.149
Petiole diameter	0.426[a]±0.005	0.517[b]±0.005	0.623[c]±0.005
Petiole length	9.100[a]±0.055	7.883[b]±0.108	6.850[c]±0.098
Total of petiole stripe	13.000[a]±0.301	12.667[a]±0.333	12.083[a]±0.358

Note: Based on the Duncan Multiple Range Test (DMRT), the numbers followed by the different letters in same row are significantly different, P<0.05

Table 5. Leaf morphology of *Cucurbita moschata* observed at three different level altitudes

Leaf characters	Altitude (m asl.) I (1-350)	II (351-750)	III (751-1050)
Shape	Orbicularis	Orbicularis	Orbicularis
Apex	Acuminatus	Acuminatus	Acuminatus
Dentateness	Serratus	Serratus	Serratus
Basal	Emarginate	Emarginate	Emarginate
Venatio	Palminervis	Palminervis	Palminervis
Colour	Dark green	Dark green	Light green
Nervus lateralis	Very clear	Clear	Clear
Hairness	Smooth, dense	Pretty rough, pretty dense	Rough, very dense

Table 6. Flower characters *Cucurbita moschata* examined at three different level of altitudes

Flower characters (cm)	Altitudes (m asl.) I (1-350)	II (351-750)	III (751-1050)
Pedicellus length	8.433[a]±0.068	15.933[b] ±0.068	4.600[c]±0.086
Diameter	13.308[a]±0.096	13.600[b]±0.082	10.067[c]±0.08
Calyx length	5.083[a]±0.078	4.167[b]±0.061	3.608[c]±0.104
Length of petal	9.225[b]±0.056	9.267[b]±0.072	7.100[a]±0.070

Note: Based on the Duncan Multiple Range Test (DMRT), the numbers followed by the different letters in same row are significantly different, P<0.05

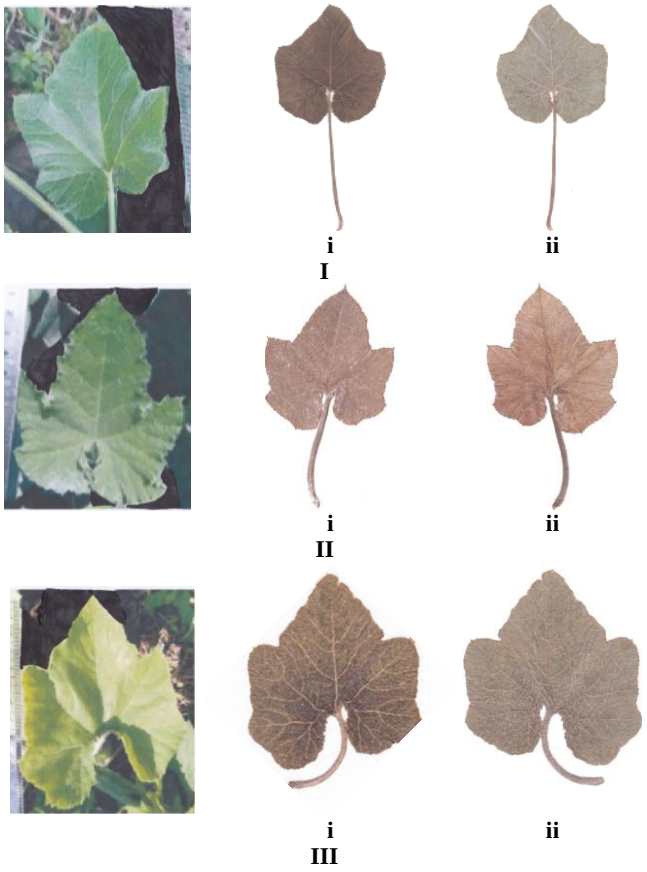

Figure 2. Morphological character of *Cucurbita moschata* leaves taken from three different levels of altitudes. Note: I (1-350 m asl.), II (351-750 m asl.), III (751-1050); (i) upper surface of leaves, (ii) lower surface leaves

Figure 3. Flower character of *Cucurbita moschata* at different level of altitudes. Note: I (1-350 m asl.), II (351-750 m asl.), III (751-1050 m asl.)

Table 7. Flower characters *Cucurbita moschata* examined at three different level of altitudes

Flower character	Altitude (m asl.)		
	I (1-350)	II (351-750)	III (751-1050)
Colour	Yellow	Yellow	Yellow to violet
Total number of calyx	5	5	5
Total number of petal	5	5	5

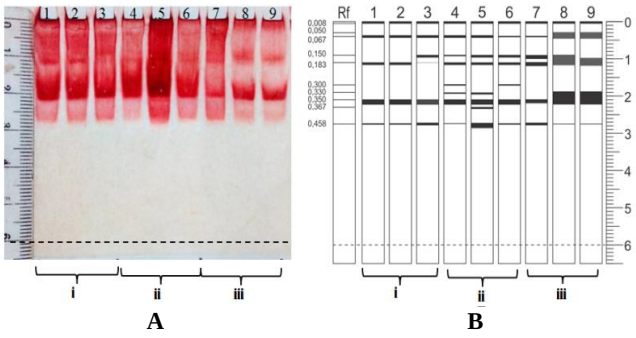

Figure 4. Peroxidase isozyme of leaf samples of *Cucurbita moschata* collected from three different lands of altitudes. Note: 1. 130 m asl., 2. 267 m asl., 3. 330 m asl., 4. 411 m asl., 5. 560 m asl., 6. 615 m asl., 7. 756 m asl., 8. 880 m asl., 9. 962 m asl. i. lower, ii. middle, iii. high. A. Band pattern, B. Zymogram

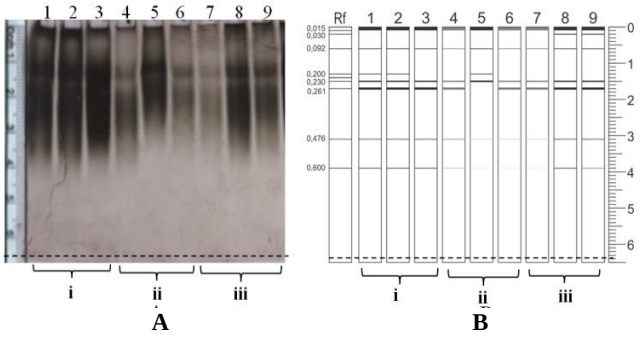

Figure 5. Esterase isozyme of leaf samples of *Cucurbita moschata* collected from three different lands of altitudes. Note: 1. 130 m asl., 2. 267 m asl., 3. 330 m asl., 4. 411 m asl., 5. 560 m asl., 6. 615 m asl., 7. 756 m asl., 8. 880 m asl., 9. 962 m asl. i. lower, ii. middle, iii. high. A. Band pattern, B. Zymogram

The longest petiole was found at the second altitude (15.933 ±0.068 cm). In this altitude, the widest of diameter of flower (13.600±0.082 cm) was also found. Meanwhile, the longest calyx length was recorded at the first altitude (5.083±0.078 cm), and the shortest was noted at the highest altitude (3.608±0.104 cm). It is interesting to note that the flower color of this pumpkin plant turns out to be violet (yellow to violet) when grown at the highest altitude. But the other characteristics were found no different (Table 7).

Isozyme analysis

The results of peroxidase isozymes analysis show differences in the banding pattern formed at three altitudes (Figure 4). The Rf values formed to range from 0.008 to 0.458. As many as 10 Rf, that was 0.008, 0.050, 0.067, 0.150, 0.183, 0.300, 0.330, 0.350, 0.367, 0.458 formed from a range of 1-350 m altitude to 751-1050 m asl. Rf formed at lowest altitudes (1-350 m asl.) and highest (751-1050 m asl.) altitudes had a similar pattern of band appearance with the value of Rf 0.008, 0.067, 0.183, 0.350, and 0.458. However, the thickness of the band at high altitudes thicker than the low altitude. There is one additional Rf, which is at Rf 0.150 at 330 m asl. and 756 m asl. Bands formed in the middle altitude are the most varied, i.e., the formed bands increase at Rf 0.300 (411 m asl. and 615 m asl.), 0.330 (411 m asl., and 560 m asl.), and Rf 0.367 (560 m asl.). The pattern of esterase isozyme bands (Figure 5) formed less varied than that of the isozyme peroxidase (Figure 4).

The bands produced by esterase isozyme were 8 (eight) bands, ranging from 0.015 to 0.600, such as Rf 0.015, 0.030, 0.092, 0.200, 0.230, 0.261, 0.476, and 0.600. Two additional bands were recorded at lower altitude (0.200 for the location of isozyme 130 m asl. and 267 m asl.). There was no band pattern obtained from esterase leaf samples in the middle altitude especially 560 m asl. at Rf 0.0261. However, the band at Rf 0.200 was detected. Based on the thickness band from esterase samples of the middle altitude, it showed that band at 0.600 was very painful. At the highest altitude, particularly at 880 m asl. and 960 m asl, the presence of band was only at Rf 0.030.

Discussion

The axis level of altitude locations will produce different light intensity. The highest altitude would almost always produce low level of light intensity. Therefore, the temperature will also very low and relative humidity would eventually go up. The lower temperature will influence the lowest of soil temperature. Other environmental factors such as oxygen level, soil condition, type of soil, as well as soil porosity, would contribute to the plant morphological appearance (Yuliani et al. 2015). Those all environmental factor would produce different morphological characteristics such as stem, leaf, flower both quantitatively and qualitatively. It is generally accepted that plants would adapt to the environmental condition both physiology and morphology (Gong et al. 2018) and it is recorded that plasticity of plant has been recorded as a good way of plant in responding the different environmental conditions (Frei et al. 2014). In addition, the plant plasticity of stem will be influenced by light intensity (Ye at al. 2017). This phenomenon has been shown by the length and diameter of stem particularly at middle altitude as compared to other altitudes. Yuliani et al. (2015) reported that plant in the family Asteraceae possessed more stem length at middle altitudes compared to the lower and highest altitudes. This occurrence was confirmed by Kofidis et al. (2007) in which the plant would grow maximally under favorable conditions and would not depend on the highest or lowest habitat.

In general, the color of stem of *C. moschata* particularly grew at middle altitudes was darker compared two other altitudes. This could be due to the optimum of light intensity produced, and this could influence the anthocyanine pigment on the stem, so that it will influence the quality of stem color (Cruz et al. 2012).

Plant growing at lower altitude usually has a narrower leaves as compared to middle and highest altitudes. Pan et al. (2013) recorded that the leaf shape of plants growing at highest altitude is normally bigger. This might interpret as a result of limited nutrient availability and this resulted in very slowly transportation, and this wider leaves needed to catch the sun in order to fulfill the plant nutrition. This condition will also influence the plant petiole. In this condition usually, plants have also very small petiole. In addition, plant grow at the highest altitude usually have leaf color younger than the other altitudes. It is noted that the quality of light intensity at higher altitude (III) could not be maximized of plants for their petiole development due to the big leaf diameter. Good quality of light intensity would result in producing the content of chlorophyll A and B. This chlorophyll would derivate the plant photosynthesis, and this would eventually influence the growth of plant normally (Samuoliene et al. 2007; Abidi 2012).

Morphological character particularly the length and diameter of flower at middle altitude gave the highest influence on plant metabolism especially carbohydrate in the leaf during reproduction phase (Bhandari et al. 2016). Besides, plant has ability to response the lower or higher temperature changes by remodeling lipid membrane and defencing unsaturated lipid or change both lipid membrane or unsaturated (Zheng et al. 2011). The smallest value of pedicels length and flower diameter was recorded at the highest altitude. Yaqoob and Nawchoo (2015) noted that length and diameter of pedicels tended to be smaller at the high altitude. This could cause the plant flowering earlier at lower altitude (Frei et al. 2014). The length of petal at the lower and middle altitude significantly different with the highest altitude. Dierig et al. (2006) reported that flowering time and the change of air temperature at lower altitude influence the growth and development of plant.

The flower colors at the highest altitude usually look brighter when they were compared with other altitudes. This occurrence may be due to different light intensity could influence the distribution of color pigment on the plant (Cruz et al. 2012). Moreover, Shrestha et al. (2014) said the difference in flower color could be influenced by pollinator richness within a plant habitat. The morphological diversity of leaf, stem, and flower in three different level of altitudes may need further evidence such as electrophoretic isozyme to make sure whether this could be genetically induced (Lo Sciavo et al. 1983).

The use of isozyme banding pattern has been used to differentiate the variation of two type of *C. moschata* (Wu and Cao 2008). Varied band was recorded to lower altitude to the higher altitude. The highest total number of bands were recorded at middle altitude rather than lowest or highest altitude. This peroxidase which is included into oxidoreductase enzyme could act as an antioxidant on the plant (Rothe 1994). This occurrence evidence confirmed that middle altitude has resulted in the optimization of enzyme peroxidase metabolism.

Gulen and Eris (2014) noted that sensitivity of peroxidase would be influenced by the difference of temperature. Different altitude with the alteration of air temperature influences the appearance of peroxidase banding pattern. This occurrence could be observed from the appearance of peroxidase isozymes in both of these altitudes in which showing thicker bands compared to the middle area (Rf 0.0183 and 0.350 at Figure 4).

The isozyme banding pattern of esterase showed its high activity at both lowest and highest altitudes compared to the middle one. This activity was quite different as the peroxidase did. This phenomenon may indicate that every single enzyme would response different to the different environmental condition (Ivachenko et al. 2016).

Subramani et al. (2011) recorded that this esterase, as a hydrolytic enzyme which has function in the seed germination and plant maturation. Thus, at both altitudes of highest and lowest, plant adapt to the unfavorable conditions and this could be expressed by more detected in additional bands or was thicker bands (Figure 5).

Based on the morphological appearance of leaf, stem, and flower as well as isozymes banding pattern, *C. moschata* tended to be better growing at middle altitude, rather than other altitudes. The warm temperature would eventually enhance the growth and development of the plant. And this three different altitudes could be used as on early evidence in using the environmental factor in characterizing the pumpkin plant.

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The grouping system and local distribution pattern analysis of Javan green peafowl (*Pavo muticus muticus*, Linnaeus 1758) population in Baluran and Alas Purwo National Parks, East Java, Indonesia

JARWADI BUDI HERNOWO♥, HADI SUKADI ALIKODRA♥♥

Ecology and Wildlife Management Laboratory, Department of Forest Resource Conservation and Ecotourism, Faculty of Forestry, Institut Pertanian Bogor. Kampus IPB Dramaga, Jl. Lingkar Akademik, Bogor 16680, West Java, Indonesia. Tel.: +62 251-8621244, Fax.: +62 251-8621244, ♥email: jblina11@yahoo.com, ♥♥halikodra@gmail.com

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Abstract. *Hernowo JB, Alikodra HS. 2018. The grouping system and local distribution pattern analysis of Javan green peafowl (Pavo muticus muticus, Linnaeus 1758) population in Baluran and Alas Purwo National Parks, East Java, Indonesia. Biodiversitas 19: 1690-1695.* The Javan green peafowl population lives in a group system. The population applies a small size group system. The distribution of the birds in Java Island is randomly fragmented and isolated in several types of habitat and each has a small number of individuals in every group. Baluran and Alas Purwo National Parks, East Java, Indonesia as part of Javan green peafowl (*Pavo muticus muticus*, Linnaeus 1758) distributions have been selected for the study on the grouping system and the analysis of local distribution. The research was aimed at obtaining data and information on the grouping system and local distribution of Javan green peafowl population in Baluran and Alas Purwo National Park. The number of individuals and groups was counted by applying a transect method and a concentration method on every type of habitat where peafowls are present. The distribution pattern data were analyzed by using a formula (Ludwig and Reynolds 1988). The results indicate that Javan green peafowl population is living in small groups (2-4 birds). There are 5 types of Javan green peafowl groups in Baluran National Park (BNP) and Alas Purwo National Park (APNP). The dominant group is adult female group consisting 3 individual members. The leader of the group is a female bird. Adult males live in solitary. The group system among Javan green peafowl populations is a strategy of the birds. Local distribution of Javan green peafowl populations in Baluran and Alas Purwo National Parks is mostly in the form of clumped dispersion.

Keywords: Alas Purwo, Baluran, group, Javan green peafowl, local distribution

INTRODUCTION

Javan green peafowls are distributed and scattered into fragmented habitats with small populations in several types of habitats (Balen et al. 1995; Hernowo 1995; Hernowo and Hernawan 2003; Hernowo and Wasono 2005). Green peafowls are present in protected areas such as sanctuary reserves, game reserves as well as national parks, and also in unprotected areas such as forest plantations or estate plantations. Alas Purwo and Baluran National Parks are two of the distribution sites of Javan green peafowls at the tip of the eastern part of Java Island. Baluran National Park has typical savanna and monsoon forest habitat, while Alas Purwo National Park has more diverse types of habitats compared to Baluran National Park (Hernowo 2017). The habitat in Alas Purwo includes low land tropical rainforests, grazing areas, and teak plantations with intercropping. Baluran has a typical monsoon climate with a long dry season. This climate is heavily influenced by the southeast wind during the period of April to October, with less precipitation. The average dry season is approximately 7-8 months each year. Meanwhile, Alas Purwo has type B rainfall according to Smith-Ferguson classifications and has annual precipitation of 1279-1554 mm per year (Hernowo 2011; Hernowo 2017). The dry season in BNP begins in May and ends in November, while in Alas Purwo it is from June to October.

Javan green peafowl populations have been experiencing high pressure posed by illegal hunting of the birds' (for their eggs, train feathers, individuals) lost habitat, as well as the conversion or destruction of their habitats. Illegal hunting has led to the extinction of local populations of Javan green peafowls in their local distribution. Balen et al. (1995) stated that during the last decade the most serious problem for Javan green peafowls is poaching, which has made the birds endangered. Habitat destruction and conversion have affected the quality and quantity of their habitats, such as the availability of food, shelter, and cover. However, the reality in the field shows that the birds still exist in their local distribution. This means that green peafowls have strategies on how to overcome the obstacles. One of their strategies is to apply the grouping system among Javan green peafowls.

Green peafowls live in a group system (Delacour 1977; Hernowo 1995). There is very limited information on the grouping system of Javan green peafowls. It is interesting to analyze the grouping system of Javan green peafowl populations namely how Javan green peafowls apply their strategies to overcome the high pressure and also their local distribution.

The paper aims at analyzing the grouping system among Javan green peafowl populations and their local distribution pattern in relation to the types of habitats.

MATERIALS AND METHODS

The observation

Research was conducted in Baluran National Park (BNP) of Situbondo and Banyuwangi districts, East Java, Indonesia from June to October 2006, in 2007, March 2009, March 2010, April 2012, April 2013 and in Alas Purwo National Park (APNP), Banyuwangi District, East Java, Indonesia, from August to December 2006, in 2007, March 2009, March 2010, April 2012, and April 2013. The study focused on the local distribution of Javan green peafowls in Baluran National Park in Bekol resorts (savanna, coastal forests, and monsoon forests) and in Alas Purwo National Park in Rowobendo resort (Sadengan grazing areas, lowland forests, mixed plantation forests with intercropping area and teak plantation forest with intercropping area and teak plantation forest) (Figure 1).

The number of Javan green peafowl groups in Baluran National Park was calculated by using the transect method and concentration count method following Hernowo (1997). The sample area covered an area of approximately 4 km x 3 km (1,200 ha). Four transects were observed at the sample area approximately 3 km length of each transect. Census was carried out in ten days for every observation time and it was conducted simultaneously every month. The census started every morning at 5.00 AM and ended at 8.00 AM. Four observers went through the transect routes. The walking speed was about one hour per km in each transect. The individual number was counted based on the number of Javan green peafowl in fixed area (1,200 ha) and direct visual contact with the birds during the census. After the census, the observers came together to make correction to avoid double counting. In addition to the census, additional observation with concentration count was conducted at water holes for the number of groupings of Javan green peafowls.

In Alas Purwo National Park (APNP), the census of Javan green peafowls was conducted by using the concentration count method. The sample area for the concentration of the birds focused on five places namely Sadengan grazing area, Rowobendo intercropping area, Gunting intercropping area, Sumber Gedang teak plantation forest, and Ngagelan teak plantation forest. Five observers recorded the number of green peafowls at the concentration area at each observation time. The census was carried out in ten days for every observation time and was conducted simultaneously every month. The census started every morning at 5 00 AM and ended at 8.00 AM.

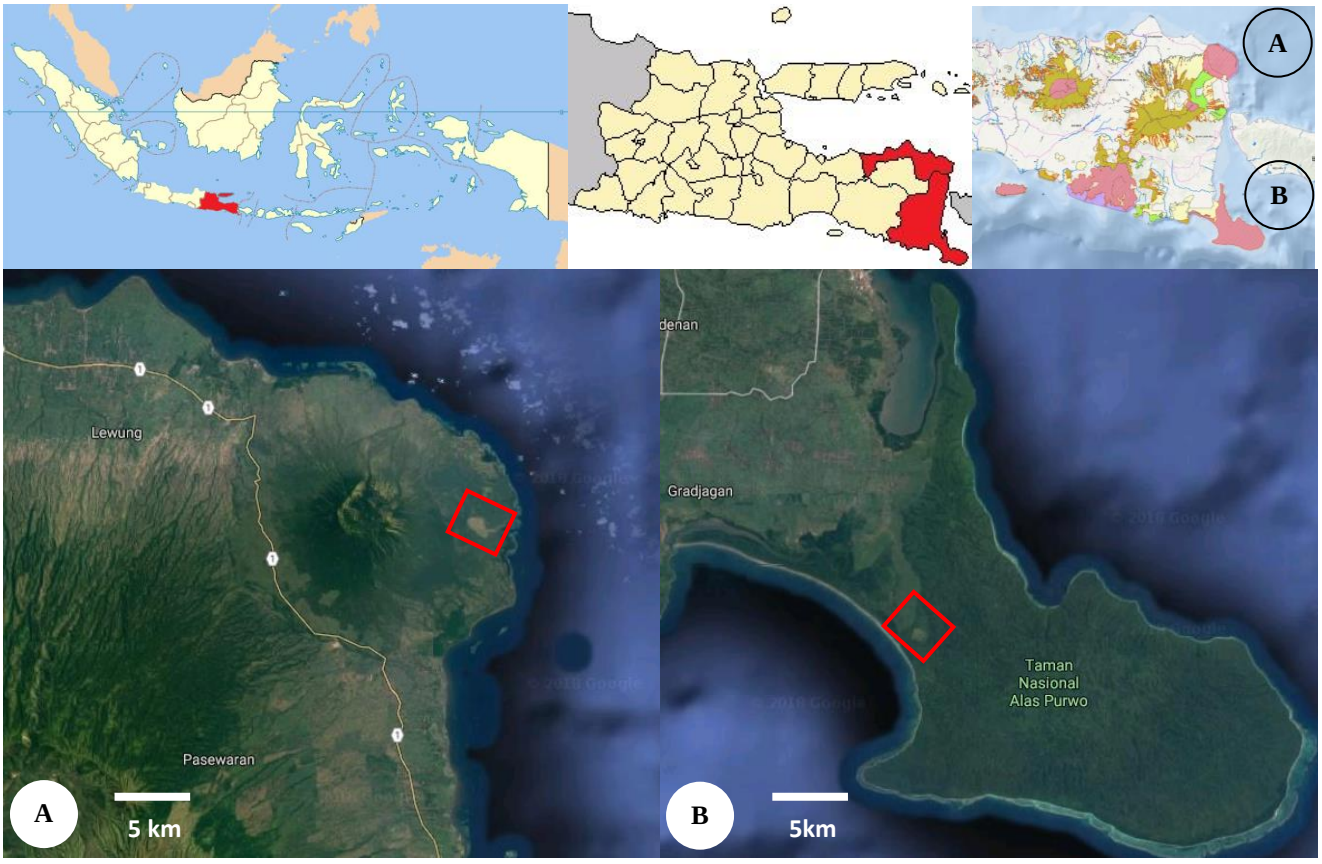

Figure 1. Study sites of Javan green peafowl (*Pavo muticus muticus*, Linnaeus 1758) population in East Java, Indonesia. A. Bekol resort of Baluran National Park, B. Rowobendo resort oft Alas Purwo National Park. Red squares indicate location of sampling plots.

Green peafowls are classified into adults, sub-adults, or young birds according to Delacour (1977) criteria. The local distribution pattern was analyzed by using the following formula (Ludwig and Reynolds 1988).

$\sigma^2 = \mu$ random distribution pattern,
$\sigma^2 > \mu$ clumped distribution pattern
$\sigma^2 < \mu$ systematic distribution pattern,

Where:
σ^2 predicted by S^2, and
μ predicted by x = average

The number of Javan green peafowls in groups in several types of habitats in BNP and APNP was tested by using *chi-square test* in order to know if the different numbers of the groups are caused by the types of habitats.

RESULTS AND DISCUSSION

Group system of the Javan green peafowl population

Based on field observation, the results show that Javan green peafowls in BNP and APNP live in groups, and the groups that were found at BNP and APNP have 5 types, not so much different from what Hernowo (1995) and Hernowo et al. (Hernowo 2011) found. The Javan green peafowl group types are as described below:

Group of Peahen and their chicks. The group consists of adult females and 1-4 chicks. Their relation is very close; they stay together when they are feeding, drinking, sheltering, roosting, and everywhere. They also come easily together with other adult females, with adult males, or with adult female groups or other groups. The leader of this group is peahen (mother). The group of Peahen and their chicks is only 3.33% of the total number of group Javan green peafowls in BNP, but in APNP it is 3.23%.

Group of adult females (Figure 2.A). The group consists of adult females of 2-5 individuals. This group can stay together with other female groups or sub adult female groups or groups of peahen and chicks or with a peacock and other groups. The leader of this group is a female. This group is 43.33% of the total number of Javan green peafowl group in BNP, but in APNP it is around 35.14%.

Group of sub-adult females (Figure 2.B). The group consists of sub-adult females of 2-4 individuals. This group could be formed from the same clutch. The group also easily relates with a peacock, or adult female groups, the group of peahen and chick or with other same groups. The leader of this group is a female. This group is 6.67% of the total number of Javan green peafowls group in BNP and APNP.

Group of sub-adult or young mixed (female and male) (Figure 2.C). The group consists of sub-adult females of 2-3 individuals and 1 sub adult male. This group might be from the same clutch. They can come together with another group, but are rather loose with adult males if a sub adult male has matured. The leader of this group is a female. Sub-adult mixed group is only 3.33% of the total number of Javan green peafowl groups in BNP, and in APNP it is 3.23%.

Solitary groups (Figure 2.D). The adult male bird (peacock) does not have a group and also a sub-adult male is frequently not living in groups. A male bird, if it has matured, will live in solitary. Solitary group is only 3.33% of the total number of Javan green peafowl groups in BNP, and in APNP it is 3.23%.

According to Hernowo (1995), Javan green peafowls in BNP live in a group system with a small number of members (2-4 individuals), except adult male birds which live in solitary (1 individual). The Javan green peafowl groups found in BNP and APNP are classified into 5 types of group systems (GAF, GSAF, GPC, MG, and SG). The dominant group found in BNP and APNP is adult female group (GAF) comprising 3 individuals. The small number of individuals in each group is supposedly related to effective and efficient managing of their group system. In relation to the management of the group system, the size of the birds' body has influenced the members of the group. Javan green peafowls belong to big size birds (Hernowo 1995). If big size birds work with a large number of individuals in a group system, they will face obstacles or difficulties in managing or controlling individuals in the group.

The abundance of the group number (%) in Javan green peafowl population is influenced by the process within the group. The female will separate from the adult female group (GAF) if individually a member of the group is ready for the mating process. The field observation shows that the Group of Peahen and Chick (GPC) will perform when an adult female has a chick. This condition is related to post-breeding process (rearing). The chick will appear at the beginning of the rainy season. Usually, GPCs in Baluran are active after November, but in Alas Purwo they are active after October. Those conditions are influenced by the rainy season. In Alas Purwo the rainy season comes earlier than in Baluran.

If GPC has enough process for developing the chick, the chick will separate from the peahen. The developing process of a chick takes a period of approximately 3-4 months. The chick will form a new group, and the group is the sub adult female group (GSAF) or the young mixed group. The group types will depend on individual members of sex found there (male or female). If all members are females, the type of the group system will be a group of sub-adult females (GSAF), but if the members of the group consisting of females and males, the group system will be young mixed (female and male) group (MG).

The leader of the group in BNP and APNP is a female bird. Hernowo (1995) found that the leader of the group in BNP is a female bird. The role of a female bird as a leader indicates that all Javan green peafowl groups are mostly dominated by female birds. The members of the group may be from the same clutch size. The female bird can easily move its group, especially when the group meets another group. If the group leader is a male bird, it will be difficult for another group. Hernowo (1995) states that the territories of Javan green peafowls are marked by a fight or expulsion. The females seem to have no territories. It is not

clear whether the males occupy territories or not, but adult males keep quite a clear distance from each other. Hernowo et al. (Hernowo 2011) explain that male Javan green peafowls may be close to each other, but the distance is around 25-75 m from each other during the mating season. Keeping a distance between adult male Javan green peafowls does not only happen during the mating season but also out of the mating season (almost a year long). Even though adult males feed together in open areas, they always keep a distance among them. When they are drinking in drinking sites, they cannot be close to one and another (not less than 3 m). It has never been found two or more adult peafowls staying together on one roosting tree. This explains why male peafowls have never become leaders in their group system.

The number of groups observed in Baluran National Park is listed in Table 1. Javan green peafowl groups recorded in several habitat types are dominated by adult female groups. The highest number of birds in BNP is in Bekol savanna habitat. The number of Javan green peafowl groups observed in APNP is also indicated in Table 1. Adult birds dominate in habitat types. However, the birds are more distributed in Sadengan grazing area and Gunting intercropping teak plantation area habitats.

The chi-square test indicates that the abundance of Javan green peafowl groups differs by habitat type (χ^2 = 29.05, $p < 0.01$) in BNP and (χ^2 = 38.92, $p < 0.01$) in APNP. This means that the number of groups is influenced by habitat types in both national parks (BNP and APNP). Chi-square test does not only indicate a significant difference by habitat type, but also by seasons (indirectly). In addition to the significant difference by habitat, the number of groups is also affected by seasons in both national parks (BNP and APNP). Such a difference in the number of groups is caused by the availability of supporting factors, especially food resources in each habitat type. There are more supporting factors in Bekol savanna in BNP and Sadengan grazing area in APNP compared to other habitat types (Hernowo 1999; Hernowo and Wasono 2006; Hernowo et al. 2011a,b). Seasons are also related to the availability of food resources. It is not directly affecting the number of groups, but directly affecting the individual number of the birds.

Figure 2.A. Adult female group, and B. Sub adult female group at Bekol water hole, Baluran National Park, East Java, Indonesia. C**.** Sub adult mixed group at Bekol waterhole, Baluran National Park, East Java, Indonesia. D. Adult male (peacock) solitary group in Sadengan, Alas Purwo National Park, East Java, Indonesia

Javan green peafowls in BNP and APNP live in small groups of individuals (2-4 birds). The dominant group is the adult female group comprising 3 individual birds. The leader of an adult female group is a female Javan green peafowl. Female birds are also leaders of sub-adult female groups, sub-adult mixed groups, peahen, and chicks or young groups of Javan green peafowls. Male Javan green peafowls are not leaders in the bird group system. The advantages of applying the group system among Javan green peafowls are (i) easy to find resources (food, shelters, covers, nest sites, rost sites, and rest sites), and (ii) easy to detect disturbances (predators and any other disturbances).

The results indicate that the number of group systems of Javan green peafowls is significantly different by habitat types in both national parks (BNP and APNP). This means that habitat type affects the number of groups present in each type of habitat. It is caused by habitat condition which supports the availability of food, shelter, cover, nesting site, and water (Hernowo 1999; Hernowo and Hernawan 2003; Hernowo and Wasono 2006).

Table 1. The number of Javan green peafowl groups observed in Baluran and Alas Purwo National Parks, East Java, Indonesia

Type of habitat	Number of Javan green peafowl groups	
	Observation I	Observation II
Baluran		
Bekol Savanna	20 (4AM1, 2GAF2, 2GAF4, 6GAF3, 1MG3, 2SAM1, 2GSAF3, 1GPC4)	17 (4AM1, 2GAF2, 2GAF4, 4GAF3, 1MG3, 2SAM1, 1GSAF2, 1GPC4)
Bama-Manting Beach Forest	3 (1AM1, 1GAF3, 1MG3)	4 (1AM1, 1GAF3, 1GAF2, 1MG3)
Bekol Monsoon Forest	3 (1AM1, 1GAF2, 1GAF3)	5 (1AM1, 2GAF3, 2GAF2)
Bekol Evergreen forest	3 (1AM1, 1GAF4, 1GAF2)	4 (1AM1, 2GAF3, 1GAF2)
Alas Purwo		
Sadengan grazing area	13 (5AM1, 1GAF4, 1GAF3, 1MG3, 2SAM1, 1SAM4, 1GPC4)	14 (5AM1, 1GAF4, 3GAF3, 1MG3, 2SAM1, 1GSAF3, 1GPC4)
Rowobendo mixed plantation and intercropping	4 (1AM1, 1GAF2, 1GAF3, 1SAM1)	6 (1AM1, 2GAF3, 2GAF2, 1SAM1)
Gunting intercropping teak plantation	16 (1AM1, 3SAM1, 2GAF4, 5GAF3, 1GAF2, 2GSAF4, 1GPC4, 1MG3)	12 (1AM1, 3SAM1, 1GAF4, 4GAF3, 1GSAF3, 1GPC4, 1MG3)
Sumber Gedang teak plantation	2 (1AM1, 1GAF2)	2 (1AM1, 1GAF2)
Ngagelan teak plantation	2 (1AM1, 1GAF2)	2 (1AM1, 1GAF2)

Note: Observation I (June-October 2006 and 2007). Observation II (March 2009, March 2010, April 2012, April 2013). AM (Adult Male) = (SG Solitary Group), GAF (Group of Adult Female), MG (Mix Group), SAM (Sub Adult Male), GSAF (Group of Sub Adult female), GPC (Group of Peahen and Chick). 1,2,3,4 values behind of group is an individual number at group. The number at front of group is the number of groups; the number behind of the group is the member's number of the group, value (21, 18, 5, 4, and 3) front of bracket is the number of groups

Table 2. The local distribution pattern of Javan green peafowls in Baluran National Park, East Java, Indonesia

Habitat type	Variance square (S2)		Average number (X)		Distribution pattern
	Observation I	Observation II	Observation I	Observation II	
Baluran					
Savanna Bekol	64.84	45.60	43.40	50.80	Clumped
Beach Forest Bama-Manting	12.84	8.72	6.80	8.50	Clumped
Monsoon Forest Bekol	6.01	10.68	5.30	10.30	Clumped
Evergreen Forest Bekol	6.40	8.46	6.20	8.30	Clumped
Alas Purwo					
Sadengan Grazing Area	25.88	31.17	30.50	25.10	Clumped
Mix Plantation Intercropping Rowobendo	6.62	12.10	6.20	11.90	Clumped
Teak Plantation Intercropping Gunting	44.77	30.01	44.10	29.70	Clumped
Teak Plantation-Back Mangrove Sumber Gedang	2.49	2.71	2.40	2.60	Clumped
Teak Plantation Ngagelan	3.21	1.96	2.90	1.80	Clumped

Note: Observation I (August-December 2006 and 2007); Observation II (March 2009, March 2010, April 2012, and April 2013)

Local distribution pattern of the Javan green peafowl populations

The local distribution pattern of Javan green peafowls, which is estimated based on the results of the census (the number of individuals) in BNP and APNP, is listed in Tables 2. The local distribution pattern of Javan green peafowls in BNP is of clumped type. The local distribution pattern of the birds in this National Park is closely related to the availability of resources in every habitat type. The birds are distributed in various habitat types in BNP, such as savanna, monsoon forests, coastal forests, and evergreen forests. In general, the local distribution of Javan green peafowls in APNP was of clumped type. Such local distribution pattern of Javan green peafowls in this National Park is closely related to the availability of resources in every habitat type. The birds are distributed in various habitat types in APNP, such as lowland tropical rainforests with grazing areas, intercropping areas, and teak plantations.

The local distribution of the Javan green peafowl population in various habitat types in BNP and APNP is of clumped type. Such clumped local distribution is probably related to the availability of food resources, water, covers, shelters, roost sites, and nesting sites. Several observers of the Javan green peafowl population in BNP stated that the distribution of the birds is concentrated in savanna and monsoon forest in BNP (Pattaratuma 1977; Hernowo 1995; Hernowo 1999; Yuniar 2007; Risnawati 2008). The local distribution of Javan green peafowls in APNP is concentrated in Sadengan grazing area and teak plantation with intercropping area (Supratman 1998; Hernowo and Wasono 2006). Meanwhile, Hernowo and Palita (2004) reported that the Javan green peafowl population has clumped the local distribution in open areas surrounded by forests or coffee and rubber plantations in Meru Betiri National Park. Hernowo and Hernawan (2003) recorded that the distribution of Javan green peafowls is concentrated in teak plantation with intercropping area in BKPH Buah Dua and Songgom KPH Sumedang. Sumbara (2006) reported that the distribution of Javan green peafowls is concentrated in open areas surrounded by pine forests, sub-mountain forests, and horticultural areas in Cikuray pine forests of BKPH Boyongbong KPH Garut.

In conclusion, the populations of Javan green peafowls live in a group system with a small number of individual members (2-4 birds). The Javan green peafowl populations in BNP and APNP have 5 group types. The dominant group is adult female birds (GAF) comprising 3 individuals. The Javan green peafowl populations are found in abundance in savanna habitat at BNP and are distributed in Sadengan grazing area and intercropping teak plantation in APNP. The local distribution of Javan green peafowls in BNP and APNP is of clumped type.

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Insect diversity in post-mining areas: Investigating their potential role as bioindicator of reclamation success

DAMAYANTI BUCHORI[1], AKHMAD RIZALI[2,♥], GILANG ADITYA RAHAYU[1], IRDIKA MANSUR[3,4]

[1]Department of Plant Protection, Faculty of Agriculture, Institut Pertanian Bogor. Jl. Kamper, Kampus IPB Dramaga, Bogor 16680, West Java, Indonesia
[2]Department of Plant Pests and Diseases, Faculty of Agriculture, Universitas Brawijaya. Jl. Veteran, Malang, 65145, East Java, Indonesia. Tel./fax.: +62-341-575843. ♥email: arizali@ub.ac.id
[3]Department of Silviculture, Faculty of Forestry, Institut Pertanian Bogor. Jl. Lingkar Akademik, Kampus IPB Dramaga, Bogor 16680, West Java, Indonesia
[4]Southeast Asia Regional Centre for Tropical Biology (SEAMEO BIOTROP). Jl. Raya Tajur Km 6, Bogor 16134, West Java, Indonesia

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Abstract. *Buchori D, Rizali A, Rahayu GA, Mansur I. 2018. Insect diversity in post-mining areas: Investigating their potential role as bioindicator of reclamation success. Biodiversitas 19: 1696-1702.* Reclamation can be a pivotal process to return an ecosystem to its condition prior to human disturbance, by recreating a landscape so that its structure and function closely resemble a natural community. Unfortunately, there is a lack of empirical data as to whether reclamation efforts successfully establish sustainable of the ecosystem or not. The objective of this research was to study insect diversity in post-mining areas and investigate their potential role as bioindicators of reclamation success. An ecological research was conducted in post-mining reclamation areas managed by PT. Berau Coal in Binungan, East Kalimantan. We selected sub-areas that had been subject to reclamation efforts for varying periods, ranging from 2 to 10 years, for observation. We also used an area of undisturbed natural forest as a comparison. Inside each of these subareas of different reclamation age, insects were sampled using pitfall traps and malaise traps along a 100-meter transect. Our results showed that insect diversity differed in areas of different reclamation age. Based on CCA revealed that environmental factors i.e. pioneer tree age, vegetation diversity and soil chemistry (N total) affected the diversity of insects in the reclamation area. In particular, NMDS analysis showed different species composition in ant communities found in subareas of varying reclamation age. We conclude that ants are the most useful potential bioindicator to assess reclamation success in post-mining areas.

Keywords: Ant, Berau, reclamation area, revegetation, species composition

INTRODUCTION

As a country with significant mineral resources and extensive mining, Indonesia's government has enacted regulations related to reclamation of landscapes previously subject to mining (PP No.78, Year 2010), with a goal to protect ecosystems from collapse after mine closures. Mining companies' restoration activities in closure areas generally consist of planting pioneer trees, with the presumption that in time the ecosystem will fully recover functionality and structure (Ge et al. 2010). Unfortunately, there is little data as to whether this reclamation strategy will succeed in producing a sustainable ecosystem. Therefore, ecosystem assessments are needed to provide crucial evidence of reclamation outcomes.

One indicator of reclamation success is new soil formation. In mining areas, such as for coal, the soil layer is removed as a result of exploration activity. Assessing the success of soil regeneration post-mining is challenging because soil development is extremely complex and the details are poorly understood. Soil development itself is a product of both physical and biological processes which link abiotic and biotic variables (Walker and del Moral 2003). However, we do know that the reclamation age of an area (chronosequence) is an important factor for soil development. Different aged reclamation areas will presumably manifest different stages of ecological succession which encompasses not only environmental conditions but also biotic factors (Stevens and Walker 1970).

Biotic factors, particularly the composition of plant and animal communities present, are the most important element in the development of new soil in depleted areas. Plants selectively concentrate soil nutrients, transport water from the soil to the atmosphere, and add organic matter when they decay. In addition, animals use plants for food, nest sites, and protection, and plants and animals mutually influence each other as animals seek out and use these resources. Through these mechanisms, animals alter soils by burrowing, feeding, defecating and dying. Hence, interactions between plants and animals play an important role in soil formation as well as influencing ecological succession in reclamation areas (Walker and del Moral 2003). In this study, we measured soil chemistry and composition in order to determine the health and ecological status of soil in our study sites. In general higher levels of organic carbon and nitrogen correlate to increased soil formation.

The changes in species composition of plant communities during succession are accompanied by changes in animal communities as well. All of these groups mutually interact in feedback loops, so that changes in one

group of organisms influence other groups. Consequently, attempting to distinguish between cause and effect may be difficult or impossible. In studies of these relationships, animals are often treated as passive responders to changes in plant succession (Bradshaw 1983). Plants provide the primary food source and habitat structure without which most animals cannot survive. The course of plant succession is intimately linked to animals that disperse seeds, pollinate flowers and eat various plant parts, as well as redistribute nutrients and improve soil structure. Animal succession may respond to changing plant resources, abiotic conditions or interactions among animals themselves. However, animal succession may be more dependent on vegetation cover and structure than on plant species composition, resulting in a partial uncoupling of plant and animal community compositions related to succession (Gallé 1991).

Animal succession can be influenced by diversity in local flora or in abiotic conditions, which affect the structure and availability of habitat and the functional ability of animals to act as seed dispersers and pollinators. In the succession process, herbivores cannot colonize an area until plant resources are present and available, providing a form of obligatory facilitation. Similarly, predators and parasites must colonize either simultaneous to, or after, the arrival of their prey and hosts (Edwards 1988). This dependency on habitat variables (e.g., the presence of food or predators) has been described by a model of habitat accommodation and applied to situations such as colonization of ants on mined lands in Brazil (Majer 1992). In this case, animals become important as bioindicators of succession in restoration habitat.

Certain groups of animals may be used as surrogates for species diversity or assemblage composition of other taxa, to understand the effect of habitat disturbance (McGeogh 1998). Observation of arthropods can potentially reveal restoration status, as shown in studies with beetles (McGeoch et al. 2002; Pearce and Venier 2006), spiders (Pearce and Venier 2006), grasshoppers (Bazelet and Samways 2011) and ants (Gollan et al. 2011). Nevertheless, the use of insects as indicators of restoration success has largely been overlooked, with most studies focused only on vegetation (Colloff et al. 2010; Déri et al. 2011). Insects can be particularly useful as indicator species because they are extremely sensitive to environmental change (Rosenberg et al. 1986; Peck et al. 1998).

The objective of this research was to study insect diversity in post-mining areas and investigate their potential role as bioindicators of reclamation success.

MATERIALS AND METHODS

Research site

Our study was conducted in a reclamation area managed by PT Berau Coal, Berau, East Kalimantan, Indonesia (Figure 1). Sites had previously been mined for coal, and had subsequently been planted, after mine closure, with pioneer tree species such as acacia (*Acacia sieberiana*), johar (*Cassia siamea*) and sengon (*Paraserianthes falcataria*). To study the succession process, we selected several reclamation sub-areas of different reclamation age, ranging from two to ten years since the inception of reclamation efforts (planting of pioneer trees). Two plots were defined in each subarea as replication. Two plots in adjacent natural undisturbed forest were also selected to compare insect diversity there to that of study reclamation sites (Table 1).

Insect sampling

The sampling methods were adapted from Majer et al. (2007). A 100 m transect was laid out in a representative part of each subarea of different reclamation age (Figure 2). Along each transect, we placed ten plastic glasses (6.5 cm diameter × 9.5 cm depth) in the ground as pitfall traps. The traps were located at 10 m intervals and left open for six consecutive days and nights. Two malaise traps were also placed on each transect and left for 3 days. Pitfall traps capture insects on the soil surface in order to estimate abundance and richness. Malaise traps capture flying insects primarily and allow measurement of airborne insect diversity within each reclamation subarea. Insect sampling was conducted between July and August 2012.

Insects collected from pitfall traps and malaise traps were placed in plastic vials filled with 70% alcohol. Then, in the laboratory, collected specimens were sorted and identified to broad taxonomic levels (order or family level) using a reference key such as Borror *et al.* (1996). Some specimens were further identified to genera or morphospecies level (e.g., identification key of Bolton (1994)).

Vegetation and soil analysis

Plant and vegetation diversity were surveyed within 100 meters on either side of the transects laid for insects sampling. Vegetation was surveyed once during the study period, with visual identification of the species *in situ* and measurement of species abundance along each transect. Plants that could not be identified in the field were collected for later identification in the lab. Vegetation analysis showed that the pioneer trees most commonly planted in the transect areas were acacia (*A. sieberiana*), johar (*C. siamea*) and sengon (*P. falcataria*). Plant diversity tends to increase with subarea reclamation age (Figure 3, Table 1). Therefore, in studying the relationship between insect diversity and reclamation age, we were able to use reclamation age as a proxy for plant diversity and successful succession.

To obtain data about soil chemistry and composition, soils samples from each transect were analyzed in the laboratory. The results showed no relationship between reclamation age and soil chemistry (Figure 4a, Table 1) or soil composition (Figure 4b, Table 1). Therefore, we cannot assume that reclamation age can proxy for soil characteristics and ecosystem function of soils.

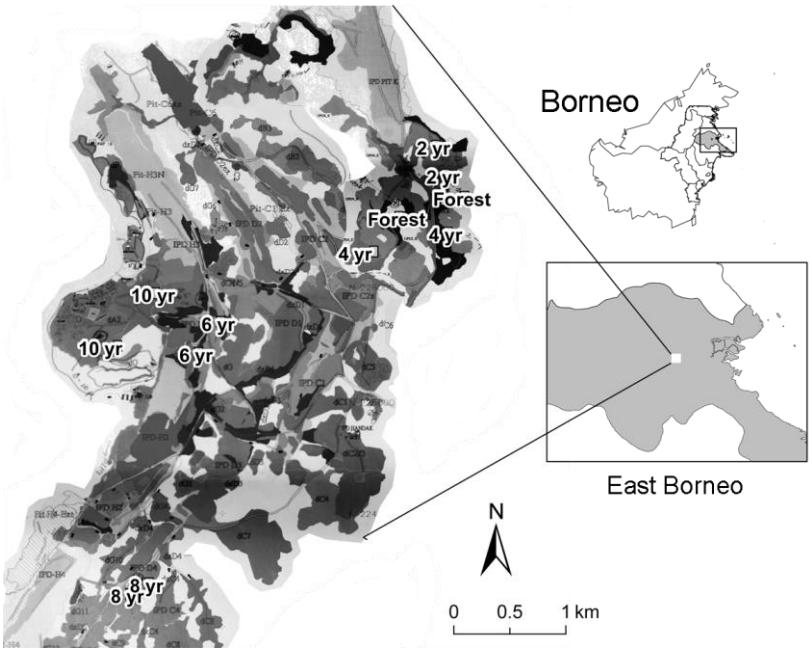

Figure 1. Map of the reclamation area managed by PT. Berau Coal in Binungan, Berau District, East Kalimantan, Indonesia. The reclamation age of study sites ranges from 2 to 10 years of active restoration activity

Table 1. Geographic coordinates, vegetation diversity and soil chemistry and composition in each subarea of the Berau, East Kalimantan, Indonesia reclamation area

Code (year)	Plot	Ordinate		Vegetation (# individuals)		Soil chemistry				Soil composition (%)		
		Latitude	Longitude	Tree	Scrub	pH H_2O	pH $CaCl_2$	Organic C	N total	Sand	Dust	Clay
PIT (2yr)	P1	2.053807	117.458412	4	18	4.5	3.8	1.01	0.04	34.6	17.7	47.7
	P2	2.056141	117.457633	2	15	5.3	5.0	2.74	0.06	42.3	25.5	32.2
D1 (4yr)	P1	2.049139	117.458613	2	22	4.8	4.1	0.66	0.08	30.9	19.8	49.3
	P2	2.047614	117.452660	2	15	4.4	3.8	1.41	0.15	13.0	29.0	58.0
H1 (6yr)	P1	2.041938	117.440290	5	17	4.6	4.1	2.74	0.24	15.8	34.3	49.9
	P2	2.039421	117.438549	3	14	4.8	4.3	1.34	0.16	13.5	55.1	31.4
D4 (8yr)	P1	2.020904	117.434565	4	16	4.2	3.9	1.46	0.17	32.8	22.5	44.7
	P2	2.020052	117.433025	2	12	4.5	3.9	1.68	0.15	19.5	24.6	55.9
A2 (10yr)	P1	2.039995	117.432074	11	8	4.6	3.8	1.46	0.16	19.7	23.2	57.1
	P2	2.044273	117.435080	10	17	5.1	4.4	1.11	0.13	59.2	13.5	27.3
Forest	P1	2.050399	117.454673	40	8	3.9	3.4	2.83	0.20	35.5	27	37.5
	P2	2.052015	117.459928	68	6	4.4	3.7	1.22	0.13	29.3	34.1	36.6

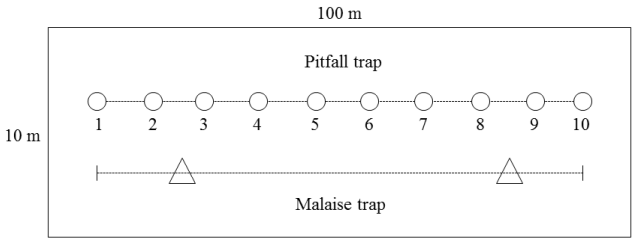

Figure 2. 100 m transect laid within each subarea of different reclamation age

Statistical analysis

Species richness and abundance of sampled insects within the measured taxonomic level were summarized for each transect or reclamation subarea. Shannon (H') and Simpson (1/D) diversity indices (Magurran 2004) were measured to compare the diversity of insects between reclamation age. Pearson's correlation analysis was used to measure the relationship between insect species diversity, reclamation age, and environmental variables. In addition, the assemblage composition across various taxa was compared between each of the reclamation subareas and the natural forest sample sites, using the Bray-Curtis index, followed by non-metric multidimensional scaling (NMDS),

which is recommended as a statistical tool for bio-indicator assessment (Smith and Mather 2012). Analysis of similarity (ANOSIM) was used to compare species composition between subareas of different reclamation age. The relationship between insect species composition and environmental variables were analyzed using ordistep method within canonical correspondence analysis (CCA) (Borcard et al. 2011). All analyses were performed using R statistics (R Core Team 2017) and vegan package (Oksanen et al. 2015).

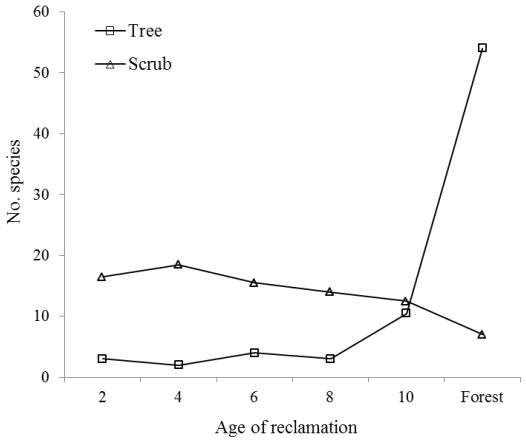

Figure 3. Relationship between subarea reclamation age and vegetation diversity (tree and scrub)

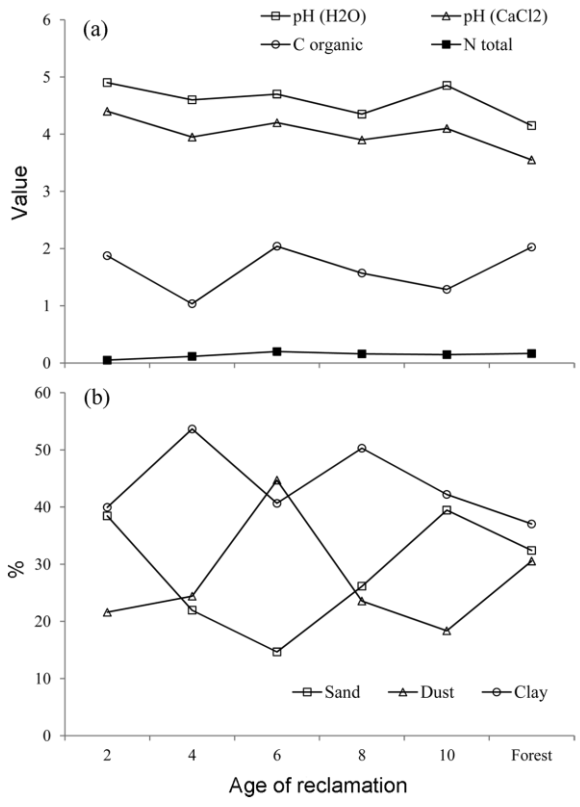

Figure 4. Relationship between subarea reclamation age and (a) soil chemistry (pH H_20, pH $CaCl_2$, Organic C and N total) and (b) soil composition (sand, dust, and clay)

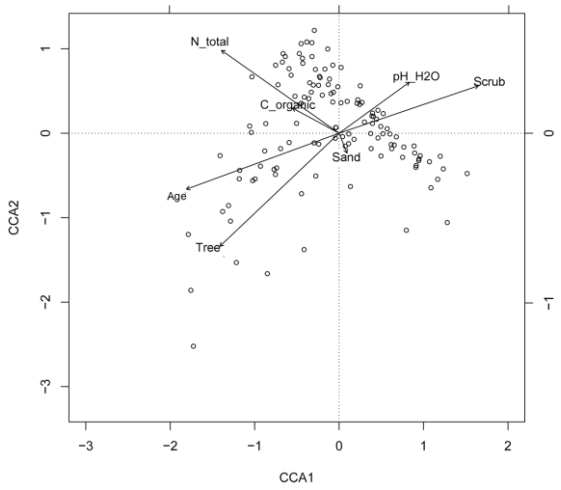

Figure 5. Ordination of canonical correspondence analysis (CCA) for data on insects and other arthropods in the reclamation area. Arrows represent environmental variables and arrow length indicates the relative importance and direction of correlation, of the variable to the axes. The orthogonal projection of a species point on an environmental arrow represents the approximate center of the species distribution along that particular environmental gradient. Species are indicated with circle points and labeled. Some species are not labeled in order to avoid congested graph

RESULTS AND DISCUSSION

Insect diversity in the PT. Berau Coal reclamation area

Results showed that insect assemblage diversity measured within the Berau Coal reclamation area differed between subareas of different reclamation age. Sixteen arthropod orders were identified from the total combined insects collected from pitfall traps and malaise traps, in all subareas including the natural forest control site (Table 2). Some arthropod orders, including Coleoptera, Hymenoptera (especially Formicidae), Orthoptera, Acarina, and Collembola, were found in every research plot but exhibited different species richness and abundance in plots of different reclamation age. Based on Shannon and Simpson diversity indices showed that insect diversity tended to increase with increasing reclamation age. The highest diversity was found in 8 years-old reclamation area and then decrease or prone to be stable in 10 years-old reclamation area, approximately similar to forest (Table 2).

Implication of environmental change on insects

As a reflection of the different duration of reclamation efforts (reclamation age) in reclamation subareas, subarea environmental conditions were expected to vary in terms of tree age, vegetation diversity (tree and scrub), soil chemistry (pH, organic C, and N total) and soil composition (Table 1). However, although we did find such differences between subareas, our analysis revealed no significant relationship between reclamation age and characteristics of soil chemistry and soil composition. Nevertheless, there was an indication that the age of reclamation area was marginally correlated with changes in C/N ratio (r= 0.512, P= 0.089).

Table 2. Species richness (S) and abundance (N) at the order level, of insects, collected from sites (P1 and P2) within subareas of different reclamation age in the PT. Berau Coal reclamation area.

Order		PIT (2yr)		D1 (4yr)		H1 (6yr)		D4 (8yr)		A2 (10yr)		Forest	
		P1	P2	P1	P2	P1	P2	P1	P2	P1	P2	P1	P2
Insects													
Blattodea	N	2	7		5	1	4	2	1	1		2	2
Coleoptera	S	8	11	15	11	15	7	10	9	12	12	8	13
	N	14	13	15	13	15	7	10	9	15	16	8	18
Dermaptera	N									1	1		
Diptera	N	6	7	5	8	10	10	10	7	16	12	10	9
Hemiptera	N	6	6	7	8	10	9	9	7	5	5	5	9
Hymenoptera													
a. Formicidae	S	27	24	25	28	30	24	15	15	22	31	21	18
	N	118	106	79	94	58	59	25	18	53	101	61	29
b. Non Formicidae	N	9	7	2	5	2	5	7	1	8	8	10	6
Isoptera	N			1		1		1		3	2	5	1
Lepidoptera	N	6	1	2	1	3	7	5	4	2	3	3	1
Odonata	N										1		
Orthoptera	S	16	10	13	17	11	7	13	4	6	1	3	11
	N	30	19	23	27	13	13	26	4	6	1	4	12
Phthiraptera	N				1								
Psocoptera	N		1		2			4	1			1	
Thysanoptera	N	2	1				4		2	3	1	2	
Other Arthropods													
Acarina	S	1	2	4	3	9	2	1	5	5	10	1	1
	N	1	6	8	3	25	3	1	16	17	36	1	1
Arachnida	N	8	10	9	8	9	13	9	10	10	12	9	6
Collembola	S	7	6	7	5	7	6	7	8	11	10	6	11
	N	39	37	36	34	51	25	41	40	47	48	23	47
Shannon index (H')		1.612		1.733		1.975		2.104		1.825		1.867	
Simpson index (1/D)		3.170		3.785		5.206		6.211		4.362		4.531	

Table 3. The effect of environmental variables on community composition of insects and other arthropods in the PT Berau Coal reclamation area. Table shows the results of a forward selection procedure within a canonical correspondence analysis (CCA) using ordistep method with 1000 permutations. Significant codes: 0 = ***, 0.001 = **

Variable	df	AIC	F	N.Perm	P
Age	1	72.608	1.6493	999	0.001***
Tree	1	72.759	1.5038	999	0.001***
Scrub	1	72.800	1.4647	999	0.005**
N total	1	72.838	1.4288	999	0.005**
pH H_2O	1	73.284	1.0117	999	0.394
Sand	1	73.479	0.8337	999	0.821
Organic C	1	73.518	0.7992	999	0.851

Results of ordistep analysis within CCA showed that certain environmental factors significantly affect the characteristics of insect communities in our sample areas (Table 3, Figure 5). Tree age, vegetation diversity (tree and scrub) and N total significantly influence insect diversity in the reclamation area. In this case, increased age of pioneer trees was closely correlated with the increase in vegetation diversity, indicating that the older reclamation areas provide suitable habitat for insects.

Relationship between insect diversity and age of reclamation area

Ants (Hymenoptera: Formicidae) were the predominant insect group found throughout the reclamation area, in terms of both in species richness and abundance (Table 2, Figure 6a). This is expected given that ants are the most dominant insect in terrestrial ecosystems earth wide (Wilson 1990) and are often caught in pitfall traps. Ants were found in all subareas regardless of reclamation age. However, ant species richness was fluctuated and not correlated with age of reclamation area (P>0.05, Figure 6a). Specifically, ant species richness increases with reclamation age until 6 years, whereupon it declines steeply before rising again in sites of 8 years and declining less steeply in areas where 10 years.

Different patterns were observed for sampled insects of beetle (Order Coleoptera) and grasshopper (Order Orthoptera). Although not significantly correlated (P>0.05), species richness for both of these insect orders tended to decrease with increasing age of reclamation area (Figure 6a). In contrast, species richness for sampled insects of termite (Order Isoptera) tended to increase with increased reclamation age (r=0.704, P=0.011, Figure 6a). The presence of termites is very important in reclamation areas because they have a pivotal role in decomposing organic materials (decomposer). Their presence and community characteristics are therefore closely correlated

with vegetation conditions (r=0.706, P=0.010). Increased vegetation diversity facilitated the presence of termites in reclamation areas.

Species diversity for other arthropods, i.e. mites (Acarina) and springtails (Collembola) showed a similar pattern to that of termites, and their species diversity tended to increase with increased reclamation age (Figure 6b). A longer reclamation duration may allow for increased leaf litter on the soil surface, which serves as the primary habitat for Collembola. In addition, the presence of Collembola may affect other insect populations due to their role as saprophage in the ecosystem and also as primary prey for insect predators.

Effect of reclamation age on insect species composition: the role of insect as bioindicator

Results from NMDS analysis showed no significant difference in community composition in relation to reclamation age, for all insects (Figure 7a, ANOSIM statistic R=0.031, P=0.430) and other arthropods (such as Collembola) (Figure 7d, ANOSIM statistic R=0.342, P=0.080). This suggests that using composite of these organisms is not effective as bioindicators, nor will they serve as an effective tool to assess reclamation success. As also suggested by McGeogh (1998), to utilize the insects as bioindicator require to select certain taxa or groups based on a priori suitability criteria.

Unlike composite of all insects as well as other arthropods mentioned above, two taxa did show significant differences in community composition depending on reclamation age i.e. coleopteran (Figure 7b, ANOSIM statistic R=0.372, P=0.020) and ants (Figure 7c, ANOSIM statistic R=0.408, P=0.010). Community composition for coleopteran and ant groups clearly varied in sites of different reclamation age. However, the species composition of coleopteran found in the natural forest control site vs. that composition in the 2-year-old reclamation subarea, was found to be quite similar, more so than that of the ant groups in the same comparison (Figure 7b). This suggests that coleopteran is not an effective indicator of reclamation success, despite findings in earlier research that some groups of coleopteran have such potential (McGeoch et al. 2002). Our findings indicate that, of the arthropod groups we studied, ants are the most effective potential bioindicator to evaluate reclamation success in the PT Berau Coal reclamation area. Previous research by Majer *et al.* (2007) also supports the use of ants as the best bioindicator of reclamation success in post-mining areas.

Ant communities in the Berau reclamation area were dominated by tramp species, which always co-exist with humans (McGlynn 1999). Human activities in mining areas may be conducive to maintaining ant populations, by providing additional food resources for ants (Kaspari and Majer 2000). In addition, the proximity of intact natural forest surrounding the mining area in Berau may be especially helpful for recovery of ant diversity as well as other species. Such natural habitat plays an important role as a reservoir and source of indigenous insect taxa that can repopulate nearby disturbed habitat (Rizali et al. 2002).

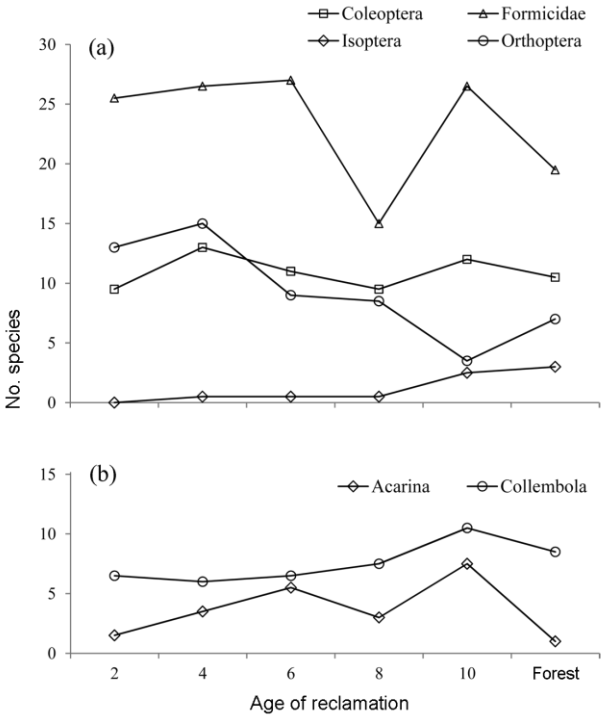

Figure 6. Pattern of species richness of (A) insects (Order Coleoptera, Isoptera, Orthoptera and Hymenoptera (Formicidae)) and (B) other arthropods (Acarina and Collembola) in areas of different reclamation age

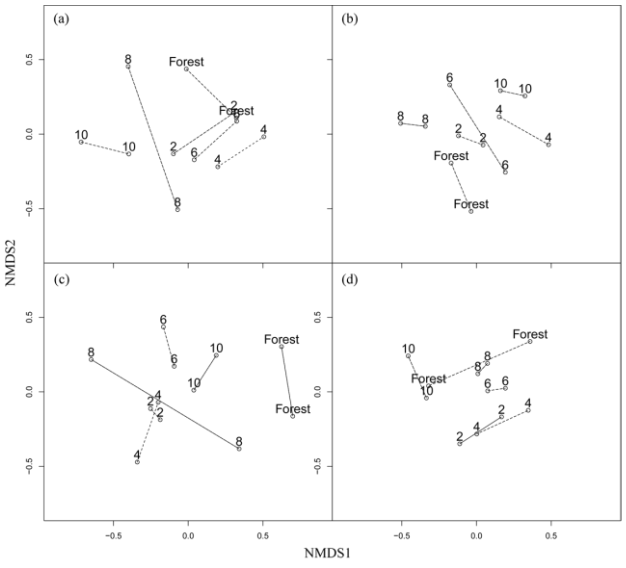

Figure 7. Non-metric multidimensional scaling analysis (NMDS) based on similarity of insect species composition across sites of different reclamation age, using Bray-Curtis index. (A) Insect and other Arthropod, stress=0.145, (B) Coleoptera, stress=0.048, (C) Ant, stress=0.138, and (D) Collembola, stress=0.086

Trends for changes in ant diversity and composition in mine reclamation areas may differ from that found in another habitat types. In agricultural ecosystems, for example, rice fields, only ant abundance, but not richness

and composition of ant species, were influenced by the age of the crop (Settle et al. 1996; Setiani et al. 2010). For annual crops such as cacao raised in agroforestry plots, the age of cacao trees affected ant species composition despite the fact that habitat conditions were not otherwise altered (Rizali et al. 2013).

In conclusion, the key factors to assess reclamation success with respect to biodiversity recovery include of time elapsed since reclamation began (reclamation age), vegetation diversity and soil chemistry (N total). Although insects have potential to serve as bioindicators of environmental disturbance, not all insects are effective bioindicators of reclamation success and biodiversity recovery. Ants show the greatest potential as a bioindicator of reclamation success in post-mining areas.

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Karst vegetation in the natural habitat of sandalwood (*Santalum album*) at various altitude places in Timor Island, Indonesia

FRANSISCA XAVERIANA SERAFINA LIO♥, MARIA PAULIN SARI DEWI
Biology Education Program, Faculty of Teacher Training and Education, Universitas Katolik Widya Mandira. Jl. San Juan, Penfui, Kupang, East Nusa Tenggara, Indonesia. Tel.: +62-380-833395, ♥email: fransiscaserafina@gmail.com

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Abstract. *Lio FXS, Dewi MPS. 2018.* Karst vegetation in the natural habitat of sandalwood (*Santalum album*) at various altitude places in Timor Island, Indonesia*. Biodiversitas 19: 1703-1713.* Sandalwood (*Santalum album* L.) was the superior commodity In South Central Timor District, but it tends to decrease in number at present. South Central Timor has been highly regarded for the quality and former abundance of its sandalwood stocks and it has been one of the most productive sources for sandalwood on Timor Island. It grows in the karst ecosystem at altitude of 450-1044 m asl. This study aims to assess karst vegetation in the natural habitat of sandalwood at three elevation sites in South Central Timor. A total of 4 plots were placed in the middle land zone (307-382 m asl.), 7 plots in the upland zone (784-1031 m asl.), and 4 plots in the highland zone (1665-1782 m asl.). Data were sampled using a square plot measuring 20 x 20m for tree, and the sub-plot of 1 x 1m for the ground vegetation category. Ecological data were measured together with the sampling of vegetation data, while the soil quality was assessed in the laboratory of Nusa Cendana University. The results showed that the higher a place from the sea surface, the less number of species was found. The highest number of species was found in the upland zone, but it tended to decrease in the highland zone. Dominant species placed at each zone were also different, which in the middeland zone the dominant species were *Acacia mangium, Tectona grandis,* and *Eleusine indica*; in the upland zone, they were *Lantana camara, Gmelina arborea, Senna siamea* and *Cyperus rotundus,* whereas in highland zone was dominated by *Senna occidentalis, Portulaca oleracea, Eucalyptus urophylla,* and *C. rotundus*. The results of multiple regression analysis also showed that environmental factors did not affect the species number in research sites. Ecological condition and soil quality in research sites indicated that those conditions are suitable for sandalwood's growth.

Keywords: vegetation, karst, sandalwood forest, South Central Timor

INTRODUCTION

The sandalwood (*S. album* L.) grows in karst ecosystem with the thin layer of soil and poor nutrients. Karst has a unique topography as a result of the dissolution of soluble rocks and characterized by the presence of grooves and strains, had a high porosity of rocks that could not accommodate large amounts of water (Heilman et al. 2009; Liu et al. 2012; Lu et al. 2014). It was very susceptible to annoyance. Sandalwood was an endemic plant species to East Nusa Tenggara which had high economic value, and also as a symbol of community unification and local wisdom. Currently, sandalwood grows both wild and cultivated status in Timor Island (Kupang, South Central Timor and North Central Timor Districts) and Sumba Island (East Sumba and West Sumba Districts) at a dry region with agro-climate condition of D3, D4 and E4 (McWilliam 2005).

Sandalwood was the superior commodity in the past, but it tends to decrease at present in South Central Timor. The district had been highly regarded for the quality and former abundance of its sandalwood stocks and it has been one of the most productive sources for sandalwood on Timor Island (Pulonggo 1995; McWilliam 2005). According to the Government of East Nusa Tenggara, there were 300,000 sandalwood trees in Timor, Alor, and Sumba in 2010. In fact, in 2000 there were still around 1,000,000 sandalwood trees in the area (Nurcahyani 2017).

Kaho (2011) conducted a study on the distribution of sandalwood at various altitude places in South Central Timor, and his research area was only in Kuanfatu Village, Kuanfatu Sub-district at altitude 450 m asl.; Haunobenak Village, Kolbano Sub-district at altitude 750 m asl.; Anin Village, South Amanatun Sub-district at altitude 755 m asl.; Oelbubuk Village, Central Mollo Sub-district at altitude 997 m asl., and Eonbesi Village, North Mollo Sub-district at altitude 1044 m asl. In North Central Timor District, sandalwood grows at altitude of 100-730 m asl. At least, 21 species of associated species of sandalwood were found in lowland, 34 species in middleland and 68 species in upland (Lio 2015).

Plant species are associated with sandalwood include: *Acacia leucophloea, Amorphophallus variabilis, Annona squamosa, Artocarpus heterophyllus, Brucea javanica, Cajanus cajan, Capsicum annuum, Chromolaena odorata , Euphorbia hirta, Imperata cylindrica, Lantana camara, Leucaena leucocephala, Oryza sativa, Pleiogynium timoriense, Psidium guajava, Pterocarpus indicus, Schleichera oleosa, Senna siamea, Sesbania grandiflora, Swietenia macrophylla, Tectona grandis,Tridax procumbens*, and *Ziziphus mauritiana* (Kaho 2011). According to Surata (2006) sandalwood is a hemiparasite

plant that requires other plants as hosts.The primary host species are *C. cajan, Capsicum annuum, L. leucocephala* and *S. grandiflora.* The secondary host species include *Acacia villosa, Casuarina equisetifolia, I. cylindrica,* and *S. siamea*.

The number of vegetation species that compiled sandalwood forest community showed that the higher a site from the sea level, the more number of associated species were found. There were several different patterns of plant species diversity due to varying responses to altitude i.e. (i) plant diversity decreased with increasing altitude; (ii) plant diversity increased with increasing altitude; (iii) "swells" in the middle-altitude; (iv) plant diversity decreased in the middle-altitude; or (v) no relationship with altitude (MacArthur 1972). The present study aimed to assess karst vegetation in the natural habitat of sandalwood at three elevation sites in South Central Timor.

MATERIALS DAN METHODS

Study areas

The research has been carried out from January to February 2018. The research sites were divided into 3 zones in South Central Timor District, East Nusa Tenggara Province, Indonesia, i.e., zones of middle land, upland, and highland (Figure 1). According to Degroot (2009), the lowland ranges at 0-199 m asl., middle land at 200-499 m asl., upland at 500-1499 m asl., and highland at >1499 m asl.

The middle land zone was located in Puna Village, Polen Sub-district at altitude of 307-382 m asl. The upland zone was located in Villages of Eonbesi, Oelbubuk, Oehala, and Binaus at altitude of 784-1031 m asl. Gunung Mutis Natural Reservation in Fatumnasi Village at altitude of 1665-1782 m asl. The data were not collected in lowland, because the sandalwood was found only in the middle to highland zones (Kaho 2011).

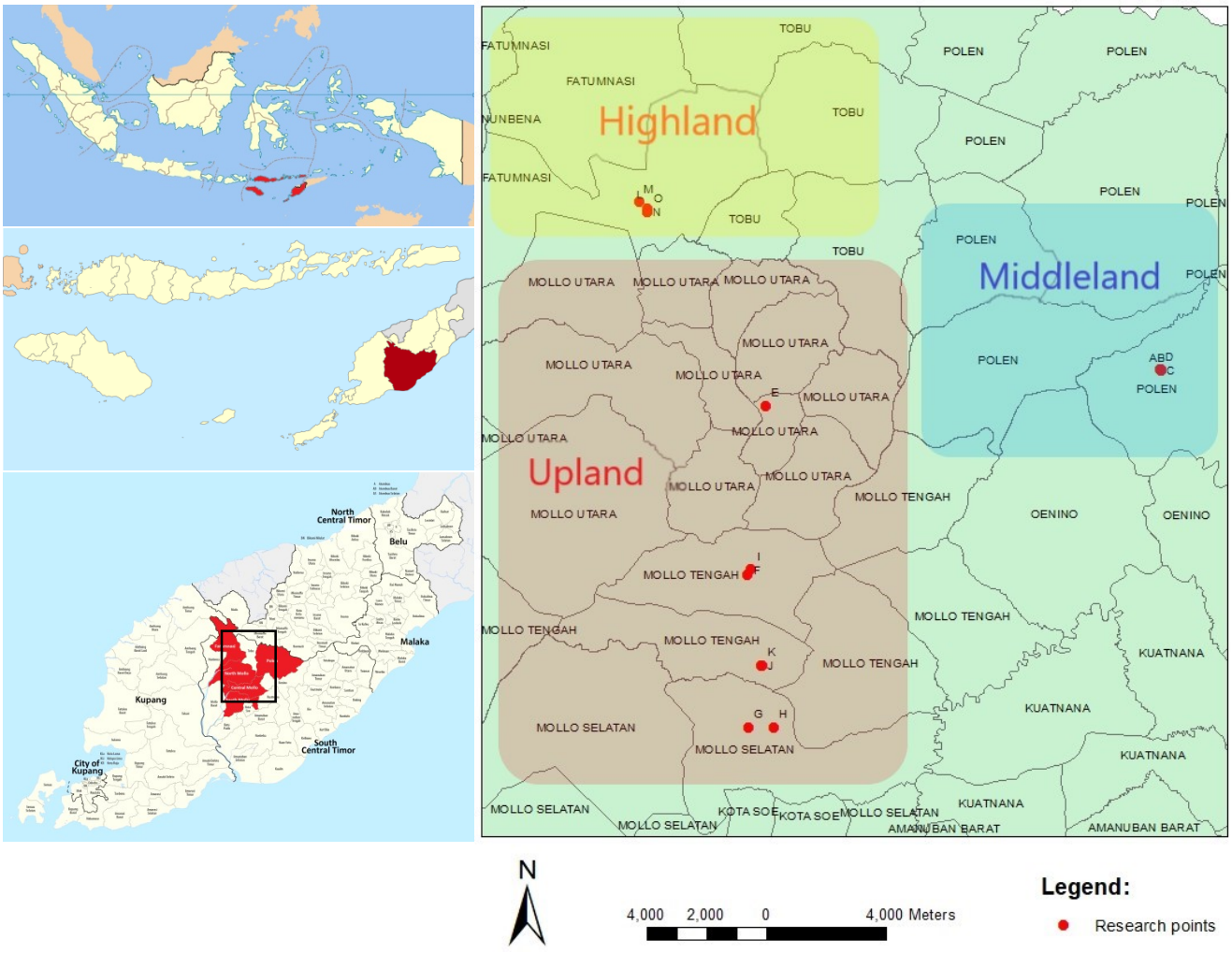

Figure 1. Study sites in South Central Timor District, East Nusa Tenggara Province, Indonesia. The ABCD points, representing 4 plots were placed in the middle land zone. The EFGHIJK points, representing 7 plots were placed in the upland zone, and the LMNO points, representing 4 plots were placed in the highland zone

Procedures

Vegetation

Vegetation data were recorded using a plot of size 20 x 20m (Barbour et al. 1987). The data sampled on each plot were vegetation (stand, shrubs, herbs and floor vegetation), ecological condition, and soil chemistry. Herbs are plants with soft trunk (not woody) (Brewer 1993). Shrubs are clumping plants with short stems, creeping, a few centimeters to approximately 1.5 m (Lenard 2008). The stand is a distribution of the number of trees per unit area (ha) in various layers of its diameter class (Meyer et al. 1961).The stand consisted of a tree (clump circular> 31.4 cm), sapling (clump circular between 6.28-31.4 cm), and seedling (clump circular< 6.28 cm).It categorized as the stand if a species had1 m height above, meanwhile if it was 1 m height under, it categorized as floor vegetation (Relva et al. 2008). Growth form parameter of stand, shrub, herbs, and floor vegetation measured the involved number of plant species, individual number of each plant species, each species density, stem circular, a canopy length and width, and clump circular (Zhao et al. 2005). To record the data of floor vegetation, it used subplot of 1m x 1m (Figure 2). Vegetation data ecological condition and soil quality in the highland zone were taken from 4 plots; 7 plots in upland zones, while in the middle land zone was placed 4 plots.

Ecological condition and soil quality

Ecological condition such as air temperature and humidity, light intensity, soil temperature, moisture, and pH were measured and recorded in research sites. Those parameters of each quadrant in the plot were measured with 5 replications. Data of soil quality were obtained in 5 replications (Figure 3). Each replication will be taken as much as 100 g and then mixed until homogeneous. The layer of soil was taken at a layer of 0-20 cm. Each zone would be obtained 1 composite soil derived from the composite soil of the whole plot (Mahler and Tindal 1990). For analysis, 200 gr (composite soil per zone) were taken to the laboratory for the study of Nitrogen, P_2O_5, Fe, potassium, and calcium concentrate.

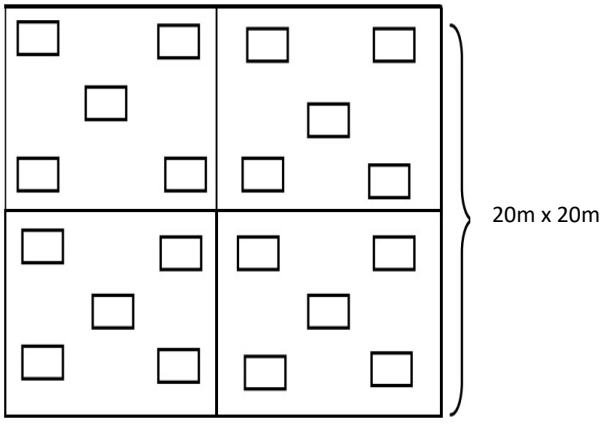

Figure 2. Sub-plot to calculate the floor vegetation (Bexter 2014)

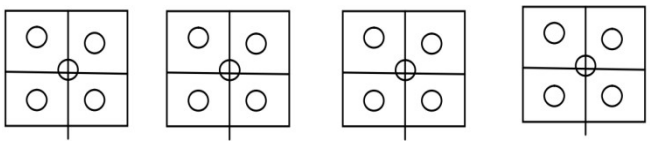

Figure 3. Soil composite sampling (Mylavarapu and Won 2002)

Data analysis

Vegetation data were tabulated and inserted into MS Excel as a calculating tool. The calculations for obtaining the Important Value (IV) (Barbour et al. 1987) were as followed:

$$\text{Density of species A} = \frac{\text{individual count of species A}}{\text{Area wide}}$$

$$\text{Relative density of species A} = \frac{\text{a total count of species A}}{\text{density of whole species}} \times 100\ \%$$

$$\text{Frequency of species A} = \frac{\text{number of plots found species A}}{\text{number of whole plot}}$$

$$\text{Frequency relative of species A} = \frac{\text{frequency of species A}}{\text{frequency of whole species}} \times 100\ \%$$

$$\text{Wide of basal area of species A} = \pi(\text{stem radius of species A})^2$$

$$\text{Wide of relative basal area of species A} = \frac{\text{wide of basal area of species A}}{\text{wide of basal area of whole species}} \times 100\%$$

$$\text{Canopy wide of spesies A} = \pi\ (\text{lengthsx width}) \text{ canopy}$$

$$\text{Relative canopy wide of species A} = \frac{\text{canopy wide of species A}}{\text{canopy wide of whole species}} \times 100\%$$

IV of species A= Relative density of species A + Relative frequency of species A + Wide of basal area of species A + Relative canopy wide of species A

To examine the effect of environmental physical factors on the number of species found in each study plot, a multiple regression analysis was conducted with the help of SPSS software.

The formula of it described as follow (Uyanik and Nese 2013):

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + \ldots + b_6X_6 + \varepsilon$$

Where :

Y : Number of plant species in each plot

b_0 : The value of plant species number when whole free variable counted as zero

b_1-b_6 : The coefficient of regression variable

ε : Error

X_1 : Air temperature

X_2 : Air humidity

X_3 : Soil temperature

X_4 : Soil moisture

X_5 : Soil pH

X_6 : Light intensity

RESULTS AND DISCUSSION

The number of species on each elevation

Twenty-seven species were recorded in the middle land zone, 79 species in the upland zone, and 35 species in the highland zone. The results indicated that altitude was not affecting to the number of species in South Central Timor. If it had reached the highland zone, then the number of species tended to decrease. This result might be due to temperature being the limiting factor of the presence of various species of vegetation. According to Lio (2015), there were 21 species found in the lowland zone. Based on the present study, there were 27 species found in middle land, 79 species in upland, and 35 species in highland (Figure 4).

The diversity of plant species due to altitude patterns had been the focus of attention from ecologists such as Whittaker (1960), Mac Arthur (1972), and Walter (1979). The altitude and slopes primarily determined microclimate conditions and in the broader scale determined the spatial distribution and vegetation pattern and cover (Jin et al. 2008). There were several varying patterns of plant species diversity due to different responses to altitude i.e. (i) plant diversity decreased with increasing altitude; (ii) plant diversity increased with increasing altitude; (iii) "swells" in the middle-altitude; (iv) plant diversity decreased in the middle-altitude; or (v) no relationship with altitude. MacArthur (1972) revealed lowland regions in the tropics and sub-tropics had the highest biodiversity, in where species diversity decreased with increasing altitude. However, the hypothesis lacked of evidence (Colwell and Hurtt 1994). More references of the effect of altitude on species diversity in tropical and sub-tropical mountain ecosystems suggested that the elevation gradients had a significant impact on plant distribution; the highest diversity tended to be in the middle land zone (Lomolino 2001). However, there was another study reported that there was no diversity differentiation with increasing altitude (Wilson and Sydes 1988). At different altitude, growth form as shrub and herb had different diversity (Whittaker 1960).

Based on those studies, it assumed that the number of species did not increase with increasing of altitude. Climate change due to deforestation caused various species adapted to a variety of habitat conditions and the highest number of species found at upland (Zhao et al. 2005). Present study showed that the upland zone is an optimal habitat for the growth of various species. It means habitat conditions in upland zones with a variety of environmental factors exist were still able being tolerated by the species. Areas below and above the upland zone were only be occupied by species that had wide tolerance ranges with habitat conditions that might be said to be a little bit extreme. However, it needed to be further studies, especially for tropical monsoon climatic regions such as Timor in particular and East Nusa Tenggara in general.

The influence of ecological factors on the number of species

Number of species and ecological conditions, such as air temperature and humidity, soil temperature, moisture, and pH, and light intensity were performed in Table 2.

The relationship between species number and environmental factors (eliminate the interactive effects among species number and environmental factors) that used F test and t-test analysis were presented in Table 3 and 4. F test analysis used to understand the simultaneously ecological factors with the appearance of species number in each plot, whereas t-test analysis used to understand the effect of ecological factors partially on the number of species (Zhao et al. 2005).

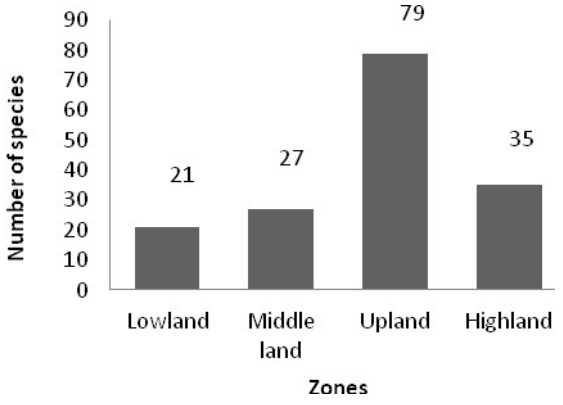

Figure 4. Number of species found in the study sites

Table 1. Ecological data and number of plant species

Environmental physical factors	Zones														
	Middle land				Upland							Highland			
	Plots														
	1	2	3	4	1	2	3	4	5	6	7	1	2	3	4
	Altitude (m asl.)														
	307	364	377	382	948	1031	784	790	1005	832	825	1655	1702	1662	1683
Air Temp. (°C)	37.5	36.75	42.25	41	26.13	24.75	24.88	25.38	27.88	32.88	32.75	17	18.75	19.75	19.75
Air Humidity (%)	49.75	45.5	44	48.8	81	85.13	87.38	86.5	59	59.75	66.25	95.5	92.75	92	95.25
Soil Temp. (°C)	32.5	33.5	32.5	31.5	23.88	20.63	24.88	24.25	22.63	27.13	26.13	17.5	17.25	17.5	17.75
Soil Moisture (%)	4.1	4.15	4	4.1	7.8	8.525	6	6.625	7.975	7.975	8.05	7.025	6.05	6.975	7.05
Soil pH	7.5	7.125	7.175	7	7	7.05	6.6	7.05	7.05	6.975	7.025	7	6.88	6.98	6.78
Light Intensity (Cd)	325	40	45	375	10	22.5	10	25	25	37.5	32.5	0	0	0	0
Number of species	17	20	17	20	20	13	20	21	32	26	24	10	13	16	22

Note: Light intensity valued zero in the highland zone because there were fogs during the sampling data taken

Table 2. F test analysis result of environmental factors to species number

ANOVA

Model		Sum of Squares	Df	Mean Square	F	Sig.
1	Regression	220.989	6	36.831	1.426	.313b
	Residual	206.611	8	25.826		
	Total	427.600	14			

Note: a. Dependent Variable: Species Number (Y); b. Predictors: (Constant), Light intensity (X6), Soil pH (X5), Soil moisture (X4), Air moisture (X2), Soil temperature (X3), Air temperature (X1)

Table 3. The result of t-test analysis of environmental factors to species number

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	SE	Beta		
1	(Constant)	101.879	83.817		1.215	.259
	Air temp. (X1)	-.436	.856	-.652	-.510	.624
	Air moisture (X2)	-.354	.253	-1.267	-1.400	.199
	Soil temp. (X3)	.310	.908	.330	.342	.741
	Soil moisture (X4)	2.318	1.260	.682	1.839	.103
	Soil pH (X5)	-9.566	9.433	-.337	-1.014	.340
	Light intensity (X6)	.002	.017	.050	.134	.896

Note: a. Dependent Variable: Species number (Y)

Ecological data and number of plant species were performed in Table 1. F test analysis showed that the sig value is 0.313> 0.05, it means, there was not any influence of environmental factors simultaneously (together) to the number of species in each plot (Table 2). The results of t-test analysis showed that each environmental factor, which consists of: (1) the air temperature had a sig value of 0.624> 0.05; (2) the air humidity had a sig value. 0.199> 0.05; (3) the soil temperature had a sig value of 0.741> 0.05; (4) soil moisture had sig value of 0.103> 0.05; (5) soil pH had sig value of 0.340> 0.05; and (6) the light intensity had a sig value of 0.896> 0.05, had not any significant effect to number of species (Table 3).

Plant species diversity might be affected by air temperature and precipitation, which highly depended on altitude and sunlight. The distribution of species had a significant association with spatial location, where altitude showed a lot of vegetation data, followed by latitude and longitude. The largest number of species was found at 1200 m asl, and fewer were found at altitude below 1200 m asl or above 1200 m asl (Zhao et al. 2005). The number of plant species in a particular community was also influenced by the availability of resources on a broad scale, the broad ecological niche of a species, or also affected by the time of evolution and the environmental succession (Pianka 1966). Environmental factors were the main factors caused biodiversity compared to biological factors (Huang 1994). The alteration of altitude, together with the changing in temperature, rainfall and sunlight conditions changed the distribution of vegetation (Jhonson et al. 1986). Habitat diversity and human activity were able to change water and temperature and caused vegetation diversity regarding altitude patterns. Since flora in the tropics was generally tropical, low temperatures limited the distribution of tropical plants along with increasing altitude, therefore plant species diversity decreased with increasing altitude (Zhao et al. 2005).

According to Rohde (1992), there was no a consistent correlation between species diversity, environmental stability, environmental predictability, productivity, abiotic rarefaction, physical heterogeneity, aridity, seasonality, number of habitats, and latitudinal ranges. The ecological and evolutionary time hypotheses, as usually understood, also could not explain the gradients, nor did the temperature dependence of chemical reactions permit predictions on species richness. Only differences in solar energy were consistently correlated with diversity gradients along latitude and altitude. It was concluded that greater species diversity is due to higher "effective" evolutionary time (evolutionary speed) in the tropics, probably as the result of shorter generation times, faster mutation rates and selection at higher temperatures.

The habitat condition of sandalwood

The total of 208 individuals grouped into the category of the stand and 4105 individuals categorized of floor vegetation of 27 species (both stand and floor vegetation) grouping to 13 families were found in the natural habitat of sandalwood in the middle land zone. The highest density for the seedling category was *Acacia mangium* with density of 27 individuals/ 0.24 ha; the highest Importance Value (IV) was *A. mangium* with IV of 20.9%. For the sapling category, the highest density was *Tectona grandis* with the density of 77 individuals/ 0.24 ha, which was also the species with the highest IV of 77.9%. While for the tree category was *T. grandis* with density of 22 individuals/0.24 ha, with IV equal to 60.4%. For the group of ground vegetation was occupied by *Eleusine indica* with density of 2267 individuals/0.24 ha and IV equal to 60.9%. All species were recorded in study sites were performed in Table 4.

Acacia mangium was planted as a marginal land plant. It could fix nitrogen significantly that helped to rehabilitate the soil. It was suitable to grow at an altitude of 0-400 m asl, at warm temperatures with very low to high rainfall and soil conditions with the range of pH 4-9. It could grow well under degraded soil conditions, tolerant to environmental stress, on barren land, clay, high salt soil or inundated soil; and suitable to be raised in various tropical and sub-humid regions (Aguiar et al. 2014; Tanaka et al. 2015). *T. grandis* could grow and develop well in moist areas compared with dry areas, with average temperatures during the daylight was 27-36°C and at night it was from 20-30°C. It also required soil with high calcium level with

the range of pH 6.5-7.5 (Kaosa-Ard 1989). According to An et al. (2014), *E. indica* was one of the herbs that had a high level of fecundity and a wide tolerance to various environmental factors that vary in certain habitat. The total species found in study sites were the list in Table 5.

In the upland zones were recorded 379 individuals of stand category, and 8462 individuals of floor vegetation from 79 species grouped into 31 families. The highest density and IV for seedling category were *Lantana camara* with density of 24 individuals/0.28 ha and IV of 10.4% respectively. For the sapling category was *Gmelina arborea* with density of 39 individuals/0.28 ha and IV of 28% respectively. While for the category of trees was *Senna siamea* with density of 27 individuals/ 0.24 ha and IV of 40.6% respectively. For the group of floor vegetation was *Cyperus rotundus* with density of 2889 individuals/0.28 ha and IV of 40.0% respectively. Priyanka and Joshi (2013) suggested that *L. camara* had the wide distribution in various habitat conditions and on various soil types (Sharma et al. 2005; Sankaran 2007). It was found from open areas, vacant lots, coastal ridges, tropical rainforests, and disturbed forests due to human activities such as burning and logging. Human activities further exacerbated the spread widely of the species. Sulaiman and Lim (1989) revealed that *G. arborea* could survive, easily reproduced, and its seed could grow in various climatic conditions. According to Joker (2000), *S. siamea* was a species that able to grow in various climatic conditions, from lowland areas with monsoon climate. It was also growing in degraded habitats, infertile soil and resistant to wind shocks because it had a shallow root system.

In the highland zone were recorded 63 individuals of stand category and 7201 individuals of floor vegetation from 35 species and grouped into 20 families. For seedling category, *Portulaca oleracea* had the highest density of 3 individuals/0.24 ha and *Senna occidentalis* had the highest IV of 12.8%. For the sapling category, *Spondias pinnata* had the highest density and IV of 10 individuals/0.24 ha and 23.3% respectively. The highest density and IV for tree category was *Eucalyptus urophylla* with a value of 27 individuals/0.24 ha and IV of 249.9% respectively. While for the group of floor vegetation was occupied by *C. rotundus* with density and IV of 2253 individuals/0.24 ha and 29.69% respectively. According to Sein and Mitlöhner (2011), *E. urophylla* was naturally found in Adonara, Alor, Flores, Lembata, Pantar, Timor and Wetar at an altitude of 180-3000 m asl. It was also found in hilly, clay areas, in open areas, secondary forests, and mountain forests with deep soil conditions, moisture, and well-drained soils. It also did not require so much soil nutrition. So, it was suitable for reforestation both on floodplains and dry lands in tropical lowland areas. Important value is needed to determine the composition of a forest community. According to Barbour et al. (1987), IV was a relative contribution within a community. The IV described the importance of the role of a vegetation species in the ecosystem (Kalaba et al. 2013). The IV at each growth rate would describe the important species at that level.

The dominant species at study sites were different. This difference was due to each species dominating a different region. The dominating species meant having a more extensive range tolerance of environmental factors if compared to the other species (Barbour et al. 1987). The environmental conditions led to competing among species with other species. Competition would increase the fighting power to alive, so that strong species will prevail and suppress other species. Losing species became less adaptive and caused low reproductive rates and were found in small amounts (Aerts 1999). Each plant species had a minimum, maximum and optimum condition of the existing environmental factors.

In the highland zone was recorded several species of orchids, fungi, mosses, and lichens. Fungi species was found included *Pleurotu sostreatus*, *Ganoderma applanatum,* and *Pleurotus cystidiosus*. Species of moss were found highlands such as *Pellia endiviifolia* , *Anthoceros punctatus*, and *Polytrichum abbreviatum*. *Usnea* sp. and *Parmalia* sp. were found too. The orchid species was found living commensalism to ampupu plants, as well as with moss and lichens. Solikin (2015) revealed that the vegetation in the Gunung Mutis Natural Reservation was dominated by ampupu, especially in the savannas. The orchid species found in the Gunung Mutis Natural Reservation included *Bulbophyllum ovalifolium, Bulbophyllum odoratum, Ceratostylis radiate, Dendrobium kuhlii, Eria retusa, Eria rhynchostyloides,* and *Pholidota rubra.*

Sandalwood was found in all research sites. This finding is supported by the living species associated with sandalwood such as *A. leucophloea, A.variabilis*, *A. squamosa*, *A. heterophyllus*, *B. javanica*, *C. cajan, C. annuum, C. odorata*, *E. hirta, I. cylindrica, L. camara L. leucocephala*, *O. sativa, P. Timoriensis, P.guajava*, *P. indicus, S. oleosa*, *S. siamea*, *S. grandiflora*, *S. macrophylla*, *T. grandis*, *T. procumbens*, and *Z. mauritiana* (Kaho 2011). The results are similar as expressed by Surata (2006) that sandalwood is a hemiparasite plant that requires other plants as hosts. The primary host species are *C. cajan*, *C. annuum*, *L. leucocephala*, and *S. grandiflora*. The secondary host species include *A. villosa*, *C. equisetifolia*, *I. cylindrica*, and *S. siamea*.

Based on the analysis, it is known that the number of species found on each plot in the middle land zone was 17 and 20 species (Figure 5). It means that the number of species found in the four observation plots was not different. In the upland zone, the smallest amount of species was found 13 species on plot 2, whereas the most was on plot 5. While in the highland zone was found 10 species on plot 1 and the most were found 22 species on plot 4. According to Pausas and Austin (2001), the variety of environment condition such as physical, chemical and interactions between species along the gradient of research sites caused the variety of species that live in it. This phenomenon would turn different if the environmental condition relatively homogeny. A relative homogeny of micro situ condition would be lived by individual from same species because it naturally developed adaptation mechanism tolerant to its habitat (Barbour et al. 1987). The number of species most commonly found was floor vegetation. It was because the study area was a savanna,

with rare stand condition and more found in floor vegetation. This condition was also supported because the vegetation is in a position with less density. The gaps formation created a microhabitat for underlying vegetation and provided opportunities for sapling, seedling, and floor vegetation to develop (Whitmore and Burnham 1984; Barbour et al. 1987; Campello et al. 2007; Naaf and Wulf. 2007). This condition also caused sunlight able to penetrate the forest floor so floor vegetation that generally light-requiring able to grow and develop (Campello et al. 2007).

Ecological condition

The average of ecological conditions was recorded in 15 plots presented in Table 5. The measurement result was a description of the condition in the field during the research.

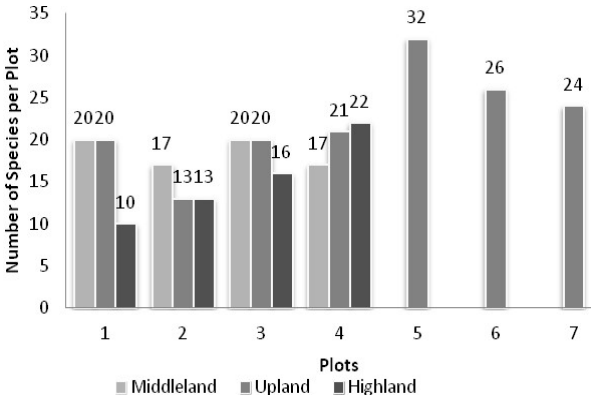

Figure 5. Number of species on each plot in study sites

Table 4. Species were recorded in study sites

Local name	Species	Family	Location			Habit
			Middle land	Upland	High land	
Akasia	*Acacia mangium* Wild.	Leguminosae	+	+	-	Stand
Alang-alang	*Imperata cylindrica* (L.) Raeusch	Poaceae	-	+	+	Shrub
Alpukat	*Persea americana* Mill.	Lauraceae	-	+	+	Stand
Ampupu	*Eucalyptus urophylla* S.T.Blake	Myrtaceae	-	+	-	Stand
Bakoma'a	*Hyptis capitata* Jacq.	Lamiaceae	-	+	-	Herb
Bandotan	*Ageratum conyzoides* (L.) L.	Compositae	-	+	+	Herb
Batang sirih hutan	*Peperomia luisana* Trel. & Standl.	Piperaceae	-	-	+	Herb
Baunoet	*Spigelia anthelmia* L.	Loganiaceae	-	+	-	Herb
Biduri	*Calotropis gigantea* (L.) Dryand	Apocynaceae	+	+	-	Herb
Bonsai	*Duranta erecta* L.	Verbenaceae	-	+	-	Shrub
Bunga kuning	*Tribulus terrestris* L.	Zygophyllaceae	-	+	-	Herb
Bunga mayana	*Coleus aromaticus* Benth.	Lamiaceae	-	-	+	Herb
Bunga putih	*Hippobroma longiflora* (L.) G.Don	Campanulaceae	+	+	+	Herb
Bunga putih terompet	*Hymenocallis acutifolia* (Herb. ex Sims) Sweet	Amaryllidaceae	-	+	-	Herb
Bunga ungu	*Stachytarpheta acuminata* DC. ex Schauer	Verbenaceae	+	+	+	Herb
Caliandra	*Calliandra biflora* Tharp	Leguminosae	+	+	-	Stand
Cemara	*Casuarina junghuhniana* Miq.	Casuarinaceae	-	+	-	Stand
Cendana	*Santalum album* L.	Santalaceae	+	+	+	Stand
Ceri hutan	*Syzygium cumini* (L.) Skeels	Myrtaceae	-	-	+	Stand
Cocor bebek	*Bryophyllum pinnatum* (Lam.) Oken	Crassulaceae	-	-	+	Herb
Cyperus	*Cyperus rotundus* L.	Cyperaceae	-	+	+	Herb
Damar hutan	*Agathis dammara* (Lamb.) Rich. & A.Rich.	Araucariaceae	-	+	-	Herb
Daun bentuk jantung	*Mikania micrantha* Kunth	Compositae	-	+	-	Herb
Daun dewa	*Gynura divaricata* (L.) DC	Compositae	-	-	+	Herb
Daun mint	*Plectranthus amboinicus* (Lour.) Spreng	Lamiaceae	+	+	+	Herb
Daun sendokan	*Plantago major* L.	Plantaginaceae	-	+	+	Herb
Daun tempel baju	*Desmodium rhytidophyllum* Benth.	Leguminosae	+	-	-	Shrub
Daun Ubi	*Manihot esculenta* Crantz	Euphorbiaceae	-	+	-	Herb
Delima	*Punica granatum* L.	Lythraceae	-	-	+	Stand
Euporbia	*Euphorbia heterophylla* L.	Euphorbiaceae	-	+	-	Herb
Fua koti	*Phyllanthus urinaria* L.	Phyllanthaceae	-	+	-	Herb
Gamal	*Gliricidia sepium* (Jacq.) Kunth ex Walp.	Leguminosae	-	+	-	Herb
Haukase	*Sambucus javanica* Blume	Caprifoliaceae	-	+	-	Herb
Huk kau	*Eleusine indica* (L.) Gaertn.	Poaceae	+	-	-	Herb
Huk pisu	*Axonopus compressus* (Sw.) P.Beauv.	Poaceae	+	+	+	Herb
Ito	*Barringtonia asiatica* (L.) Kurz	Lecythidaceae	-	+	-	Stand
Jahe merah	*Zingiber officinale* Roscoe	Zingiberaceae	-	+	-	Herb
Jambu	*Psidium guajava* L.	Myrtaceae	+	+	-	Stand
Jambu air	*Syzygium aqueum* (Burm.f.) Alston	Myrtaceae	-	+	-	Stand
Jamur kayu	*Ganoderma applanatum* (Pers.) Pat.	Ganodermataceae	-	-	+	Fungi
Jamur payung cokelat	*Pleurotus cystidiosus* O.K.Mill.	Pleurotaceae	-	-	+	Fungi
Jamur payung putih	*Pleurotus ostreatus* (Jacq.) P. Kumm.	Pleurotaceae	-	-	+	Fungi

Jati	*Tectona grandis* L.f.	Lamiaceae	+	+	-	Stand
Jati putih	*Gmelina arborea* Roxb.	Lamiaceae	+	+	-	Stand
Johar	*Senna siamea* (Lam.) H.S.Irwin & Barneby	Leguminosae	-	+	-	Stand
Kabesak	*Acacia leucophloea* (Roxb.) Willd	Leguminosae	+	+	-	Stand
Kacang hutan	*Senna occidentalis* (L.) Link	Leguminosae	-	-	+	Stand
Kangkung hutan	*Ipomoea pes-caprae* (L.) R.Br.	Convolvulaceae	-	-	+	Herb
Kapok	*Ceiba pentandra* (L.) Gaertn.	Convolvulaceae	-	+	-	Stand
Kate emas	*Euphorbia heterophylla* L.	Euphorbiaceae	-	+	-	Herb
Kayu putih	*Melaleuca leucadendra* (L.) L.	Myrtaceae	+	+	+	Stand
Kedondong hutan	*Spondias pinnata* (L.F.) Kurz	Anacardiaceae	-	-	+	Stand
Keladi	*Colocasia esculenta* (L.) Schott	Araceae	-	+	+	Herb
Kemiri	*Aleurites moluccanus* (L.) Willd.	Euphorbiaceae	-	+	-	Stand
King rass	*Pennisetum purpureum* Schumach.	Poaceae	-	+	-	Herb
Kiun'ut	*Cosmos caudatus* Kunth.	Compositae	-	+	+	Herb
Kuk nefo	*Tridax procumbens (*L.) L.	Compositae	-	+	-	Herb
Kunyit	*Curcuma longa* L.	Zingiberaceae	-	+	-	Herb
Kusambi	*Schleichera oleosa* (Lour.) Merr.	Sapindaceae	+	+	-	Stand
Lengkeng	*Dimocarpus longan* Lour.	Sapindeceae	-	+	-	Stand
Mahoni	*Swietenia macrophylla* King	Meliaceae	+	-	-	Stand
Mahoni hutan	*Swietenia* sp	Meliaceae	-	+	-	Stand
Maleku	*Oxalis corniculata* L.	Oxalidaceae	-	+	-	Herb
Masi	*Bauhinia purpurea* L.	Leguminosae	+	+	-	Stand
Nabasbot	*Hibiscus trionum* L.	Malvaceae	-	+	-	Herb
Name	*Diospyros abyssinica* (Hiern.) F. White	Ebenaceae	-	+	-	Stand
Nangka	*Artocarpus heterophyllus* Lam.	Moraceae	-	+	-	Stand
Nombesa	*Talinum fruticosum* (L.) Juss.	Talinaceae	-	+	-	Herb
Paku tanduk rusa	*Platycerium bifurcatum* (Cav.) C.Chr.	Polypodiaceae	-	+	-	Fern
Pangkase	*Lantana camara* L.	Verbenaceae	-	+	-	Shrub
Papa'i	*Sida cordifolia* L.	Malvaceae	-	+	-	Herb
Papo'e	*Arachis pintoi* Krapov. & W.C Greg	Leguminosae	-	+	+	Herb
Paria hutan	*Tithonia diversifolia* (Hemsl.) A.Gray	Compositae	-	+	-	Herb
Pegagan	*Centella asiatica* (L.) Urb.	Apiaceae	-	+	+	Herb
Papih	*Syzygium aromaticum* (L.) Merr. &L.M. Perry	Myrtaceae	+	-	-	Stand
Petes	*Leucaena leucocephala* (Lam.) de Wit	Leguminosae	+	+	-	Stand
Petikan Kebo	*Euphorbia hirta* L.	Euphorbiaceae	+	+	+	Herb
Pohon A	*Saraca indica* L.	Caesalpinaceae	-	-	+	Stand
Pohon B	*Pachystachys lutea* Nees	Acanthaceae	-	-	+	Stand
Pohon daun sirih	*Melastoma malabathricum* L.	Melastomataceae	-	-	+	Stand
Pulai	*Alstonia scholaris* (L.) R.Br.	Apocynaceae	-	+	-	Herb
Put'puta	*Polygala paniculata* L.	Polygalaceae	-	+	-	Herb
Putri malu	*Mimosa pudica* L.	Fabaceae	+	+	-	Shrub
Reo	*Pleiogynium timoriense* (A. DC.) Leenh.	Anacardiaceae	-	+	-	Stand
Rumput gajah	*Poa trivialis* L.	Poaceae	-	+	+	Herb
Rumput mutiara	*Oldenlandia corymbosa* L.	Rubiaceae	-	+	-	Herb
Rumput sarang buaya	*Lophatherum gracile* Brongn.	Poaceae	-	+	+	Herb
Sapotili	*Ficus natalensis* Hochst.	Moraceae	-	+	-	Stand
Spesies A	*Portulaca oleracea* L.	Portulacaceae	+	+	+	Herb
Spesies C	*Emilia sonchifolia* (L.) DC. ex DC.	Compositae	-	+	-	Herb
Spesies E	*Sida rhombifolia* L.	Malvaceae	-	+	-	Herb
Suf muti	*Chromolaena odorata* (L.) R.M.King & H.Rob.	Compositae	+	+	-	Shrub
Taduk	*Brucea javanica* (L.) Merr.	Simaroubaceae	-	+	-	Shrub
Tapak liman	*Elephantopus scaber* L.	Compositae	+	+	+	Herb
Temulawak	*Curcuma aeruginosa* Roxb.	Zingiberaceae	+	-	-	Herb
Terong	*Solanum melongena* L.	Solanaceae	-	+	-	Herb
Timu	*Timonius timon* (Spreng.) Merr.	Rubiaceae	+	+	-	Stand
Usmusi	*Aleurites moluccanus* (L.) Willd.	Euphorbiaceae	-	+	-	Stand

Note: + = exist, = not exist

Table 5. The analysis results of physical parameter

Zone	Parameters					
	Air temp. (°C)	Air humidity (%)	Soil temp. (°C)	Soil moisture	Light intensity (Cd)	Soil pH
Middle	39.4	47	32.5	4.1	380 lux	7.2
Upland	27.8	75	24.2	7.5	21.78 lux	7.8
Highland	18.8	93.8	17.5	6.7	-	6.9

The analysis result of physical condition showed that the air temperature in study sites was in the range of 39.4-18.8°C; Air humidity was in the range of 47.0-93.8%; (Table 5). Surata (2006) revealed that the air temperature that supports the growth of sandalwood was 10-35°C with air humidity conditions ranging from 50-60%.

The air humidity in the upland and highland zones were in the range of 75-93% due to the position of the existing location with an altitude of more than 500 m asl. Humidity would be high if the air temperature decreased and the light intensity was low. It also due to the density of the canopy that made few light intensity so that the air temperature was also low and the air humidity became high. According to Barbour et al. (1987), in the shade, temperature turned low than outside the shade. The lowest air humidity and temperature were found in the middle land zone. Since the location was only in the range of 300 m asl., the canopy was not tight and light intensity was high. The high light intensity was caused the air temperature increased so that the humidity was decreased. Withmore and Burnham (1984) revealed that the moisture was affected by air temperature and light intensity. If the light intensity was low, then the air temperature was low and the air humidity would be high. The analysis results showed that the air temperature and humidity in the middle land and highland zones were above the optimal range of sandalwood growth.

Soil pH analysis showed that the study sites had soil pH ranged from 6.9 to 7.8. It means that soil conditions in it were in a neutral condition. According to Surata (2006), soil pH conditions suitable for sandalwood growth were neutral-alkalis. These results were in line with Lio (2015) in which the soil pH of the natural forest of sandalwood in North Central Timor ranged from 6.56 to 6.9. Soil conditions were at a slightly acidic pH to neutral, in which under such circumstances sandalwood was able to live naturally. Other physical condition such as soil temperature, soil moisture, and light intensity respectively were 17.5-32.5°C; 4.1-7.5; 21.78-380 lux. This condition was allegedly optimal for sandalwood growth because it was still found in all study sites with soil temperature conditions classified as moist to heat, dry soil, and low to high light intensity. In North Central Timor, on soil conditions with hot soil temperature, wet soil, and high light intensity, sandalwood still found (Lio 2015).

In the highland zone, the light intensity was undetectable due to the fog that covers the forest and the tight canopy cover and the broad basal area of *E. urophylla*. According to Ediriweera et al. (2008), light intensity decreased with increasing canopy cover and basal area of the tree. Diaz et al. (2017) revealed that the fog (natural) as a water form of condensed water presence had a very significant impact on various environmental factors such as climate globally and regionally, air quality, water, flora, fauna, and others. It also caused the air humidity relatively high and the air temperature became low. It would appear along with altitude and slowly disappear in the sunshine, so that the heat raised.

Soil quality

Middle and upland zones had a soil texture class in the form of sandy clay while in the highland zone was clay sandy loam (Table 6). Almulqu et al. (2018) explained that the Gunung Mutis Natural Reservation is the wettest area on the Timor Island with rainfall almost every month throughout the year, with an average of 1500 up to 3000 mm/year. the soil texture in the nature reserve is also different from the other zones in East Nusa Tenggara, and sandalwood grew primarily in volcanic calcareous rocky terrain, shallow rocky soil, texture of clay soil from lime parent material, black soil color and red-brown. Soil types were generally lithosol, Mediterranean and complex soil (Hamzah 1976 in Surata 2006). Sandalwood requires fertile soil with good drainage (generally in dry land), and deep-souled soil solum (In shallow, rocky and under-fertile, it grew and produced wood with good quality (Surata 2006).

Table 6. Analysis result of soil quality

Zone	N (%)	P_2O_5 (ppm)	Fe	K (me/100g)	Ca	Fraction composition (%)			Texture class
						Sand	Dust	Clay	
Middle land	0.35	101.01	14.23	1.12	37.55	72.00	12.00	16.00	Sandy clay
Upland	0.26	99.75	17.61	0.89	30.07	73.00	11.00	16.00	Sandy clay
Highland	0.30	85.13	15.88	0.97	32.23	68.00	11.00	21.00	Clay sandy clay

Table 7. Criteria of soil chemistry assessment according to the Bogor Research Center (Soepraptoharjo 1983)

Nature of the soil	Very low	Low	Moderate	High	Very high
N (%)	< 0.10	0.10-0.20	0.21-0.50	0.51-0.75	> 0.75
P2O5 HCl (me/100 g)	< 10	10-20	21-40	41-60	> 60
K2O HCL 25% (me/100 g)	< 10	10-20	21-40	41-60	> 60
Ca (me/100 g)	< 2	2-5	6-10	11-20	> 20
Mg (me/100 g)	< 0.4	0.4-1.0	1.1-2.0	2.1-8.0	> 8.0

Sandalwood required high of Fe, Calcium, and Potassium from the soil (Nasi 1994 in Surata 2006). Based on the Criteria of soil chemistry assessment according to the Bogor Research Center (Soepraptoharjo 1983), the results of this study indicated that all study sites had a medium category of N and very high of P_2O_5 (Table 7). According to Kaho (2011), sandalwood could grow under low of N level and able to survive on high P level soil. To produce good sandalwood, it required moderate levels of N, medium-high P_2O_5 and high amounts of Fe, Ca and K. According to Mcwilliam (2005), it was known as hemi-parasitic plants or had semi-parasitic roots and required host suitable for nutrition and moisture intake for its self. Its seedling will only live if its roots were attached to the roots of the host plant, while the sandalwood tree was able directly to obtain nutrients from the soil (Fallick 2009).

According to Lio (2015). In the lowland, middle land and upland zones of North Central Timor included total N, total P, and total K at the ranged of 0.1-0.78%; 77.11-101.12 ppm; and 0.87-1.01 me/100g respectively and sandalwood was also still found that grew naturally. Based on those studies above, it could be concluded that all study sites are suitable and had an optimal soil nutrient condition for sandalwood growth.

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Genetic diversity of mastitis cow's milk bacteria based on RAPD-PCR

APON ZAENAL MUSTOPA[1,♥], INES FERDIANA PUSPITASARI[2], FATIMAH[3], LITA TRIRATNA[1], GINA KARTINA[1]

[1]Research Center for Biotechnology, Indonesian Institute of Sciences, Jl. Raya Bogor Km 46, Cibinong, Bogor 16911, West Java, Indonesia. Tel.: +62-21-8754587, Fax. +62-21-8754588. ♥email: azmustopa@yahoo.com
[2]Department of Biochemistry, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Bogor 16680, West Java, Indonesia
[3]Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development. Jl. Tentara Pelajar No. 3A, Cimanggu, Bogor 16111, West Java, Indonesia

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Abstract. *Mustopa AZ, Puspitasari IF, Fatimah, Triratna L, Kartina G. 2018. Genetic diversity of mastitis cow's milk bacteria based on RAPD-PCR. Biodiversitas 19: 1714-1721.* Mastitis in cow is caused by several pathogenic bacteria, including antibiotic-resistant bacteria. Identification of the pathogenic bacteria's diversity that is contaminating cow's milk needs to be done. The aim of this research was to conduct molecular identification in mastitis cow's milk bacteria through RAPD-PCR (Random Amplified Polymorphism DNA-Polymerase Chain Reaction) analysis. Bacteria from mastitis cow's milk were enumerated using selective media. Based on the result of media selection, there were 72 isolates of bacteria from mastitis cows in Ciguha, Guranteng, Cikarenceng Village (Pagerageung, Tasikmalaya), and Warnasari Village (Pangalengan, Bandung), West Java, Indonesia. The genomes from these isolates were extracted and then subjected to RAPD-PCR analysis. The results of RAPD-PCR analysis showed 8 clusters of dendrogram which 4 dominant clusters were selected. Identification of 4 dominant clusters, which contained representative strains, using 16s rRNA showed the isolates BPA-12, MHA-6, L-4, and XLDA-8 were identified as *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, and *Enterobacter bugandensis.*

Keywords: 16S rRNA analysis, mastitis cow's milk bacteria, RAPD-PCR analysis

INTRODUCTION

Mastitis in dairy cow is an important problem in Indonesia's dairy industries. Mastitis subclinical gives about 75-83% of cases until 2006 (Sudarwanto et al. 2006). Rahayu (2009) reported that the prevalence of subclinical mastitis increased by 85% and caused reduction in milk production by up to 15%. Ditjennak (2006) also reported that 80% of lactation cows in Indonesia are mastitis subclinical. It becomes the main problem for animal farmer because of milk production decline to 20%. In East Java, the prevalence of subclinical mastitis case is low, only 51.8% (Winarso 2008), compared to the same case in small dairy farm in Java Island that reached 85% in term of 2008-2010 (Nurhayati and Martindah 2015).

Ditjennak (2006) stated Indonesia's dairy population reach to 368,470 cows with milk production of 6.5-8.5 L/cow/day. While, ideal dairy production in Indonesia is 14-16 L/cow/day. Low milk production is analogous with 60-90% cows affected by mastitis in Indonesia, which most of cases caused by subclinical mastitis. Almost all cattle ranchers are unaware about subclinical mastitis because there is no physical change in milk and udder (Nurdin 2007). Economically, milk contamination gives disadvantage to producer and consumer because of low milk production and low quality. Milk quality is the most important factor for consumer and can determine the quality of production during processing (Collins et al. 2010). Normally, fresh cow's milk can last for 4 hours. Subclinical and clinical mastitis can contaminate milk and accelerate damage in milk.

However, treatments for this contamination in Indonesia is still not effective. They usually use broad-spectrum antibiotics without analyzing the specific causal agent. Improper mastitis treatment and using large-scale antibiotics could rise antibiotic resistance in certain bacteria (Sandholm and Pyorala 1995). It is necessary to test the mastitis-causing agent and consult with veterinarian in determining the type of drug and antibiotic to be used, so the treatment will be more effective (Waldner 2007).

There are 137 pathogens causing mastitis. The most common types of pathogens that infect large animal groups, are *Staphylococcus aureus*, *Streptococcus agalactiae*, other *Streptococcus* species and coliforms. Mastitis is often associated with other organisms such as *Actinomyces pyogenes*, *Pseudomonas aeruginosa*, *Nocardia asteroides*, *Clostridium perfringens*, *Mycobacterium*, *Mycoplasma*, *Pasteurella*, and *Prototheca*. Cases that occur are mostly caused by *Staphylococcus*, *Streptococcus*, coliforms, and *A. pyogenes* (Herlina et al. 2015).

Random Amplified Polymorphism DNA-Polymerase Chain Reaction (RAPD-PCR) technique is a type of molecular marker widely used in molecular biology research and diagnostic. The analysis results can be stored computerized and allowing rapid identification of unknown isolates. In addition, this technique is simpler, easier in its preparation, gives faster results, and produces unlimited characters that can help to analyze bacterial genetic

diversity. RAPD is an inexpensive yet powerful method to study diversity (Mustopa and Fatimah 2014; MacGowan et al. 1993; Williams et al. 1990).

This study aimed to analyze genetic diversity of mastitis cow's milk bacteria in West Java, Indonesia by using RAPD-PCR technique. The results of this study are expected to provide information of polymorphism pattern and obtained phylogenetic tree from pathogenic bacteria in mastitis cow's milk in Indonesia.

MATERIALS AND METHODS

Study area

Samples were collected at cattle farms in Ciguha, Guranteng, Cikarenceng Villages (Pagerageung, Tasikmalaya) and Warnasari Village (Pangalengan, Bandung), West Java, Indonesia. The research was conducted in Laboratory for Applied Genetic Engineering and Protein Design, Research Center for Biotechnology, Indonesian Institute of Sciences, Bogor, West Java, Indonesia. The research was begun from March to September 2017.

Procedures

Sample preparation

About 10 to 15 ml of milk samples were milked from cows infected with mastitis in 4 different villages. The milk sample was tested by Mastitis Test, homogenized sample for negative mastitis and slimy or dense sample for positive mastitis. Then, fresh milk samples were diluted and enumerated by total plate count (TPC) method (Yunita et al. 2015). As much as 100 µL of bacterial suspension were removed from each dilution into petri dishes containing media selection of Baird Parker Agar (BPA) (Baird-Parker 1962), Mueller Hinton Agar (MHA) (Mueller and Hinton 1941), Xylose Lysine Deoxycholate Agar (XLDA) (Zajc-Satler and Gragas 1977), and Listeria Oxford Agar Base (LOAB) (Lee and McClain 1986). Suspension was spread into agar medium in petri dish. The petri dish was incubated at 37°C for 24-48 hours. The growing colony is transferred into Nutrient Broth (NB) medium, incubated at 37°C overnight and stored at 4°C.

Genomic DNA extraction

The DNA genome from the growing colony (bacteria isolate) was extracted by the method developed by

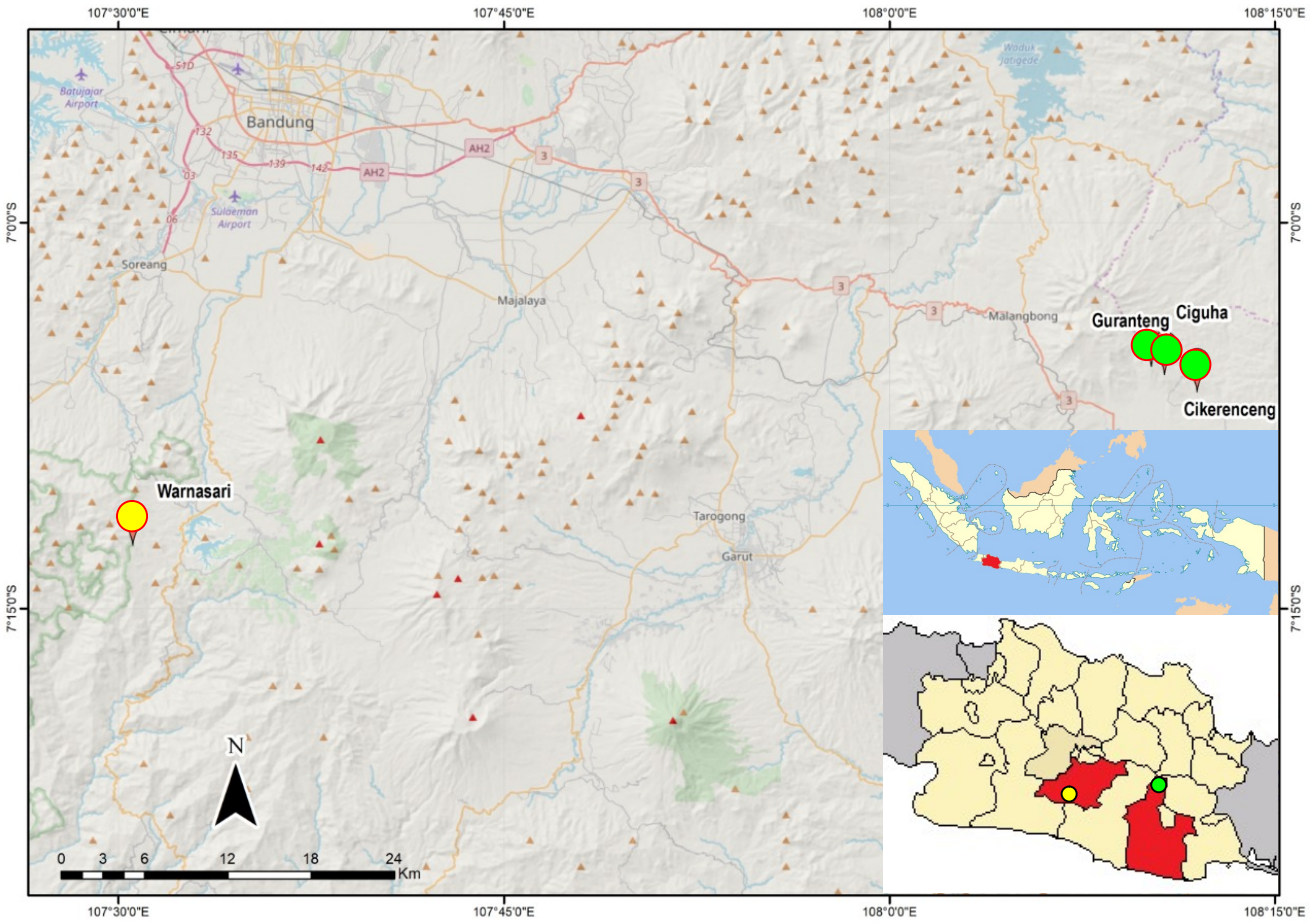

Figure 1. Location of sample collection in Pagerageung Sub-district (●) of Tasikmalaya and Pangalengan Sub-district (●) of Bandung, West Java, Indonesia

Mustopa and Fatimah (2014). The isolates were transferred into 1.5 mL sterile tube and centrifuged at 11,000 x g for 10 minutes, at 4°C. The cell pellets were suspended with 540 µL of Tris-EDTA buffer and 10 µL lysozyme, then homogenized and incubated at 37°C for 60 minutes. The solution was added with 200 µL SDS 10%, 100 µL NaCl 5 M, 80 µL 10% CTAB and incubated at 68°C for 30 minutes. Then, chloroform was added 1:1 (v/v), and centrifuged at 23000 x g for 10 minutes. The top phase solution was transferred to a new microtube, then isopropanol was added with a volume ratio of 1:1 and centrifuged. The DNA pellets were added by 1 mL 70% cold ethanol and inverted. Then, centrifuged at 10000 x g for 2 minutes at 4°C. The DNA pellets were aired overnight. Dry DNA was solubilized in 27 µL of sterile water (ddH_2O) and 3 µL RNAse. The DNA solution incubated at 37°C for 30 minutes, then stored at 4°C.

RAPD-PCR

The composition of RAPD-PCR reaction of mastitis cow's milk bacteria can be seen in Table 1. The PCR was performed by pre-denaturation at 94°C, 3 mins; followed by 44 cycles of denaturation at 94°C, 1.5 mins; annealing at 36°C, 1 min; extension at 72°C, 2 mins; and final extension at 72°C, 5 mins. The PCR product further analyzed by electrophoresis on 1.5 % (w/v) agarose gel in 0.5x TBE buffer and stained with Ethidium Bromide (EtBr). Marker Universal Ladder was used as standard DNA size (Chao et al. 2008). The results of RAPD-PCR can be visualized in electrophoregram.

Identification of mastitis cow's milk bacteria

16S rRNA gene sequencing for bacterial identification was using PCR with two primers. The primers were 8F primer (5'-AGAGTTTGATCATGGCTCAG-3') and 16R primer (5'-AAGGAGGTGATCCAACCGCA-3'). The PCR mix comprised 16 µL distilled water, 1 µL DNA template, 0.2 µL 8F primer, 0.2 µL 16R primer, and 0.2 µL *Taq* (Dream). The PCR was performed using pre-denaturation at 94°C, 3 mins, followed by 30 cycles of denaturation at 94°C, 1 min; annealing at 50°C, 1 min; extension at 72°C, 2 mins; and final extension at 72°C, 5 mins. The PCR product was analyzed by electrophoresis on 1% (w/v) agarose gel in 0.5x TBE buffer and stained with EtBr. Marker 1 kb Ladder was used as standard DNA size. The 16S rRNA gene is then direct sequenced. The sequencing results were analyzed using Basic Local Alignment Search Tool (BLAST) in the NCBI Program. The phylogenetic tree is further prepared using MEGA 6.06 application (Chao et al. 2008).

Data analysis

Evaluation of the DNA bands was based on specific molecular weight. The resulting amplification fragment is the dominant DNA locus. The RAPD marker was scored manually. Scoring criteria based on the presence of locus, 1 for presence and 0 for absence. Electrophoregram was manually analyzed to be a binary number of 1-0 in excel format. The binary data further processed into dendrogram or called phylogenetic tree using NTSYS 2.02 program (Rohlf 1993).

RESULTS AND DISCUSSION

Bacterial isolation of mastitis cow's milk samples in four different regions were selected by four different selective media, which were BPA, MHA, XLDA, and LOAB. The bacterial isolation showed 72 isolates as shown in Table 2.

Table 1. RAPD amplification reaction of mastitis cow's milk bacteria

Substance	Final concentration	1X (*) (µL)
10X PCR buffer	1X	2
20 µM RAPD primer	0.3 µM	3
5 µM dNTPs	0.3 µM	2
Taq Polymerase		0.2
ddH_2O		10.7
DNA samples		2
Volume total		20

Note: * Total of samples prepared; (#) sample volume 2 µL per reaction

Table 2. Isolate code and the origin of the used isolates

Isolate code	Origin	No	Isolate code	Origin
BPA-1	Ciguha	37	L-13	Warnasari
BPA-2	Ciguha	38	L-14	Warnasari
BPA-3	Ciguha	39	L-15	Warnasari
BPA-4	Ciguha	40	XLDA-1	Guranteng
BPA-5	Ciguha	41	XLDA-2	Guranteng
BPA-6	Guranteng	42	XLDA-3	Guranteng
BPA-7	Guranteng	43	XLDA-4	Guranteng
BPA-8	Guranteng	44	XLDA-5	Guranteng
BPA-9	Guranteng	45	XLDA-6	Cikarenceng
BPA-10	Guranteng	46	XLDA-7	Cikarenceng
BPA-11	Cikarenceng	47	XLDA-8	Cikarenceng
BPA-12	Cikarenceng	48	XLDA-9	Cikarenceng
BPA-13	Cikarenceng	49	XLDA-10	Cikarenceng
BPA-14	Cikarenceng	50	XLDA-11	Warnasari
BPA-15	Cikarenceng	51	XLDA-12	Warnasari
BPA-16	Cikarenceng	52	XLDA-13	Warnasari
BPA-17	Cikarenceng	53	XLDA-14	Warnasari
BPA-18	Cikarenceng	54	XLDA-15	Warnasari
BPA-19	Cikarenceng	55	MHA-1	Ciguha
BPA-20	Cikarenceng	56	MHA-2	Ciguha
BPA-21	Warnasari	57	MHA-3	Ciguha
BPA-22	Warnasari	58	MHA-4	Ciguha
BPA-23	Warnasari	59	MHA-5	Ciguha
BPA-24	Warnasari	60	MHA-6	Guranteng
L-1	Warnasari	61	MHA-7	Guranteng
L-2	Warnasari	62	MHA-8	Guranteng
L-3	Warnasari	63	MHA-9	Guranteng
L-4	Warnasari	64	MHA-10	Guranteng
L-5	Warnasari	65	MHA-11	Cikarenceng
L-6	Warnasari	66	MHA-12	Cikarenceng
L-7	Warnasari	67	MHA-13	Cikarenceng
L-8	Warnasari	68	MHA-14	Cikarenceng
L-9	Warnasari	69	MHA-15	Cikarenceng
L-10	Warnasari	70	MHA-16	Warnasari
L-11	Warnasari	71	MHA-17	Warnasari
L-12	Warnasari	72	MHA-18	Warnasari

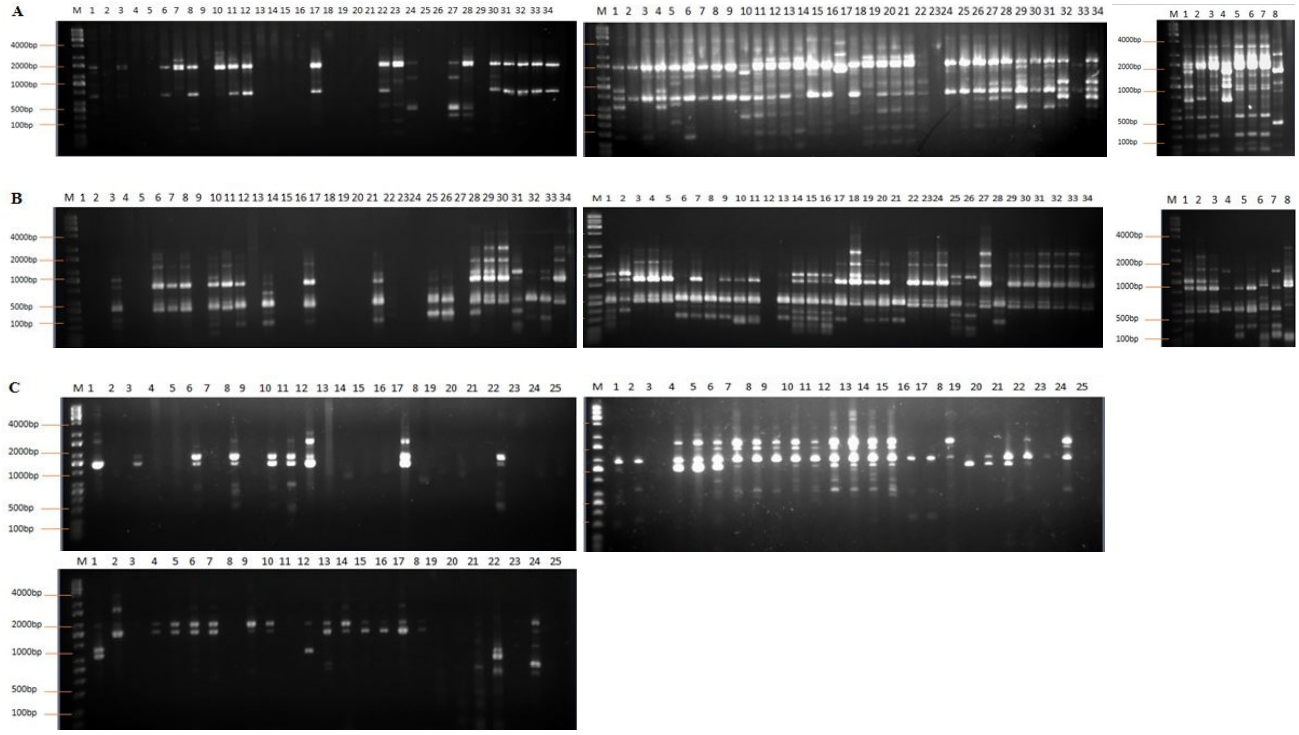

Figure 2. DNA profiling of mastitis cow's milk bacteria using RAPD PCR. A. Primer A, B. Primer B, C. Primer C

Genomic DNA extraction results

Qualitatively, the genomic DNA extraction result from 72 bacterial isolates were visualized in electrophoresis gel. The results show a band size greater than 10 kb, even though there are 4 samples that do not show DNA band (XLDA-15, L-9, L-12, and L-15). Quantitative analysis of genomic DNA showed different concentration each isolates as shown on Table 3. The purity of DNA from protein were analysed by 260/280 ratio and show great purity in 23 bacterial isolates. While the purity of DNA from polysaccharide were analysed by 260/230 and show great purity in 9 bacterial isolates.

RAPD-PCR identification and analysis of genetic relationship of mastitis cow's milk bacteria

Polymorphism identification was done with RAPD-PCR method. This PCR used 3 random primers (primer A, primer B, and primer C). The results were visualized in electrophoregram (Figure 2). The band pattern similarities on the electrophoregram showed the bacterial diversity. Electrophoregram was analyzed manually and changed into binary number 1 and 0. Then, the binary number data were analyzed in NTSYS 2.02 program to show the genetic relationship of mastitis cow's milk bacteria in dendrogram (Figure 3). The result of dendrogram was divided in 8 clusters with 4 dominant clusters (red circle).

16S rRNA analysis of mastitis cow's milk bacteria

PCR results using primer 8F and 16R primers for 16S rRNA identification can be seen in Figure 4. Positive results were indicated by the appearance of DNA bands of 1500 bp. The positive PCR results were sequenced to determine the base sequence of the 16S gene from each isolate. The gene sequence was then analyzed with Basic Local Alignment Search Tool (BLAST) using MEGA program and obtained phylogenetic tree for BPA-12 isolate (Figure 5), isolate MHA-6 (Figure 6), isolate L-4 (Figure 7), and XLDA-8 isolates (Figure 8). The identification of 16S rRNA showed that BPA-12 is *S. aureus* bacteria, L-4 is *Listeria monocytogenes* bacteria, MHA-6 is *Escherichia coli* bacteria, and XLDA-8 is *Enterobacter bugandensis* bacteria.

Discussion

Pathogenic bacteria diversity from mastitis cow's milk was evaluated. Seventy-two of pathogenic bacteria were isolated using 4 different media selections. Then, bacteria DNA was extracted and analyzed by 2 parameters, which are qualitative analysis and quantitative analysis. Qualitatively, the genomic DNA extraction results were visualized in electrophoresis gel. All samples show a band size greater than 10 kb, except XLDA-15, L-9, L-12, and L-15 sample. It might happen because its low DNA concentration, therefore, it is slightly invisible on the electrophoregram. Damerdesh et al. (2012) stated the chromosomal DNA from bacteria has a size more than 10 kb which is about 21-23 kb. Spectrophotometric analysis was used to quantitate DNA genome from the isolates by using a UV-VIS spectrophotometer that measured absorbance at 230 nm, 260 nm, and 280 nm. All ratio A260/280 results were in the range from 1,086 to 2,431. Good quality DNA will have an A260/280 ratio of 1.8–2.0 A (Sambrook et al. 1989). Lower A260/280 values may indicate protein contamination. Then, the ratio A260/230

from these isolates were in the range of 1.124-2.309. A260/230 values greater than 1.8 are typically suitable for analysis. Lower A260/230 values indicate contamination with salts or some solvents (e.g., phenol). These results showed that 22 isolates have a good level of purity (not contaminated by protein) and 8 isolates are not contaminated by polysaccharide, salt, or some solvents. It means that the majority of genomic DNA extracted from 72 isolates having un-pure DNA. According to Weishing et al. (2005), this is caused by endonuclease activity that destroys DNA, the high content of polysaccharides that increases the viscosity of the isolated product, and the inhibitor components (e.g. polyphenols and other secondary metabolites) contamination. DNA concentration was determined by measuring absorbance at 260 nm (Sambrook et al. 1989). The DNA concentrations of 72 isolates were varied from 20 ng/µL to 1607 ng/µL. BPA-2 isolate has the highest concentration (1607 ng/µL) although its purity level is low (low than 1.8). While, the lowest DNA concentration is L-4 and it can be detected by electrophoresis. XLDA-15 DNA that was not detected by electrophoresis because it has a low concentration (24 ng/µL). While L-9, L-12, and L-15, which are also not detected by electrophoresis, have concentrations of 107, 226, and 64 ng/µL, respectively.

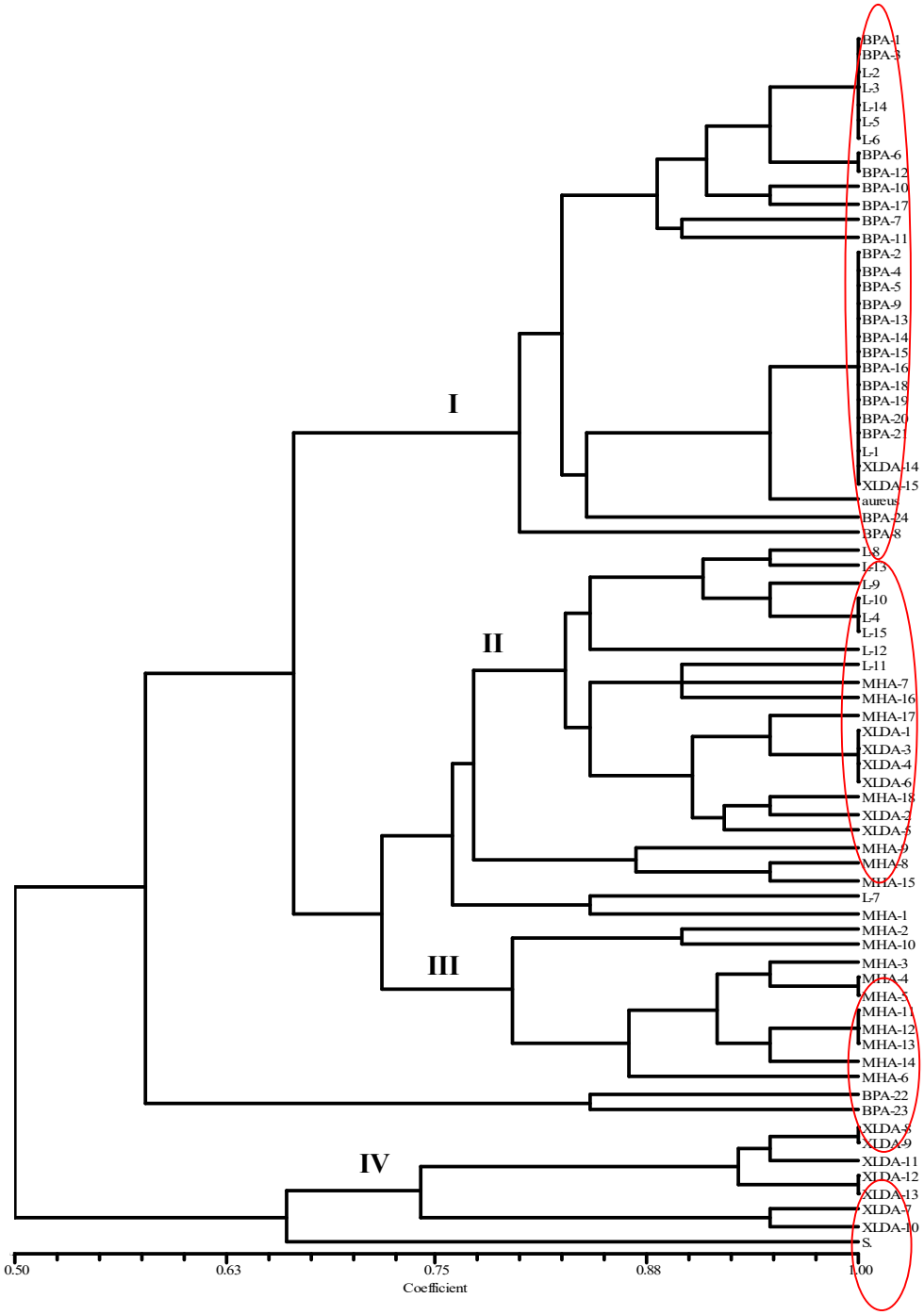

Figure 3. Dendrogram of RAPD-PCR analysis using NTSYS 2.02 program

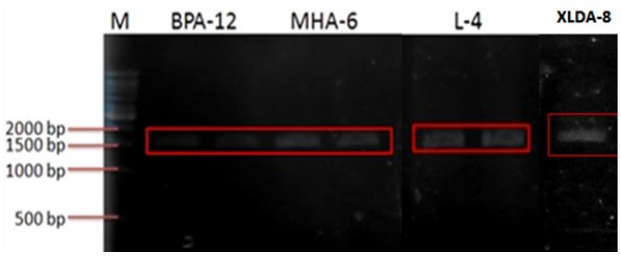

Figure 4. Electrophoregram of 16S rRNA identification result

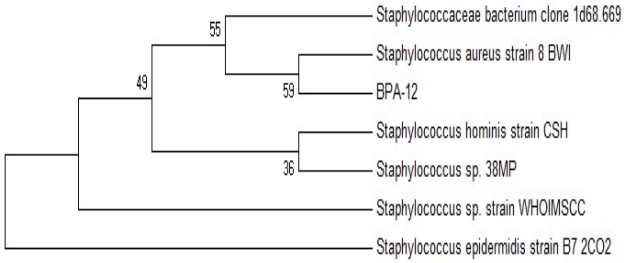

Figure 5. Phylogenetic tree of BPA-12 isolate

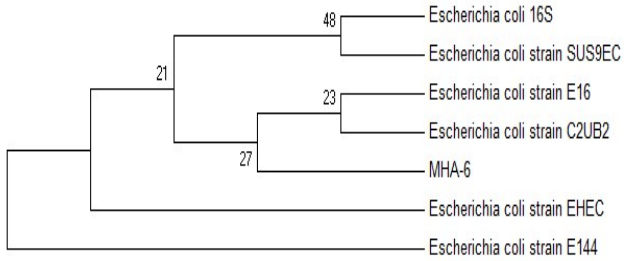

Figure 6. Phylogenetic tree of MHA-6 isolate

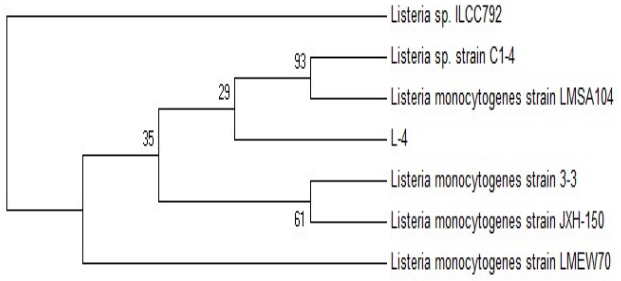

Figure 7. Phylogenetic tree of L-4 isolate

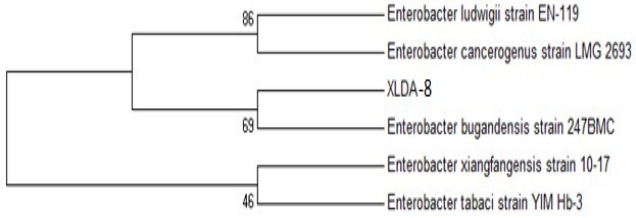

Figure 8. Phylogenetic tree of XLDA-8 isolate

Then polymorphism identification was done by RAPD-PCR method. DNA amplification is exponentially carried out with the aid of an enzyme and in vitro (Yuwono 2006). This PCR uses 3 random primers (primer A, primer B, and primer C). RAPD's excessive technique is tolerant to the level of DNA purity so it does not require high DNA purity (Prana and Hartati 2003). This technique is suitable for 72 isolates in this study which have low purity levels. Fingerprint analysis results showed the high level of polymorphisms. The polymorphism level reached 100%. This result indicates that 72 isolates from the 5 different regions are not really identical at the molecular level. Electrophoregram results showed that there is no characteristic of monomorphism, only one band appears at the same size in each isolate. Although this technique is tolerant to DNA purity level, but the intensity of the band of amplification product is influenced by the purity and concentration of DNA (Beishir 1991).

The result of the analysis of the NTSYS 2.02 program with the UPGMA method is a dendrogram that can express the proximity of each isolate (Figure 3). Dendrogram divided 72 isolates into 8 clusters with 4 main clusters (red circle). This grouping is based on the formation of the same band pattern produced by the same type of bacteria (Aqmarina 2014). Genetic relationship between bacteria was analyzed by genetic distance (Nei 1978). Cluster I consisted of 31 bacterial isolates, namely BPA-1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 24; L-1, 2, 3, 5, 6, 14; XLDA-14, and 15. The dominant isolates of cluster I was BPA isolates with 22 of 31 isolates. Cluster II consisted of 18 isolates, namely L-4, 8, 9, 10, 11, 12, 13, 15; MHA-7, 16, 17, 18; XLDA-1, 2, 3, 4, 5, and 6. Cluster II was dominated by isolate L with 8 of 18 isolates. Cluster III consisted of 10 isolates of bacteria, namely MHA-2, 3, 4, 5, 6, 10, 11, 12, 13, and 14. All isolates in cluster III are MHA isolates. Cluster IV consisted of 7 isolates of bacteria, namely XLDA-7, 8, 9, 10, 11, 12, and 13. Isolates in cluster IV were all XLDA isolates. Isolates of BPA that dominate cluster I consisted of isolates from Tasikmalaya (Ciguha, Guranteng, Cikarenceng) and Bandung (Warnasari). Isolate L which dominates cluster II came from Bandung (Warnasari). Isolate MHA that dominates cluster III came from Tasikmalaya (Ciguha, Guranteng, and Cikarenceng). Isolates XLDA that dominate cluster IV came from Tasikmalaya (Cikarenceng) and Bandung (Warnasari). Clustering is based on bacterial diversity with 80% coefficient. Isolates dominating each cluster were used as a reference in selecting bacterial isolates to be identified by the 16S rRNA technique. Four isolates were selected and had been identified namely as BPA-12, L-4, MHA-6, and XLDA-8.

PCR results using primer 8F and 16R primers for identification of 16S rRNA can be seen in Figure 4. Positive results are indicated by the appearance of DNA bands of 1500 base pair (bp). The results of 16S rRNA can be seen as phylogenetic tree for isolate BPA-12 (Figure 5), isolate MHA-6 (Figure 6), isolate L-4 (Figure 7), and XLDA-8 isolates (Figure 8). The identification of 16S rRNA showed that BPA-12 isolate is included as *S. aureus* bacteria. L-4 isolate is included as *L. monocytogenes*

bacteria. MHA-6 isolate is included as *E. coli* bacteria, and XLDA-8 isolate is included as *E. bugandensis* bacteria. While, the similarity level of each isolate with gene bank data is 97%, 94%, 98%, and 94%. Herlina et al. (2015) showed that the dominant bacteria present in mastitis cattle were *Staphylococcus* sp., *Pseudomonas aeruginosa*, *L. monocytogenes*, and *E. coli*. Epidemiological studies in Egypt found the main agents of subclinical mastitis isolated from positive CMT were *S. aureus*, *S. agalactiae*, and *E. coli*. with prevalence are 52.5%, 31.25%, and 16.25%, respectively. *S. aureus* is bacteria that causes the most subclinical mastitis. It can move quartile during the process of milking so that the transmission occurs. While the incidence of mastitis in dairy cows caused by *Pseudomonas* bacteria is very rare and sporadic (Supar and Ariyanti 2008). *E. bugandensis* is a highly pathogenic species of the genus *Enterobacter*. It is a nosocomial pathogen that can cause life-threatening infections in neonates and immunocompromised patients (Pati et al. 2018).

In conclusion, mastitis cow's milk bacteria in Ciguha, Guranteng, Cikarenceng, and Warnasari Village have 100% polymorphism value. The results of RAPD-PCR analysis showed 8 clusters from 72 bacterial isolates, with 4 dominant clusters. Coefficient of diversity and polymorphism value show bacterial isolates from 4 areas of milk sampling have a high bacterial similarity. Dominant bacterial isolates are BPA-12. MHA-6, L-4, and XLDA-8. BPA-12 are identified as *S. aureus* bacteria, MHA-6 are identified as *E. coli* bacteria, L-4 are identified as *L. monocytogenes* bacteria, and XLDA-8 are identified as *E. bugandensis* bacteria.

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Recognizing indigenous knowledge of the Karangwangi Rural Landscape in South Cianjur, Indonesia for sustainable land management

FATIYA AMELIA[1], JOHAN ISKANDAR[2,3], RUHYAT PARTASMITA[3,♥], NICHOLAS MALONE[4]

[1]Post Graduate and Environmental Study (PSMIL), Padjadjaran University. Jl. Sekeloa Selatan I Bandung 40134, West Java, Indonesia.
[2]Postgraduate of Environmental Study (PSMIL & DIL) and Institute of Ecology PPSDAL, Padjadjaran University. Jl. Sekeloa Selatan I Bandung 40134, West Java, Indonesia.
[3]Department of Biology, Faculty of Mathematics and Natural Sciences, Padjadjaran University.Jl. Raya Bandung-Sumedang Km 21, Jatinangor, Sumedang 45363, West Java, Indonesia. ♥email: rp2010rikkyo@gmail.com ; ruhyat.partasasmita@unpad.ac.id
[4]Department of Anthropology, University of Auckland, Levels 7 and 8, Human Sciences Building, 10 Symonds Street, Central Business District, Auckland 1010, New Zealand

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Abstract. *Amelia F, Iskandar J, Partasmita R, Malone N. 2018. Recognizing indigenous knowledge of the Karangwangi Rural Landscape in South Cianjur, Indonesia for sustainable land management. Biodiversitas 19: 1722-1729.* Karangwangi is a rural community on the south coast of West Java, Indonesia. The people of Karangwangi possess traditional ecological knowledge (TEK) of local landscapes through cultural inheritance and personal experiences of interacting with their environment. The people of Karangwani Village recognize various natural-cultural landscape types, including *leuweung* (forest); swidden field (*huma*); wet rice fields (*sawah*); home garden (*pekarangan*); garden (*kebun*); mixed-garden (*kebun campuran*); river (*sungai*); and sea (*laut*). These various landscapes have continuously changed over time due to people's socio-economic and cultural activities. The aim of this study was to develop an ethnoecological approach to elucidate historical changes to the Karangwangi landscapes. Toward this aim, we conducted mixed-method, qualitative and quantitative research. In addition to recognizing the various types of cultural and natural landscapes, the local people of Karangwangi are able to describe the history of landscape changes between 1950 to 2017. As identified by informants, these changes have been caused by various factors, including increases in population density, implementation of government policies and village development.

Keywords: Ethno-ecology, landscape, Karangwangi, local people, TEK

INTRODUCTION

The people of Karangwangi live in rural West Java and possess traditional ecological knowledge (TEK) of their natural surroundings. They predominantly use Sundanese as a local language and have customary habits in activities related to the natural surroundings. For example, the timing of rice farming activities are determined by observing the *bintang kidang* (orion belt), and the rain cycle called *windu* (*alim, he, early jim, je, dal, be wau and jim akhir*) associated with climate and planting time (Iskandar and Iskandar 2016). Agricultural activity is the main sector of people's livelihood in Karangwangi. The village landscape is patterned by human due to human interaction with the surrounding environment. Within the Karangwangi landscape, it is apparent that the community is required to preserve the natural environment as reflected by the various landscape units formed. Based on research conducted by Iskandar et al. (2017) it has been revealed that there are several land type categories present in Karangwangi Village, including conservation forest (*hutan konsevasi*), swidden (*huma)*, homegarden (*pekarangan*), mixed garden (*kebon tatangkalan*), dry land (*tegalan)*, community plantation, semi-technical irrigation, simple irrigation, rain-fed rice field, and tidal rice field.

Karangwangi Village administratively began to form in 1984 which is the division of the Village Cidaun (Iskandar and Iskandar 2016). The formation of the landscape in Karangwangi is in line with the development of the village due to the utilization and management of the land. It is influenced by several additional factors, including changes in *huma* land policies; increasing population; expanding infrastructural development; tourism; and reduced forest area. Furthermore, the development of a market economy caused changes in the behavior and lifestyle of the people of Karangwangi Village. These dynamic processes of transformation align with the opinion of Farina (2010) that the landscape continuously to transforms by way of religious, cultural, economic, political and environmental activity. Thus, information about the history of change and the formation of various patterns of landscape use in this village is of interest (Ramdhan et al. 2015), and can facilitate sustainable landscape utilization and management (Takeuchi 2010; Jumari 2012; Asmiwyati 2015; Ramdhan et al. 2015).

MATERIALS AND METHODS

Study Sites

This research was conducted in Karangwangi Village, Cidaun Sub-district, Cianjur District, West Java Province, Indonesia. Geographically, the village is located at 7º25'-7º30 'S and 107º23'-107º25' N. Administratively, it is an expansion of Cidamar Village with an area of 2,300.17 hectares, and located at an altitude of 200-275 m above sea level. The village is traversed by two rivers, namely Cikawung River and Cisela River. Karangwangi Village is bordered by to the north by Cimaragang Village to the west by Cidamar and Kertajadi Villages, to the east by and to the south by the Indian Ocean. In 2015, the population in Karangwangi Village has recorded 5,587 inhabitants consisting of 1,817 families (Partasasmita 2015; Iskandar et al. 2016; Partasasmita et al. 2017) (Figure 1).

Procedure

This research used an ethnoecological approach with a mixed method design (Iskandar 2012b; Albuquerque et al. 2014). The collection and analysis of qualitative and quantitative data were carried out simultaneously. Qualitative data collection techniques included participant observation, in-depth, semi-structured interviews, with key informants, selected purposively with snowball sampling. Quantitative data were collected through the use of structured interviews (questionnaire) with randomly selected respondents to support and cross-check qualitative data.

Data analysis

Qualitative data analysis of semistructured interviews is done in three stages: (i) identifying themes present across; (ii) determining salient aspects of-of the interview data and summarizing the responses of interview participants; and (iii) contextualizing the information with the relevant literature and descriptive (quantitative) analysis (Iskandar 2012b). Quantitative data analysis of structured interviews (questionnaires) will use simple statistics with percentages and descriptive analysis.

RESULTS AND DISCUSSION

Based on local knowledge, information on the history of landscape changes in this village began in the 1950s. These landscape changes are categorized into roughly one-to-two decade length periods (Figure 2).

The changes of Karangwangi landscape between 1950-1970

The history of Karangwangi village is inseparable from the history of the *leuweung* (forests) and *bojonglarang* (nature reserves) that play a major role in village formation. The Jayanti Bojonglarang forest has a total area of 750 ha. Initially, the area of 250 ha forest is utilized by the community and 500 ha is a *Hutan Perlindungan Alam* (Natural Forest) or forest that should not be disturbed. Since prior to the formation of the village, only the local people of Karangwangi only accessed this *leuweung* (forest) because they believed that *bojonglarang teu meunang digadabah* (should not be disturbed). This is because the community believes in hereditary myths which consider the *bojonglarang* to be historically sacred. For example, it is believed that the village of Karangwangi is a place of *petilasan* (once visited someone important), in this case, Raden Kian Santang, the son of Prabu Siliwangi (the former ruler of the Sundanese Kingdom of Padjajaran). Folk mythologies describe that the son of Prabu Siliwangi had intended to circumcise (*sunat*) his father. However, it did not happen because Prabu Siliwangi who was reluctant to be circumcised by his son, fled, and transformed into a tiger (*Maung Padjadjaran*) in the forest (then called *bojonglarang*).

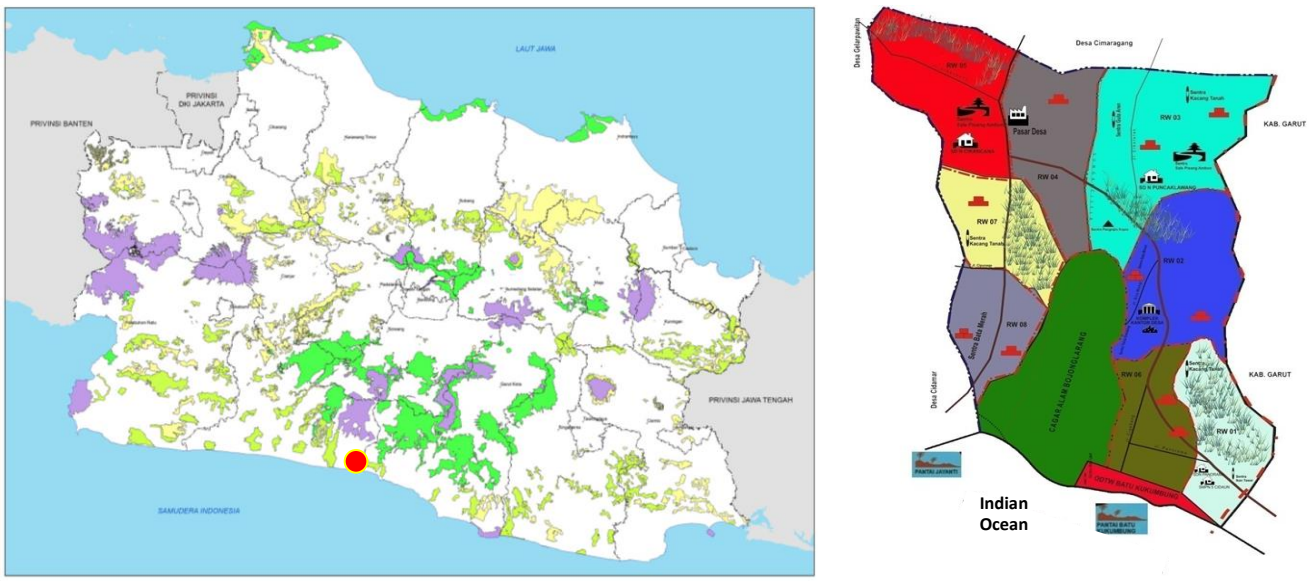

Figure 1. Research location, Karangwangi Village (●), South Cianjur, West Java, Indonesia (Iskandar et al. 2016; Partasasmita et al. 2016)

The place used for this circumcision is known as *Batu Kukumbung*, located on Cigebang Beach on the southern coast of Karangwangi Village. Batu Kukumbung is defined as a stone where *kukumbung* (gathered) the community when Prabu Siliwangi will be circumcised by his son. This traditional story has spread to other villages as well. Many people believe that Batu Kukumbung is evidence of the entry of Islam (first) to Java Island, brought by Raden Kian Santang. Presently, it is common to find at Batu Kukumbung the placement of various incense (evidence of ritual prayer) conducted by people outside Karangwangi Village. According to some scholars (Lovelace 1984; Berkes 1999; Toledo 2002; Iskandar 2012a), myths are the result of the mutual relationship between humankind and nature and strongly impact the communal management and/or exploitation of nature.

In the 1950-1960s villagers were heavily dependent on forests, because local people used it as a swidden farming site that was the main livelihood of the community at the time. Farming rice in the forest is locally known as *ngahuma* (swidden cultivation). The advantage of planting rice in the forest is the natural fertility that is available so that *pare* (rice) which is the main commodity can flourish. This is confirmed by Geertz (1963) that the *huma* system is very good for ecology because it is more integrated into the general structure of the natural ecosystem (forest), and therefore facilitates ecosystem dynamics. *Huma* system (swidden agriculture, shifting cultivation, slash-and-burn cultivation or long-fallow agriculture system) caused changes in the forest used. Therefore, in the process of formation of *huma* there are several stages, namely: (i) *jami*, secondary forest former *huma* newly abandoned or *diberakan* (fallowed) less than a year that still has the remaining straw; (ii) *reuma*, jami which is *diberakan* (fallowed) more than one year; (iii) *rungkun*, *ruyuk* or *dungus*, secondary forest overgrown with shrubs; (iv) *reuma*, if the land is old enough to be marked with old shrubs, this land can be cultivated again (Iskandar and Iskandar 2012a; Iskandar 2012b; Iskandar and Iskandar 2016; Iskandar and Iskandar 2017; Iskandar et al. 2017).

According to Arifin and Nakagoshi (2011), the conversion of primary forests into other forms of use has two forms, namely: (i) primary forest is converted to plantations; and (ii) primary forest is converted to *huma* and other uses. If forests have been converted to plantations, there will be less possibility for the land cover to return to natural forests. Conversely, if natural forest becomes *huma* it will return to secondary forest and even primary forest, but if free from human disturbance for long period of time, this second conversion, will result in abandoned or fallow forest areas and lowland rice fields. Meanwhile, the area used for *huma* can be transformed into several types of land use such as plantations (perennial crops). This form of conversion occurred at the beginning of the Karangwangi landscape changes that originated from *leuweung, followed by huma, and eventually the* formation of land use types.

The changes of Karangwangi landscape in 1970-1980

According to the informants, prior to the formation of Karangwangi Village, there were only five inhabited *kapunduhan* (hamlets). The five *kapunduhan* include Mekarlaksana, Batubentang, Puncakbayuning (currently Hegarwangi), Cimindi and Bantarwaru. The *kapunduhan* are centered in Bantarwaru which is near the Cilaki River. Then in the 1970s it developed into a further 16 *lembur* (*kampung*) consisting of Bantarwaru; Cibeledik; Puncakkukun; Datareurih; Mekarlaksana; Nempel; Cikoletak; Jati; Cikajar; Cadasleur, Gerdog; Bayuning; Puncakbayuning; Cijengkol; Batubentang; and Cimindi. With the growth of the village, the population is also increasing. This led to the start of settlement neighborhood where each family has *buruan* (homegarden), *kebon* (garden), and *huma* (swidden field). According to the local community, the *buruan* is a field around a house planted with various types of annual and perennial crops. While *kebon* is the development of the *huma* system in the form of *gundukan* (plot fields/mounds). In addition to *kebon*, the *huma* system also leaves an open field called *tegal* or *tegalan*.

In 1977, road access in the village was still a path (0.5 m). Thus, no vehicles entered the village, and crops were sold and distributed in a simple manner. At that time, the main source of sales for the Karangwangi people was the *suuk* (peanut) grown in a *kebon* or land in the Bojonglarang forest. On 16 October 1973, the Bojonglarang forest inaugurated become Bojonglarang Jayanti Nature Reserve by the Decree of the Minister of Agriculture. Yet, until the 1980s Bojonglarang has not closed. Thus, people use Bojonglarang area measuring 250 hectares to plant *suuk*. While in the forest (*leuweung*) planted with *pare* (rice) in the *huma* system, to meet the needs of households.

The changes of Karangwangi landscape in 1980-1990

In the 1980s during the transmigration policy, many villagers responded to request to migrate out of Java, but some chose to remain. The most affected by the transmigration policy were those local people without jobs or land to work. A primary resettlement location of government transmigration for rural communities is to Tulang Bawang area, Lampung, Sumatra. In the same year, discussions regarding the closing of this territory due to plans for the development of a palm oil plantation by Tien Soeharto (ex Indonesian first lady). But it did not happen. Then other issues arise to close this area, such as the relocation of the settlements because it will become the ABRI Headquarters, or potentially will be the location of a special retired state apparatus, and so on. In 1982, SD *Inpres* (Presidential Instruction) was actually built in this village. This knowledge is in line with the information sourced from Presidential Instruction No. 4 of 1982 that in 1982-1983 President Soeharto built 22,600 Inpres Elementary Schools for education in villages and towns with a small population. This school is a forerunner to the establishment of Karangwangi Village followed by the appointment of an interim official whose administrative center is located in Kampung Nempel which is part of Cidamar Village. Land clearing is done and land is legalized by Redist and SPPT. On March 23, 1984, the village was divided and the election of permanent officials in Karangwangi Village.

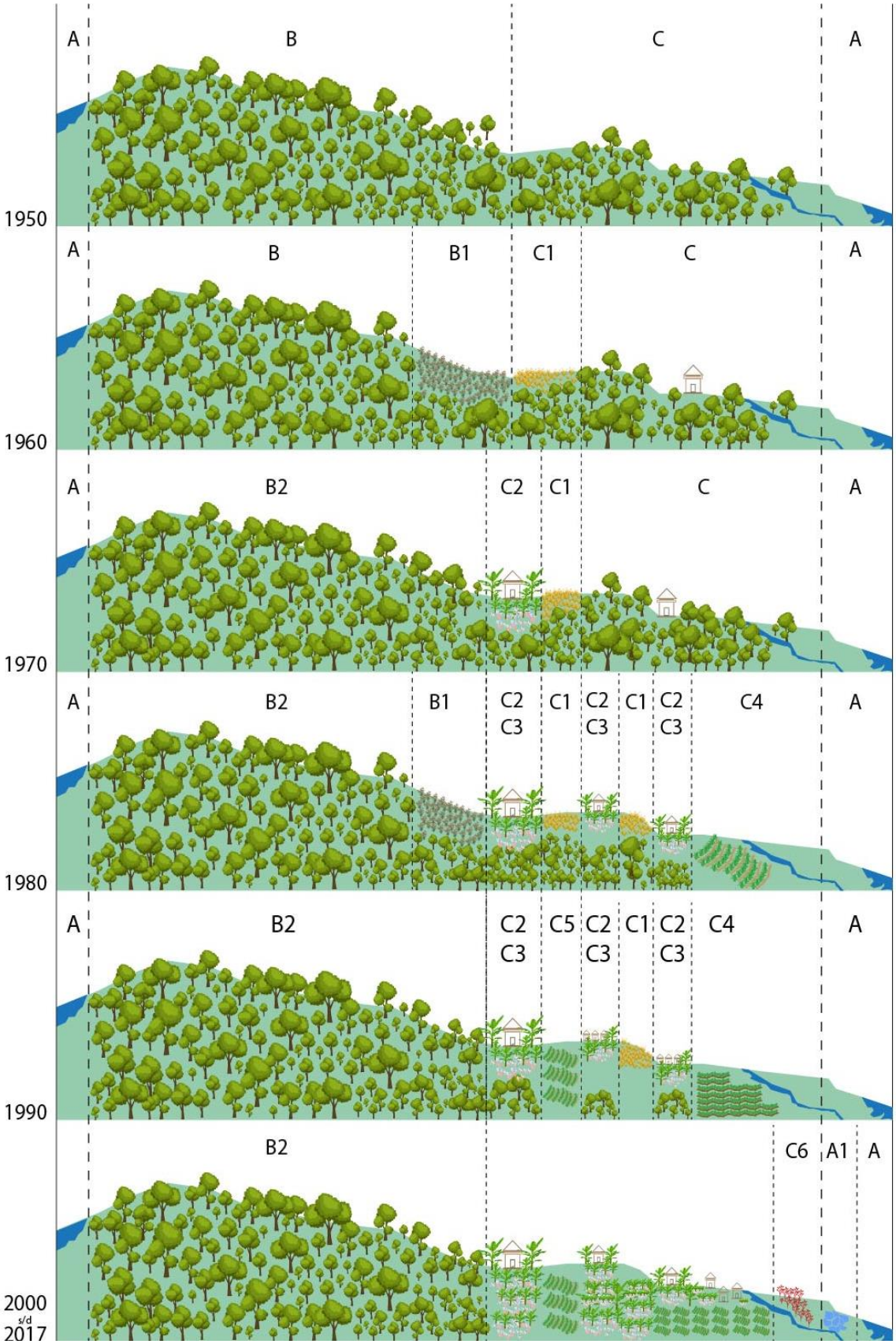

Figure 2. The illustration of the changes of Karangwangi Landscape since 1950. Note: : Punduh/dusun/kampung (hamlet), : Wahangan/sungai (river), A: Pantai (beach), A1: Tambak (fish pond), B: Bojonglarang, B1: Kebon suuk (peanut farm), B2: Cagar Alam (nature reserve) Bojonglarang Jayanti, C: Leuweng/hutan (forest), C1: Huma (swidden field), C2: Kebon (garden), C3: Buruan pekarangan (homegarden), C4: Sawah boyor (irigated rainfed), C5: Sawah tadah hujan (non-irigated rainfed), C6: Kebon cabe/kebon jagung (chili/corn rainfed)

In 1984, houses in Karangwangi were rarely dominated by *huma* and *kebon* (garden). One neighborhood unit consists of only eight families. The population number was still low and during the dry season, many people went temporary migration to other areas for obtaining off-farm jobs, including as laborer. The number of residents is also still small, and during the dry season came a lot of people who become coolies to other places. In 1986, the local people of Karangwangi began to cultivate *sawah* (rice field). This *sawah* opening is at RW I. The wetland system was originally a change from the *huma* system by providing irrigation on cultivated land. In the beginning, Karangwangi people who switch and use the rice system are very few in number. The ration of *huma* with *sawah* in this village is about 30:70. Even wetland systems are rarely found in high-income farmers. This is due to the high cost of paying the workers needed to create new fields with wetland systems. In the end, the farmer chooses to transform his own *huma* that is close to the water source to be used as a rice field. There is no striking difference in the management of rice field and *huma* system. Farmers practice an organic farming system that uses various local rice and fertilizer from manure and organic waste. The period of planting and harvesting remains the same once a year and the distance between harvesting and planting is again used by farmers for fish ponds and for planting *genjer*, or stand-alone crops.

The changes of Karangwangi landscape in 1990-2000

In the 1990s local rice began to be rarely planted in the village. Local people are starting to recognize new types of rice that can harvest three times a year. This knowledge is derived from the knowledge of the people who go to other villages or return from the transmigration sites. The root of this system comes from the introduction of the Green Revolution Program and *Panca Usaha Tani* (Five Farming Program) by the government in the 1970s in certain areas of Indonesia. This program has implications for the uniformity of farmers in making suitable planting. The five farming programs are as follows: (i) use of modern varieties paddy, such as IR, PB, and other genetically engineered seeds from laboratory; (ii) use of chemical fertilizer, like urea, TSP, etc.; (iii) improvement in land preparation (using pesticide); (iv) improvement in irrigation; and (v) improvement in crop maintenance.

Implementation of the Green Revolution Program has had a significant impact on agricultural life in Karangwangi Village. In addition to the use of modern rice seeds, also the use of pesticides is increasingly often found in the farming process of Karangwangi Village people. Another noticeable change is the changing rice cropping cycle, which was previously done only once a year, now the cropping cycle is done many times depending on the location of the rice field and the availability of water. Typically, the *musim rendeng* (rice planting) is done in November or December and the harvest is done three months later. The second planting will be farmers in March or April and harvest will be obtained in around August. For land close to the water source, a third rice planting can be done in August to be the harvested in November. As for the rice fields which are far from the water source then the rice field will be used to plant crops like corn or fallow until the next planting season. The use of pesticides and inorganic fertilizers has become very influential in the life of farmers in the village of Karangwangi. As a result, the costs of farming are higher than in the past. Another disadvantage that farmers often experience after using pesticides is the resillience of rice pests (*hama beuki meuweuh*). This is quite a dilemma, because if farmers do not use pesticides, then the brown planthopper (*Nilaparvata lugens* Stal) becomes uncontrollable and causes crop failure.

In this year, *huma* systems are less developed, and rice fields are the main farming location. According to Pranowo (1985), there are two factors that affect the duration of the cultivation of *huma:* namely, (i) forest type; and (ii) population density. Types of primary and secondary forest if used as *huma* have different cultivation times. The cultivation time in primary forest is longer in the range of 2-4 years, compared to the secondary forest that is less than two years. This is influenced by the natural content of the existing fertilizer, which is more in the primary forest than the secondary forest. In addition, population density also affects the duration of cultivation time. The greater the population in a region, the longer the cultivation time. This is due to the opportunity for forest clearing becoming smaller. The number of residents also affects the number of *huma* in Karangwangi Village, so that the number of *huma* has been reduced and replaced by rice fields.

The changes of Karangwangi landscape in 2000-2017

In 1995, village roads were *dibeko* (ground leveling from the path). In early 2000, construction began on the village road from Karangwangi to Cianjur and completed in 2005/2006. Establishment of access in Karangwangi Village had produced an influx of information, communication, and technology. One primary example is the construction of *lio* (home brick factory) to build more permanent houses, whereas before the houses in this village were constructed of bamboo with reeds for roofing material. Bamboo was obtained from *kebon awi* (bamboo garden) and *alang-alang* (reeds) coming from *huma*. Beginning in 2007, the houses of the people of Karangwangi Village have been predominantly using bricks for their walls. In 2009, the village road began to be paved, and in the same year permanent bridges over the Cikawung and Cilaki Rivers completed. In 2010, the southern highway was finished paving.

The southern road through Karangwangi, connected villages and even sub-districts and resulted in many changes. Most changes are considered beneficial for local people. Examples include: the entry of electricity and a communication tower to Karangwangi Village in 2012, produced an increase of information flow through electronics such as television, radio, mobile phone usage; changes in processing systems such as food storage in the refrigerator, rice processing with diesel engines instead of *lisung*; and the use of *sosin* to drain water. Changes are also seen in the dominant vehicles of motorcycles used by local

people in transporting crops or performing many other uses.

Farina (2010) argued that globalization and enhanced information connectivity and the spread of media (radio, television between various countries homogenizes the environment and reduce the ecological diversity. This statement is supported by changes in planting patterns in Karangwangi Village. For example, better access and information obtained from other regions have to lead to the introduction of new plant commodities such as *jati*/*jabon* (teak), chilli and albasiah/jengjen. by. Initially, albasiah (locally called *jengjen)* (*Paraserianthes falcataria* (L) Nielsen), *jabon* (*Anthocephalus* sp.), and mahogany (*Swietenia mahagoni* (L) Jacq) were known through the introduction of the Forestry Service in the 2000s. The purpose of this introduction is to reforest the formerly open fields. However, in the development of local communities Karangwangi get information that albasiah wood much in demand as building materials, furniture, and have its own market for sale to other areas. This causes the planting of albasiah in the fields such as *tegalan* (open dry field) and dominating the so-called *kebon jengjen*/albasiah. Based on Iskandar et al. (2017) with intensive *jengjen* planting in Karangwangi Village provides ecological and socioeconomic benefits. These benefits come from maintaining soil fertility through nitrogen fixing and ease of cultivation which facilitates the provision of household needs and increasing income. This development makes the growth of the higher market that causes changes in traditional agroforestry systems such as *kebon awi* (bamboo garden), *kebon kai* (wooden garden) and *talun* (forest-garden) into monoculture system that is kebon jengjen. This monoculture causes reduced species and varieties of locally grown crops, increased pests and plant diseases, and low resistance to market fluctuations (Reijntjes et al. 1992; Iskandar et al. 2017).

With access to the southern roads, it is easier for local people to sell and buy wooden seedlings for planting. In addition, in 2014 the rising prices of *cabe keriting* (chilli pepper) compel local people to grow chili pepper (*Capsicum annum* L) on their farms. Planting chili pepper is shifting rapidly to plants such as *suuk*/peanut (*Arachis hypogaea* L). The entry of investors to the village of Karangwangi is also directly related to the better access road. In 2013, the village government conducted a certification program of former fields. Therefore, the former fields and gardens in Karangwangi Village became owned and certified. Many investors purchased land in villages where in fact the land is managed by the local community. Even recently foreign investors from Korea bought tens of hectares of land near the coast. In addition to the land, sand mining on the beach near the Cilaki River also occurred in 2010. Eventually, this was stopped by the village chief at the time because of related casualties from dangerous holes on the beach that are often used for children to swim. (For illustration, see Figure 3)

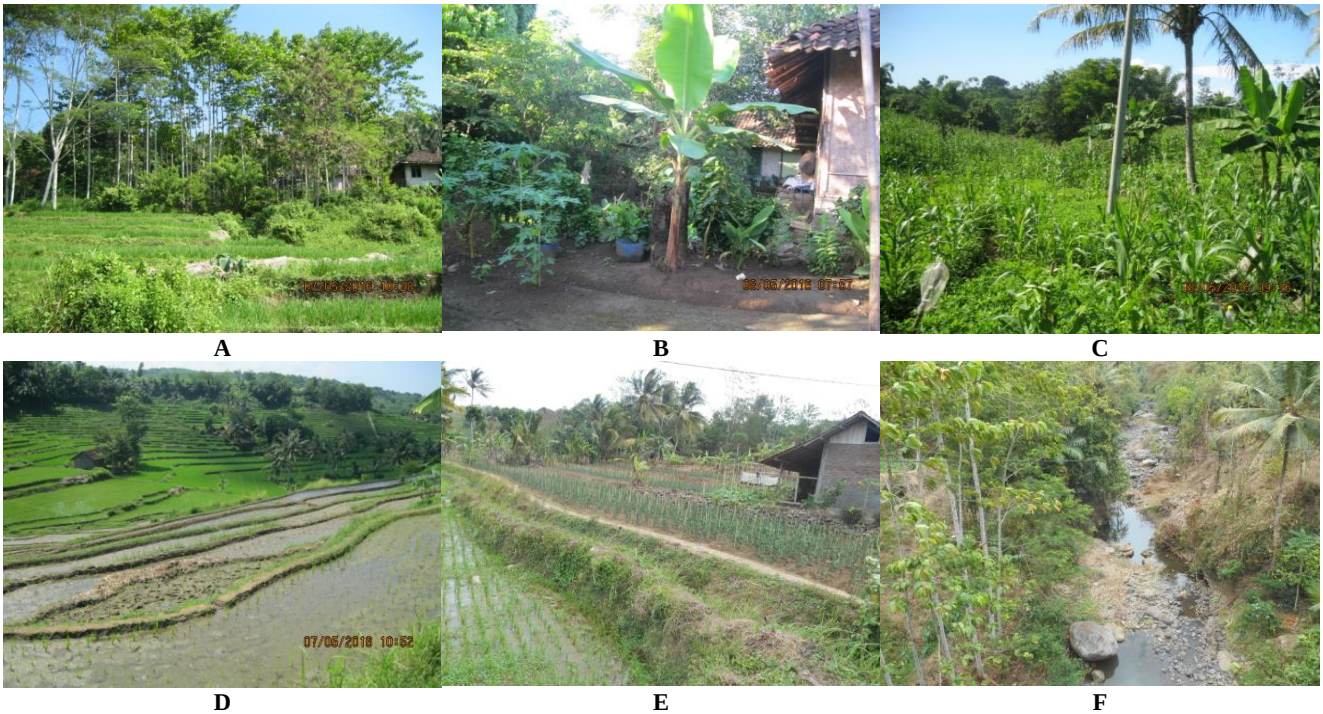

Figure 3. A. Albasiah and jabon trees are grown in tegalan, B. Homegarden is planted by various annual and perennial crops, C. Peanut crop is predominantly grown in garden, D. The wet rice field (sawah) landscape of Karangwangi, E. The chili pepper crop is one of commercial crops grown in garden or rainfed field of Karangwangi, F. Cikawudung river of Karangwangi which has a lack of water during the dry season

Strategy for sustainable rural landscape management

The changing landscape of Karangwangi Village is influenced by many factors. First, a fundamental transformation occurred whereby an increase in the population resulted in the reduction of forest area and a conversion of the huma system into a wet rice field system (*sawah*). In addition to being converted to wet rice fields, *huma* also were converted to gardens. This garden system takes place because of the adaptation of densely populated and rapidly growing market economies. Consequently, the local population selects and introduces new crops that are more profitable economically, and introduces new inputs such as inorganic fertilizers and pesticides to fit the market demand (Iskandar 2009; Mutaqin et al. 2018). The denser the population, the higher the need for settlements. This is certainly the case in Karangwangi Village with new settlements adding pressure to the land use needs of the community. To meet their needs, the community maintains gardens planted with various kinds of plants such as fruits, spices and ceremonial plants and livestock. This home-garden system is not managed intensively and is rarely attacked by pests because it is a polyculture system. The people of Karangwangi Village usually harvest their own produce from the homegarden to meet their personal or family needs. Generally, the existence of home-gardens are very good from an ecological standpoint because they are maintained without inorganic fertilizers or pesticides, and sustain diversity of local plants. Secondly, government policies such as bans on swidden cultivation system in the forest, reforestation of former land, Green Revolution programs and policies, and *Panca Usaha Tani* have all had significant impacts The introduction of modernization in the form of intensive agriculture supported by the government through the Green Revolution and *Panca Usaha Tani* have clearly degraded the quality of land in Karangwangi Village. This is caused by monoculture, pesticides and inorganic fertilizers that are increasingly used by the community. Thirdly, the improvement and development of infrastructure such as roads are the mainstream of modernization in Karangwangi Village. Soembodo (2008) argues that the processes of rural society modernization are underpinned by the construction of connecting roads and the transportation that facilitates mobilization. The development of irrigation channels and new agricultural technology, as well as the shifting of jobs from the agricultural sector to other sectors, combine to produce dramatic changes to the development trajectories of rural communities.

The aforementioned factors have changed, and will continue to alter the landscape of Karangwangi Village. Therefore, a stable strategy is needed to provide a more balanced development plan that produces benefits for the environment and social and economic prosperity for the local community. Fritz-Vietta (2017) defines two factors in sustainable land management: (i) satisfaction of human needs in the use of land and natural resources; and (ii) maintenance of the future functioning of the natural resource environment (Figure 3).

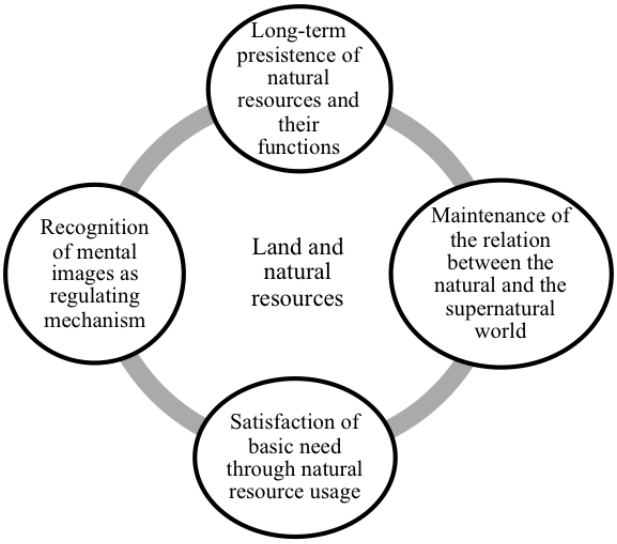

Figure 3. Elements of sustainable land management (adapted from Mahafaly Plateau region) *(*Fritz-Vietta 2017*)*

Implementation of these two factors is facilitated by identifying people who still hold tightly the mental image associated with a site, location, or resources that have a supernatural connection. *Pronoto Mongso* which is an inheritance of local knowledge in the seasonal traditional calendar in Yogyakarta plays an important role in overcoming complex socio-economic changes in the area of Karst Gunung Kidul. Pronoto Mongso teaches the relevant environmental ethics and life balance used today, such as adaptation and environmental change (Retnowati et al. 2014). In addition, Tri Hita Karana in Bali can also be used as a reference in overcoming problems in the landscape of Batukaru slopes and the associated factors of water, soil and climate throughout each year (Asmiwyati et al. 2015). In short, it can be inferred that the Karangwangi people affect, and been affected by, their ecosystem. Various landscapes of Karangwangi, and the people themselves have continuously changed over time due to the reciprocal nature of socio-economic and cultural developments.

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Genotype determination of megalocytivirus from Indonesian Marine Fishes

MURWANTOKO[1,♥], DINI WAHYU KARTIKA SARI[1], CHRISTINA RETNO HANDAYANI[2], RICHARD J. WHITTINGTON[3]

[1]Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada. Jl. Flora Bulaksumur, Sleman 55281, Yogyakarta, Indonesia. Tel.: +62-274-563062, ♥email: murwantoko@ugm.ac.id
[2]Main Center for Brackish Water Aquaculture Development Jepara.Jl. Cik Lanang, Bulu, Jepara 59418, Central Java, Indonesia
[3]Farm Animal and Veterinary Public Health, Faculty of Veterinary Science, University of Sydney. 425 Werombi Road, Camden, NSW 2570 Australia

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Abstract. *Murwantoko, Sari DWK, Handayani CR, Whittington RJ. 2018. Genotype determination of megalocytivirus from Indonesian Marine Fishes. Biodiversitas 19: 1730-1736. Megalocytivirus* is the newest genus within the family of Iridoviridae which can be divided into groups represented by red sea bream iridovirus (RSIV), infectious spleen and kidney necrosis virus (ISKNV) and turbot reddish body iridovirus (TRBIV), threespine stickleback iridovirus (TSIV). This virus caused serious systemic disease in cultured marine fishes for consumption and ornamental freshwater fishes with significant mortality. The objective of this study was to determine the genotype of megalocytivirus which infected marine fishes from Lampung, Karimun Jawa, Situbondo and Batam based on major capsid protein (MCP), ATPase, DNA polymerase, CY15 and IRB6. The liver, spleen and kidney tissues of humpback grouper (*Cromileptes altivelis*), tiger grouper (*Epinephelus fuscoguttatus*), baramundi (*Lates calcarifer*) were fixed in 10% phosphate-buffered formalin for histological and fixed in 70% ethanol for molecular analysis. Molecular analysis was performed by amplification of MCP, ATPase, DNA polymerase, CY15 and IRB6 genes and followed by sequencing. Genotype was determined by alignment of the sequences with various genotypes of megalocytivirus from Genbank. Histological examination showed that hypertrophy, inclusion body forming bearing cells were found in liver, spleen and kidney tissues. Polymerase chain reaction with MCP primer produced specific DNA bands. Those results confirmed the infection of megalocytivirus on marine cultured fish samples. The analysis from 10 isolates on five genes revealed that two genotypes of megalocytivirus as infectious spleen and kidney necrosis virus (ISKNV) and red sea bream iridovirus (RSIV) genotypes were existed in Indonesia. The ISKNV genotype was confirmed in fish samples from Lampung, Jepara, Bali; while RSIV genotype was found in fishes from Batam, and Situbondo. Interestingly, both ISKNV and RSIV genotypes were confirmed in fish samples from Karimun Jawa. This paper is the first report on the present of ISKNV and RSIV genotypes in Indonesia based on MCP, ATPase, DNA polymerase, CY15 and IRB6 genes.

Keywords: Genotype, ISKNV, marine fish, megalocytivirus, RSIV

INTRODUCTION

Megalocytivirus is the newest genus within the family of Iridoviridae along with the *Iridovirus*, *Ranavirus* and *Lymphocystivirus* genera (Kurita and Nakajima 2012). Phylogenetic analyses using major capsid protein (MCP) and ATPase genes show that the genus *Megalocytivirus* can be divided into groups represented by red sea bream iridovirus (RSIV), infectious spleen and kidney necrosis virus (ISKNV) and turbot reddish body iridovirus (TRBIV) (Kurita and Nakajima 2012), threespine stickleback iridovirus (TSIV) (Waltzek et al. 2012). The mortality caused by megalocytivirus infection varies between 30% in juvenile up to 100% in larvae stage (Eaton et al. 2007). This disease have been reported to cause mortality of 80-90% in juvenile grouper during February-August 1993 and February-April 1994 (Danayadol et al. 1996).

The Megalocytivirus was known as agents that caused serious systemic disease in more than 40 species on cultured marine fishes for consumption (Inouye et al. 1992; Chua et al. 1994; Nakajima and Maeno 1998; Gibson-Kueh et al. 2003; OIE 2009) and ornamental freshwater fishes (Anderson et al. 1993; Rodgers et al. 1997; Lu et al. 2005). Diseases caused by *Megalocytivirus* have been reported in Japan, Chinese Taipei, R.R. China, Hong Kong, South Korea, Malaysia, Philippines, Singapore, Thailand (OIE 2009), Australia (Go et al. 2006), Indonesia (Mahardika et al. 2003, 2004, 2009, Murwantoko et al. 2009), North America (Waltzek et al. 2012)

The clinical signs of megalocytivirus-infected fishes were severe anemia, red spots (petechiae) in the gills, swelling of the spleen (Nakajima and Maeno 1998) and kidney (Sudthongkong et al. 2002). Histopathological examination revealed that inclusion body forming bearing cells (IBC) were found in spleen, kidney, hematopoietic tissue and the digestive tract, while necrosis was occurred in kidney tissue (Sudthongkong et al. 2002). However Dong et al. (2017) reported that ISKNV diseases outbreak with none of the infected fish showed the presence of expected hypertrophied cells in histological examination of kidney, liver, spleen and brain tissues. Instead, kidney tissue of the affected fish exhibited hyaline degeneration in the epithelial cells of kidney tubules with notable presence of acidophilic inclusions. Multifocal coagulative

hepatocellular necrosis has been reported on TSIV infected three spine stickleback fishes (Waltzek et al. 2012).

Many studies have been conducted on megalocytivirus in Indonesia. In 2000, megalocytivirus caused mortality more than 80% in *Epinephelus coioides* in North Sumatera. The megalocytivirus has been detected and caused mortality of up to 100% on green grouper (*E. coioides*) and duskytail grouper (*E. bleekery*) in Bali during acclimation after been caught from marine (Roza et al. 2005). Others studies have been conducted on detection in orange-spotted grouper (*E. coioides*) (Mahardika et al. 2003), coral grouper (*Epinephelus coralica*) (Johnny and Roza 2009), succeptibility of humpback grouper (*Cromileptes altivelis*) (Mahardika et al. 2004), pathogenicity on coral trout grouper (*Plectrophomus leopardus*) (Mahardika et al. 2009), histopatology on experimentaly infected of humpback grouper (*C. altivelis*) (Mahardika and Mastuti 2013). Molecular study on megalocytovirus in Indonesia based on MCP gene showed the megalocytivirus from Jepara (IJP01) and Bali (IGD01) are belonged to ISKNV genotype and among those IJP01 and IGD01 isolates shared 99.8% identity in nucleotide level and 99.4% identity at amino acid level (Murwantoko et al. 2009). In this present study we determined the genotype of megalocytivirus from Batam, Karimun Jawa, Situbondo and Lampung in Indonesia based on MCP, ATPase, DNA polymerase, CY15, IRB6 DNA sequences.

MATERIALS AND METHODS

Fish samples

Marine cultures fishes as humpback grouper (*Cromileptes altivelis*), tiger grouper (*Epinephelus fuscoguttatus*), baramundi (*Lates calcarifer*) showing clinical signs of megalocytivirus infection were collected from Situbondo (East Jawa), Karimun Jawa (Central Jawa), Batam (Riau islands) and Lampung from January to October 2010. The internal organs: liver, spleen, kidney were collected and preserved in normal buffer formalin (NBF) for histological examination and in 70% ethanol for molecular analysis.

Histological examination

Liver, spleen and kidney tissues were fixed in 10% phosphate-buffered formalin for at least 24 h. The decalcifying was done on 10% EDTA in NBF for at least 5 h. The desired organs were dehydrated in a graded alcohol series before routine processing and embedding in paraffin wax. The Sections (5 mm) were stained with haematoxylin and eosin (HE) (Roberts et al. 2012).

Molecular characterization

DNA extraction

DNA was extracted from tissue following Wasko et al. (2003). Ten to 30 mg of tissue was homogenized in 400 µL TNES buffer (10 mM Tris-HCl pH 8; 125 mM NaCl; 10 mM EDTA pH 8; 0,5% SDS; 4M urea). Three µL of RNase (10 mg/mL) was added to the mixture and incubated at 42^{o}C for 1 hour. Following this, 3 µL of proteinase K (10 mg/mL) was added into the mixture and incubated at 42^{o}C for 2-6 hours. The suspension was extracted using same volume of phenol: chloroform: isoamyl alcohol (PCIAA). DNA was precipitated using 1 M NaCl and two volume of cold absolute ethanol and followed by washing with 70% ethanol.

PCR amplification

First PCR amplification for determine status on megalocytivirus infection was conducted under Mastercycler personal Thermal Cycler (Eppendorf) using primers MCP-Irido-F-Bam (ATCAGGATCCATGTCT GCAATCTCAGGTG) and MCP-Irido-R-Eco (CGTCGAA TTCGTCGACAGATGTGAAGTAG) (Murwantoko et al. 2009). Amplifications for sequencing of target genes were performed in Gradient Palm-Cycler (Corbett Research) PCR machine using primers specifically designed for several genes of megalocytivirus (Go et al. 2006) (Table 1).

Table 1. List of primers used to amplify genes of megalocytivirus (Go et al. 2006)

Name	Gene	Sequence	Tm (^{o}C)
MCPI F	MCP	TTCACAGGATAGGGAAGCCTGC	56.7
MCPI R	MCP	TCATCAGCCAGAGCAACCAG	53.2
MCP2 F	MCP	GTCTGCAATCTCAGGTGCAAAC	54.8
MCP2 R	MCP	GATCTTAACACGCAGCCACA	51.8
ATPase 2 F	ATPase	GCCACCGTAATCAGTTTGATCATC	55.7
ATPase 2 R	ATPase	ATGAACCCGCTGCACTATGC	53.8
CY15 F	CY15	TCATCTGCACGTACACCCTG	53.8
CY15 R	CY15	CGCCCACATCCAAATCTATC	48.9
DNA Pol F	DNA polymerase	CAAGGCTGTTGGATTTTGAG	49.7
DNA Pol R	DNA polymerase	AGTCCTGTCCAAGTGCAACC	53.8
IRB6 F	IRB6	AAGTAGTGAGGGCAGAAG	48.0
IRB6 R	IRB6	ATCGTAGTCGTCCATTCC	48.0

Each PCR reaction was performed in a total volume of 50 µL containing final concentration of 25 mM of each dNTP, 2 mM of each primer, PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.6, 2.5 mM $MgCl_2$, 10 mM beta-mercaptoethanol) and 2 units of Taq DNA polymerase. Optimum condition for PCR reaction with hot start (Go et al. 2006) is as follows: one cycle of initial denaturation at 94ºC for 3 min, followed by 30 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, and extension at 72ºC for 1 min. and a final extension at 72ºC for 3 min. PCR products were separated by gel electrophoresis on a 2% agarose gel containing 0.003% ethidium bromide, and compared against molecular size marker number VIII (Roche). Negative controls for the PCR mix and a negative control (water) for DNA template and DNA derived from MCIV was used as a positive control.

PCR products (2.5 µL) were examined on a 2% agarose gel containing 0.003% ethidium bromide. The PCR products were purified using a commercial silica binding column (SV Gel and PCR Clean-Up Kit, Promega Corporation), following the manufacturer's instructions. In case the non specific bands appeared, the desired band was sliced from gel and their DNA was purified using Freeze n Squeeze DNA Gel Extraction Spin Column (Biorad). The PCR product then further purified using shrimp alkaline phosphatase/exonuclease (ExoSap-IT, Amersham Biosciences) with the mixture incubated at 37 ºC for 20 min, followed by 80°C for 15 min to denature the enzyme in thermal cycler.

DNA sequencing

Sixteen µL DNA sequencing reaction contained 10 pmol of either the forward or reverse PCR primer, sterile purified water, and 50-200 pg of PCR product. All reactions were performed in a commercial supplier using BigDye Terminator version 3.1 chemistry (Applied Biosystems) and analyzed in a ABI Prism 3100 capillary Genetic Analyser (Applied Biosystems).

Data analysis

Results of DNA sequences were aligned and checked manually to resolve errors. Multiple alignments analysis with addition of Genbank collected sequences was conducted using the MEGA ver. 7.0 (Kumar et al. 2016). Cluster tree diagrams were constructed based on unweighted pair group method using arithmetic average (UPGMA) with 1000 bootstrap replicates for analysis of branch support.

RESULTS AND DISCUSSION

Clinical signs

Humpback grouper (*Cromileptes altivelis*) fishes were collected from Karimun Jawa and Lampung. The fishes were lethargy and stay solely separately from the others in sea cage. Tiger grouper (*Epinephelus fuscoguttatus*) from Batam showed stay in the bottom of tank dark color, lethargy, thin body and cloudy eyes. Lethargy was observed from baramundi (*Lates calcarifer*) collected from Situbondo. Humpback grouper from Karimun Jawa showed the progressive color change of liver to pale, enlargement of spleen, with normal size of kidney. Tiger grouper showed enlargement of spleen and pale color of liver (Figure 1). The fishes from other areas showed pale liver or enlarge spleen.

Histopathological examination

Histology examinations were conducted on liver (Figure 2.A), spleen (Figure 2.B,C), kidney tissues (Figure 2.D). Liver tissue composed of hepatocytes which are often swollen with glycogen. The pancreatic tissues are scattered in liver tissue. In spleen showed diffuse red and white pulps and erythrocytes are distributed. Kidney showed renal tubules surrounded by hematopoietic tissue. The pathological changes observed in several tissue samples such as liver and spleen of humpback grouper (*C. altivelis*) from Lampung, and the spleen and kidney of tiger grouper (*E. fuscoguttatus*) from Batam. hypertrophy, inclusion body forming bearing cells are found from those tissues. There are many pathological changes observed in the tissues, including hypertrophy, inclusion body forming bearing cells in several tissues such as liver, spleen and kidney (Figure 2)

Molecular characterisation

DNA amplification using primers MCP-Irido-F-Bam and MCP-Irido-R-Eco showed that 10 fish samples which originally came from Batam, Lampung, Karimun Jawa and Situbondo were infected by megalocytivirus as indicated by presence a DNA band at 1000 bp (data not shown). Among those samples, five samples were successfully amplified on major capsid protein (MCP) gene using designed primer (Go et al. 2006). Sequencing of those DNA could read the samples from 1266 to 1313 nucleotides and the sequences can be found in supplementary data. The sequences of MCP have been deposited in Genbank with accession number MH764414-MH764418. Cluster analysis together with data in Genbank showed that an isolate from Batam (Btm Fish_35) and three isolates from Karimun Jawa, KJw fish_23, _27 and _28 were belonged to RSIV genotypes. Alignment analysis of those sequences using BLAST showed that this isolate has 100% identity with RSIV (AB461856) and OGIV (AY 894343). Interestingly an isolate from Karimun Jawa, KJw Fish_21 was belonged to ISKNV genotype. Analysis with BLAST showed the KJw_Fish_21 had 100% identity with ISKNV (AF370008), DGIV (AY989901), MCIV (AY936203) and 99% with GSIV (JF264354). Comparing with previous study (Murwantoko et al. 2009), this Karimun Jawa isolated shared 100% nucleotide identity with Bali isolate (IGD01) and 99% nucleotide identity with Jepara isolate (IJP03) (Figure 3).

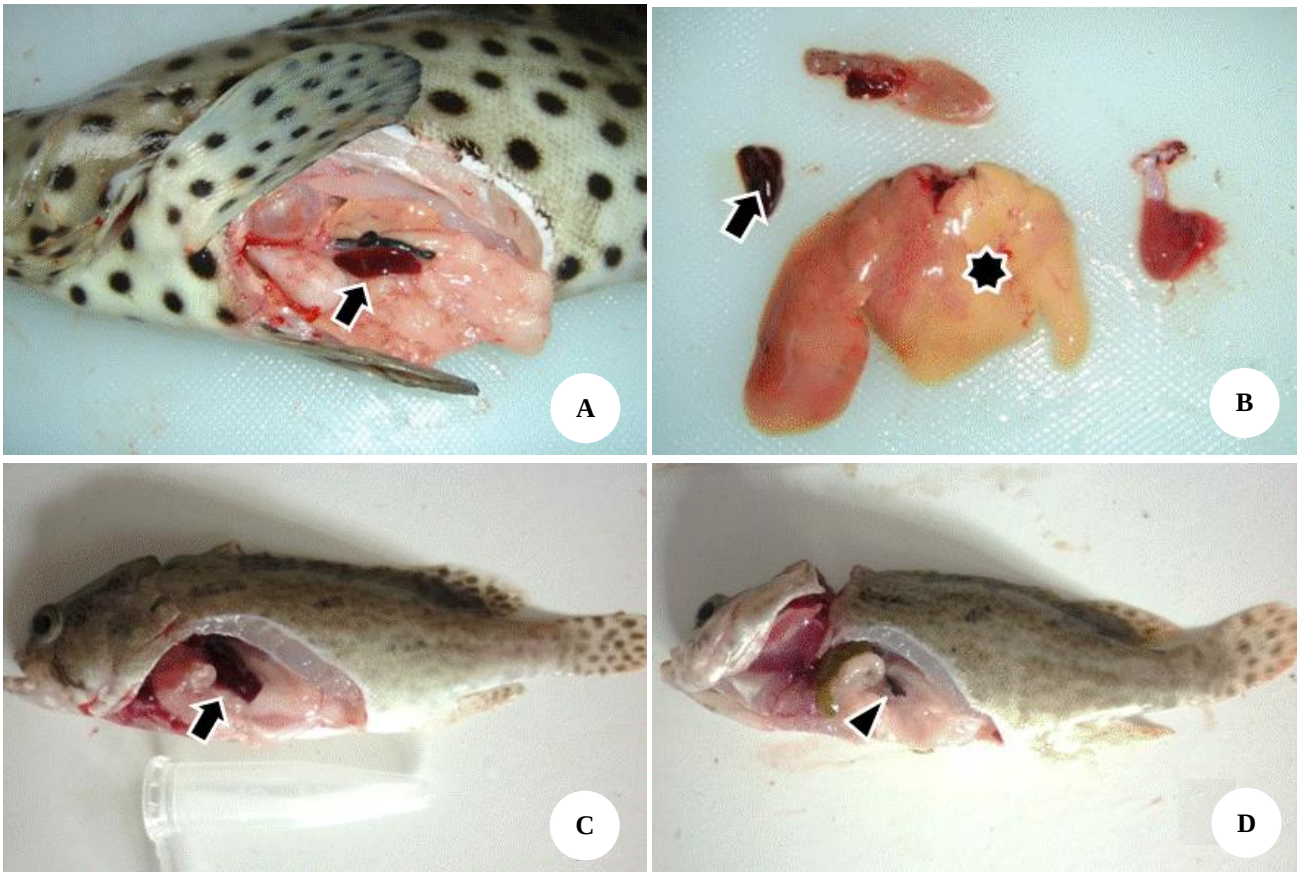

Figure 1. Gross sign of fish samples. The enlargement of spleen (arrow) and progressive color change of liver to pale (*) were found in humpback grouper *C. altivelis* (A and B) and enlargement of spleen of tiger grouper *E. fuscoguttatus* (arrow) (C) and relatively normal size of spleen of *E. fuscoguttatus* (head arrow) (D)

ATPase fragment corresponds to the central region of ATPase from eight isolates have been successfully sequenced with size range from 616 to 805 nucleotides. The sequences of ATPase have been deposited in Genbank with accession number MH764419-MH764426 and the sequences can be found in supplementary data. The sequences of ATPase were used to construct cluster tree under UPGMA, as indicated in Figure 4. Cluster analysis together with data in Genbank showed those isolates were distributed into two genotypes of megalocytivirus; the ISKNV and RSIV. Analysis using BLAST, samples form Lampung, Lpg Fish 3 and Lpg Fish 9 showed 100% identity with ISKNV (AF371960, KP292962), MCIV (AY 936204), DGIV (AY989902). Alignment analysis also showed, a sample form Batam (Btm Fish 35), and five samples from Karimun Jawa (KJw Fish 23,-26,-27,-28 and-29) were belonged to RSIV genotype and showed those sequences had 100% identity with OGIV (AY894343), GSIV (AF462344), RSIV (AP017456), and had 99% identitiy with RSIV (AB007367) (Figure 4). Consistent with the result from MCP sequences, many isolates from Karimun Jawa and isolate from Batam were belonged to RSIV.

The sequences of DNA polymerase, CY15 and IRB from Karimun Jawa (KJw fish 21, KJw Fish 23), Situbondo (Stb fish 32) and Lampung (Lpg fish 3) have been determined and those sequences can be found in supplementary data. Those sequences of DNA polymerase, CY15 and IRB6 have been deposited in Genbank with accession number MH764410-MH764413 , MH764427-MH764434. Cluster analysis under UPGMA on megalocytivirus isolates based on DNA polymerase, CY15 and IRB6 was presented in Figure 5. Cluster analysis together BLAST analysis showed the KJw fish_28 and Stb fish_32 were belonged to RSIV genotype with 100% identity against RSIV (AP017456), GSIV (KT804738) and OGIV (AY894343). The Lpg fish_3 and KJw fish_21 were belonged to ISKNV and those sequences showed 100% identity with ISKNV (AF371960). Consistent with the above results KJw fish_28 is belonged to RSIV genotype, and KJw fish_21 was belonged to ISKNV genotype (Figure 5).

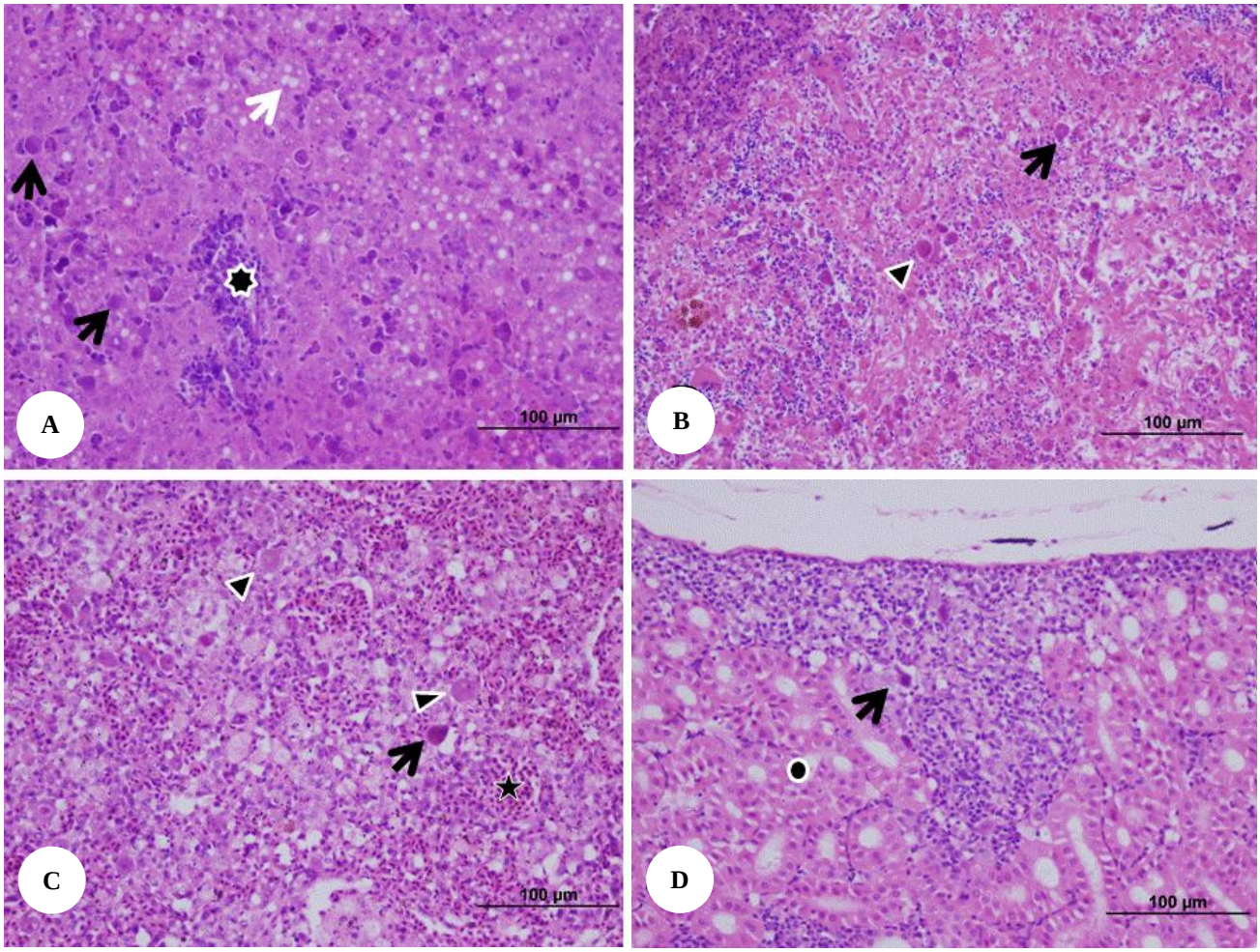

Figure 2. The histology of liver of humpback grouper (*C. altivelis*) from Lampung showed hepatocytes with glycogen (white arrow), pancreatic tissue (*), inclusion body forming bearing cells (IBC) (black arrow) (A).Histology of spleen of humpback grouper from Lampung (B) and of tiger grouper (*E. fuscoguttatus*) from Batam. (C) showed erythrocytes (star), hypertrophied spleenocytes (head arrow) and IBC (black arrow). Histology of kidney of tiger grouper from Batam showed renal tubules (dot) and IBC (black arrow)

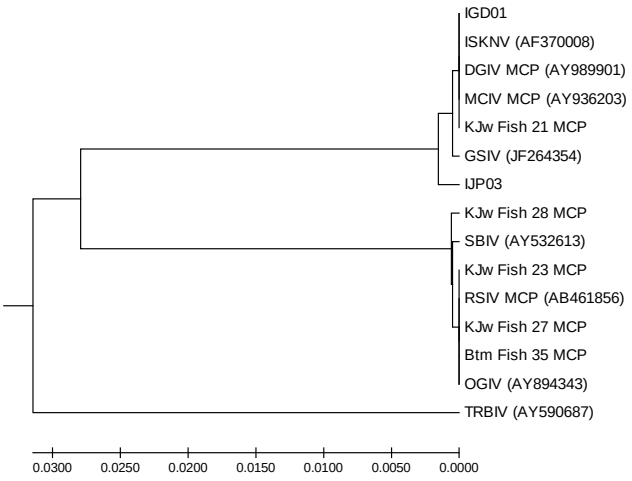

Figure 3. UPGMA dendrogram of the megalocytivirus isolates based on major capsid protein gene sequences

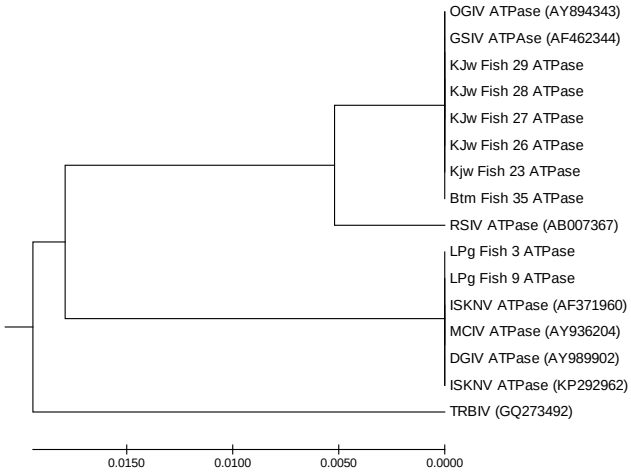

Figure 4. UPGMA dendrogram of the megalocytivirus isolates based on ATPase gene sequences

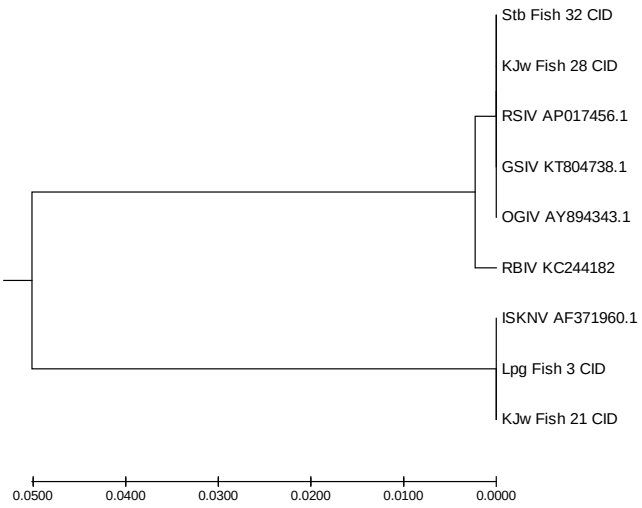

Figure 5. UPGMA dendrogram of the megalocytivirus isolates based on DNA polymerase, CY15, and IRB6 gene sequences

Discussion

The clinical signs of megalocytivirus-infected fishes are lethargic, swim helplessly, and show severe anemia, petechiae of the gills, and enlargement of the spleen (Nakajima and Maeno, 1998) and kidney (Sudthongkong et al. 2002). Our study showed that fish samples were lethargy. enlargement of spleen, pale color of liver (Figure 1). Histological examination from some samples clearly showed the presence of hypertrophy, inclusion body forming bearing cells in several tissues such as liver, spleen and kidney (Figure 2). Those result on megalocytivirus was similar with reported by Sudthongkong et al. (2002) reported the enlarged cells have been termed inclusion body-bearing cells (IBC). This IBC appearance is pathognomonic for megalocytivirus (Kurita and Nakajima 2012). Some samples on this study showed clinical signs, but the IBC was not find in the tissues. Due to the positive PCR amplification using primers MCP-Irido-F-Bam and MCP-Irido-R-Eco, we concluded that those fish samples were infected by megalocytivirus. Subramaniam et al. (2014) have reported many positive cases of ISKNV on ornamental fishes which did not show any clinical signs. Jeong et al. (2006, 2008) also found megalocytivirus in marine fish species that were externally healthy, a condition that could be called persistent or asymptomatic infection. Jeong et al. (2006) proved that he DNA concentration of the megalocytivirus in asymptomatically infected tissues was approximately 10^{-5} times of that of moribund fish infected clinically.

The previous study of Indonesian megalocytivirus using MCP sequences have identified IGD01 (from Jepara) and IJP03 (from Bali) were belonged into ISKNV (Murwantoko et al. 2009). In this study we discovered ISKNV genotype from another area in Indonesia i.e. Karimun Jawa and Lampung (Figure 3). All isolates of ISKNV genotype in this study showed 100% identity with ISKNV (AF370008). This Indonesian ISKNV isolates seems has homogenous genetic compare to the Malaysia, as Zainathan et al. (2017) reported that ISKNV isolates from Southern Malaysia showed 97-100% nucleotide identity with reference ISKNV.

Host range of ISKNV is relatively broad but freshwater and brackish water fish species are predominantly affected species (Kurita and Nakajima 2012). ISKNV diseases outbreak in freshwater fishes have been reported on ornamental fish in Germany (Jung-Schroers et al. 2016), Australia (Mohr et al. 2015; Rimmer et al. 2017), Malaysia (Subramaniam et al. 2014; Zainathan et al. 2017), and in cultured Nile tilapia (*Oreochromis niloticus*) in the US Midwest (Subramaniam et al. 2016). The results of our study showed that ISKNV could be detected from marine fish, humpback grouper (*Cromileptes altivelis*), and tiger grouper (*Epinephelus fuscoguttatus*). Dong et al. (2017) have further reported the occurrence of ISKNV diseases outbreak in farmed baramundi (*L. calcarifier)* in Vietnam.

Based on MCP sequenced, we confirmed a sample form Batam (Btm Fish 35), and five samples from Karimun Jawa (KJw Fish 23,-26,-27,-28 and-29) were belonged to RSIV genotype (Figure 3). Alignment analysis on ATPase sequences showed the KJw fish_28 and a sample from Situbondo (Stb Fish_32) were belonged to RSIV genotype (Figure 4). Several genes-other than those of ATPase and MCP-have been used to genetic analysis of megalocytivirus (Go et al. 2006). We employed a combination of DNA polymerase, CY15 and IRB genes to confirm our results using MCP and ATPase gene. The results showed samples from Situbondo (Stb Fish_32) and from Karimun Jawa (KJw Fish-28) were belonged to RSIV genotype (Figure 5). From those results we confidently confirmed that RSIV genotypes already presence in Indonesia and have been detected on fish from Batam, Karimun Jawa and Situbondo. To our knowledge, this is the first report on the presence of RSIV genotype in Indonesia.

The RSIV-type viruses can be further divided into two sub-clusters: genotype I (RSIV Ehime-1), and genotype II (majority of RSIV, grouper sleeping disease virus (GSDIV), orange spotted *grouper* iridovirus (OGIV), RBIV) (Kurita and Nakajima 2012). The sequences of MCP, ATPase, DNA polymerase, CY15 and IRB6 from RSIV Indonesian isolates showed 100% identity with orange spotted grouper iridovirus (OGIV). Those results showed that Indonesian RSIV were belonged to genotype II RSIV.

Here we confirmed the presence of RSIV and ISKNV genotypes in Indonesia. The distribution map of megalocytivirus in Indonesia are as follows: ISKNV genotype presents in Lampung, Karimun Jawa, Jepara, Bali; and RSIV genotype presents in Batam, Situbondo and Karimun Jawa. Interestingly Karimun Jawa has two genotypes of megalocytivirus, ISKNV and RSIV.

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Protein and fatty acid profile of marine fishes from Java Sea, Indonesia

SRI PRIATNI♥, DIAH RATNANINGRUM, WAWAN KOSASIH, EEN SRIENDAH, YOICE SRIKANDACE, TINA ROSMALINA, SRI PUDJIRAHARTI

Research Unit for Clean Technology, Indonesian Institute of Sciences. Kampus LIPI, Jl. Sangkuriang Bandung 40135, West Java, Indonesia.
Tel.: +62- 22-2503051, Fax.: +62-22 2503240, ♥email: sripriatni@yahoo.com

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Abstract. *Priatni S, Ratnaningrum D, Kosasih W, Sriendah E, Srikandace Y, Rosmalina T, Pudjiraharti S. 2018. Protein and fatty acid profile of marine fishes from Java Sea, Indonesia. Biodiversitas 19: 1737-1742.* Indonesia is the second largest producer of capture fisheries products in the world and the most capture fisheries production comes from marine fisheries. Marine fish is a source of protein, amino acid, saturated and unsaturated fatty acids, which are important components of diet. The objective of the study was to investigate the protein and fatty acids profile of nine marine fish samples from Java Sea of Indramayu West Java, Indonesia. The analysis data showed that the total protein content of fish samples ranged from 61.07% (*Pampus argenteus*) to 86.56% (Tetraodontidae). Meanwhile, total lipid content of fish samples ranged from 1.73% (Tetraodontidae) to 9.82% (*Leiognathus equulus*). The concentration of α-Amino Nitrogen (AN) of fish protein hydrolysate was ranging from 31 mM (*Nemipterus hexodon*) to 69 mM (*Mystacoleucus padangensis*) and% Degree of Hydrolysis (DH) was ranging from 9.33% to 20.39%. The molecular weight of protein fish samples had similar profiles primarily for almost all samples, which could be observed from a typical band with the weight around 49 kDa. The saturated fatty acid (Σ SFA) compositions of fish species ranged from 1094.03-4233.03 µg/g. Oleic acid (MUFA) content of all fish species ranged from 257.91-1216.06 µg/g. However, only three fish species contain of Poly Unsaturated Fatty Acid (PUFA) linoleic acid as the following; *Selaroides leptolepis* (171.36 µg/g), *Oxyeleotris marmorata* (249.40µg/g) and Tetraodontidae (140.35 µg/g). The highest SFA content was found in *S. leptolepis* with palmitic acid (C16:0) as the dominant saturated fatty acid (2320.88 µg/g). *S. leptolepis* also contained high oleic acid (1216.06 µg/g) and linoleic acid (171.36 µg/g).

Keywords: marine fish, Indonesia, protein, fatty-acid

INTRODUCTION

The chemical composition of marine fish is important for basic information of the research and development in fish species study included physiology, biochemistry, ecology, and conservation. Indonesia has a lot of fisheries sources and the largest archipelago, with more than 17,500 islands, which extend between the Pacific and the Indian Oceans. Fishing area in Indonesia covers 5.8 million km^2 of marine waters. In 2011, fisheries production increased to 5.7 million tonnes. After China, Indonesia was the second largest producers of capture fisheries products and the majority comes from marine fisheries. These production were attributed to some of fishing areas in Indonesia (Stobutzki et al. 2013). Fisheries production has become the big issues on ecological impact to marine biodiversity. Java Sea is one of large slight water that contributed significantly on the fish production among of fisheries manufactures in Indonesia (Nugroho et al. 2016).

Fish is important food component in the diet, not only as a source of protein but also a significant supply to the need of polyunsaturated fatty acids or omega-3. These nutrition are very beneficial to human health and stamina. Fish is consumed at several place of the world because of its high contents of protein, amino acid and saturated fatty acid. Because of the nutrition content of fish, the utilization of marine fish and its products increased significantly (S. Suvitha et al. 2014). The chemical composition of fish species is the basic importance to be applied in process production (Diniz et al. 2013). The information of the nutrient composition of some important foods is important to understand the correlation between food productions, access, nutrient intakes and innovation of production technologies to guarantee that food supply population fulfils nutrient requirements optimally (Bogard et al. 2015).

Generally, the lipid content of fish meat is lower than beef or chicken. The important nutritional components of Fish products can be used as source of energy for human life. However, the nutrition content is varied depending on species, size, sexual condition, feeding season and physical activity. Proximate analysis such as protein, lipids and moisture contents is necessary to ensure that this analysis is suitable with requirements of food regulation and commercial specification (Ondo-azi et al. 2013).

Protein from marine fish is potential as raw material of protein hydrolysate production, which can produce by acidic or enzymatic hydrolysis method. Protein hydrolysates of fish is obtained from hydrolysis reaction of peptide bonds in proteins. It causes peptides becoming shorter. It also causes amino acids could be absorbed easily by animal (Wisuthiphaet and Kongruang 2015). Peptone is one of protein hydrolysates product. Fish protein hydrolysates contain secondary protein including polypeptides, dipeptides and amino acids. These secondary protein are nitrogen source of microorganisms, which are water soluble, so that they are suitable for being used as

microbiological culture media (Al-Bahri et al. 2009).

The advantage of marine fish as dietary sources of polyunsaturated fatty acids (PUFA) and unsaturated fatty acid (HUFA), especially the omega-3 PUFA, eicosa-pentaenoic acid (EPA) and docosa-hexaenoic acid (DHA). The nutritional benefits of fish consumption containing omega-3 PUFA have been published well in the world (Dhaneesh et al. 2012). Omega-3 fatty acids such as EPA and DHA have the ability to reduce the blood serum triglycerides. Long chain PUFA can prevent the disease of human coronary artery, rheumatoid arthritis, retina improvement and brain development, asthma, inflammatory bowel, decrease the breast cancer incidence, and can regulate the prostaglandin synthesis (Suvitha et al. 2014; Bahurmiz et al. 2017) .

The comparison study of total protein, fat and omega-3 fatty acids content of raw and pressurized fish of *P. pangasius* (yellowtail catfish) and *H. macrura* (long tail shad) has been reported by Asmah et al. (2014). This study concluded that the pressurized fish is a potential source of omega-3. The Java Sea of West Java has a great variety of fish species, which are potential for fish product industries especially for protein isolate production. The objective of the study was to investigate the protein and fatty acids profile of nine marine fish samples from Java Sea of Indramayu West Java, Indonesia.

MATERIALS AND METHODS

Collection of sample

Fresh samples of fish were collected from fish market at the Java Sea of Indramayu West Java, Indonesia (Figure 1). They were kept in cold iced box and transported to the laboratory and kept in a freezer before used.

Figure 1. Fish sampling location at the Java Sea of Indramayu off shore, West Java, Indonesia

Determination of total protein and total lipid

The protein content was determined by estimating the total Nitrogen using Kjeldahl method. The protein content was calculated by multiplying total nitrogen by 6.25 factor, while lipid content in pulp was extracted using hexane in a soxhlet extractor as described by AOAC (2000).

Fish protein hydrolysates preparation

The preparation was carried out following the method of Fahraniah et al. (2002) with slightly modification. The frozen marine fish was thawed and mixed with distilled water with a ratio 1:4. Samples were blended and adjusted to pH 6.0. The hydrolysis was carried out in a water bath using 0.1% of papain at 50^{o}C for 7 h, which was then stopped by heating at 85^{o}C. The hydrolysate was allowed to stand for 15 min prior to vacuum-filtrated, which was then stored at-20^{o}C.

Analysis of soluble protein content

Soluble protein content was analyzed using a modification of Lowry method (Rahman et al. 2004). Absorbance was measured by a UV-Vis Spectrophotometer at 500 nm. A series concentration of bovine serum albumin (BSA) was used as protein standard curve.

α-AN assay was determined using formol titration

The assay was done according to the method described by Wang et al. (2012) with slight modification. 1 gram of dried fish sample was mixed with 25 mL of deionized water. The solution was adjusted to pH 8.2 with 0.1 M NaOH, and it was subsequently added with 10 mL of 35% (w/w) formalin (pH 8.2). The pH value of the solution went down due to the addition of formol. The titration using NaOH was done until the pH of the solution was 9.20. The concentration of α-AN was calculated using the following equation:

$$C\ \alpha - AN\ (mM) = \frac{\Delta V\ x\ n\ x\ 103}{V}$$

Where, ΔV is the volume in mL of NaOH used for the titration; n (mol/L) is the molar concentration of the NaOH solution; and V (mL) is the sample volume.

Determination of degree hydrolysis

Degree hydrolysis (DH) of the extract was calculated using (AOAC 1995) method. The DH was calculated using relationship between α-amino nitrogen (AN) and total nitrogen (TN) according to the following equation:

$$\%DH = \frac{\alpha - amino\ Nitrogen\ (AN)}{Total\ Nitrogen\ (TN)}\ x\ 100$$

TN was determined by the Kjeldahl method.

Molecular weight analysis

Sodium dodecyl sulphate-polyacrylamide gel electro-phoresis (SDS-PAGE) was done on all samples and protein marker on a discontinuous buffered system according to Bollag et al. (1996) method. 20 µL of each sample was added with 40 µL of sample buffer. Samples were placed in

a foam rack and heated in boiling water for 4 min. The preparation of polyacrylamide gel was placed in an electrophoresis unit. Running buffer was filled to the upper buffer chamber of the gel until the buffer reaches halfway between the tops of the short and long glass plates. 5 µL of standard protein markers and 25 µL of each samples were loaded to the polyacrylamide gel. Electrophoresis was conducted at a constant 200 V for 30 min. The gel was removed and placed in staining solution.

Methylation Preparation

During fatty acid methyl ester (FAME) preparation, pre-test was done to identify the suitable method for mehylation process. The pre-test includes sodium methoxide method, potassium hydroxide method and boron trifluoride (BF3) method. Finally boron trifluoride (BF3) was chosen while comparing the peak. Firstly 0.125 g of fish oil was put in a test tube. Secondly, 0.5 ml of boron trifluoride (BF3) in MeOH (14%) was added to the test tube. Afterward, the test tube containing the fish oil and boron trifluoride (BF3) in MeOH (14%) was incubated in an incubator shaker at 55^{o}C for 1.5 hour. 0.5 ml of saturated sodium hydrogen carbonate ($NaCHO_3$) and 0.75 ml of n-hexane was then added to the test tube. The mixture was mixed and shaken well using a vortex for about 30 second. The subsequent mixture was stored for 5 minutes under room temperature so that it will form two layers. Lastly, 0.5 ml of upper layer contain hexane was carefully transferred into a vial for Gas Chromatography (GC) analysis.

Gas Chromatography (GC) analysis

Fatty acids composition of fish hydrolysate samples were analyzed using gas chromatography (GC) (Agilent Chemstation Version 5) equipped with split-splitness injector, detector Hewlett-Packard EL-980 flame ionization detection (FID) system to separate and quantify each FAMEs components. FAMEs were separated using Ultra 1 column (25 m x 0.32 mm thickness 0.17 µm methyl siloxan film). Chromatography data were recorded and integrated using Chemstations software (version 5.0). Oven temperature was held at 70^{o}C for 3 min, which then increased to 200^{o}C at 10^{o}C/min and lastly increased to 260^{o}C, held for 3 min. Temperatures for injector and detector were set at 280^{o}C. 1 µL of sample volume was injected with split ratio of 0:50 at column temperature 110^{o}C. Carrier gases used for the system were helium gas, 1.0 ml/min controlled at 4.92 psi, hydrogen and air used for FID was held at 40 and 80 lbs/inc^2.

RESULTS AND DISCUSSION

Results

In this study, nine fish species had been collected freshly from a fish market at the Java Sea of Indramayu West Java, Indonesia. Fish samples are predominantly collected by the species with the small size fish group between10-20 cm in length. The common and species names of fish was presented in Figure 2.

Table 1 presents the total protein and total lipid content of nine species of marine fish samples. The total protein content of fish samples ranged from 61.07% (*Pampus argenteus*) to 86.56% (Tetraodontidae). Meanwhile, total lipid content of fish samples ranged from 1.73% (Tetraodontidae) to 9.82% (*Leiognathus equulus*). This data showed that Tetraodontidae contained the highest protein and the lowest lipid content.

Total nitrogen (TN), soluble protein content, alpha amino content and degree hydrolysis of nine fish species was shown in Table 2. These data represents the protein profile of fish species, which was found in Java Sea of West Java. The data showed that the content of total nitrogen, ranging from 9.95% (*P. argenteus*) to 13.81% (Tetraodontidae) based on the dry weight.

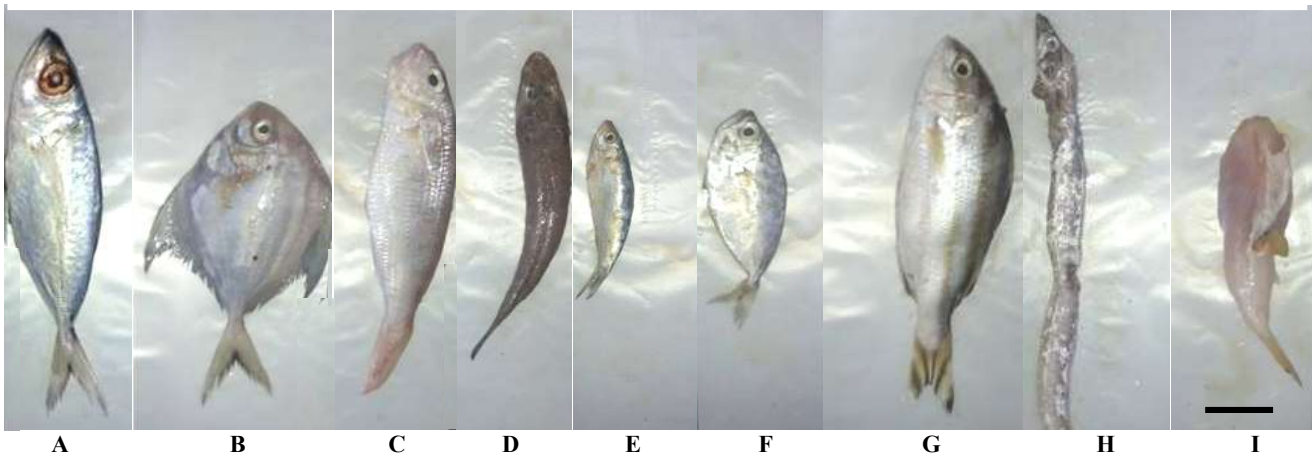

Figure 2. Fish samples; A. Selar (*Selaroides leptolepis*), B. Bawal (*Pampus argenteus*), C. Kurisi (*Nemipterus hexodon*), D. Boso (*Oxyeleotris marmorata*), E. Bilis (*Mystacoleucus padangensis*), F. Peperek (*Leiognathus equulus*), G. Kerong (*Terapon jarbua*), H. Layur (*Trichiurus lepturus*), I. Buntal (Tetraodontidae). Bar = 1 cm

Table 1. Protein and total lipid content of nine species of Marine Fishes from Java Sea of West Java, Indonesia

Species	Total protein (% w/w)	Total lipid (% w/w)
Leiognathus equulus	66.58	9.82
Mystacoleucus padangensis	74.53	6.88
Nemipterus hexodon	73.19	9.71
Oxyeleotris marmorata	78.53	4.66
Pampus argenteus	61.07	4.03
Selaroides leptolepis	73.26	5.45
Terapon jarbua	65.00	7.41
Tetraodontidae	86.56	1.73
Trichiurus lepturus	83.02	4.74

In this study, fish samples were hydrolyzed by papain enzyme. The concentration of α-AN of fish protein hydrolysate was ranging from 31 mM (*Nemipterus hexodon*) to 69 mM (*Mystacoleucus padangensis*) and the percentage (%) of DH was ranging from 9.33% to 20.39%. In our study, the hydrolysis was carried out using 0.1% of papain at 50^{o}C for 7 hours.

Table 3 presents the fatty acid composition of nine fish samples. Gas chromatography analysis of fatty acid methyl esters from the lipids of those fish samples revealed the presence of five fatty acids. The saturated fatty acid (Σ SFA) compositions of fish species ranged from 1094.03-4233.03 µg/g. Oleic acid (MUFA) content of all fish species ranged from 257.91-1216.06 µg/g, except for the species *P. argenteus* was not detected. However, only three fish species contain linoleic acid (PUFA) as follow; *Selaroides leptolepis* (171.36 µg/g), *Oxyeleotris marmorata* (249.40µg/g) and Tetraodontidae (140.35 µg/g). The highest SFA content was found in *Selaroides leptolepis* with palmitic acid (C16:0) as the dominant saturated fatty acid (2320.88 µg/g). *S. leptolepis* also contain high oleic acid (1216.06 µg/g) and linoleic acid (171.36 µg/g). However, the highest linoleic acid was found in *O. marmorata* (249.40 µg/g).

The soluble protein of marine fish samples was identified its molecular weight profile by SDS PAGE method. The result was presented on Figure 3.

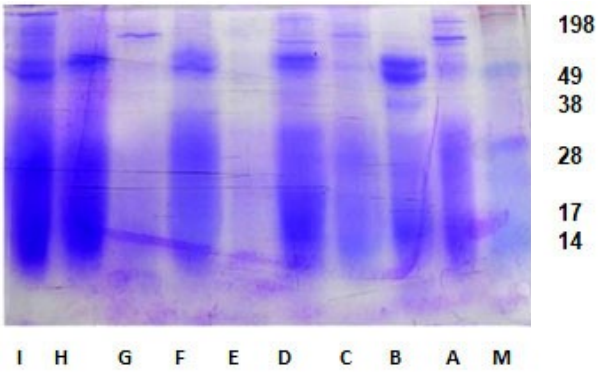

Figure 3. SDS PAGE profile of fish samples; M: protein molecular weight marker (kDa), A. Selar (*Selaroides leptolepis*), B. Bawal (*Pampus argenteus*), C. Kurisi (*Nemipterus hexodon*), D. Boso (*Oxyeleotris marmorata*), E. Bilis (*Mystacoleucus padangensis*), F Peperek (*Leiognathus equulus*), G. Kerong (*Terapon jarbua*), H. Layur (*Trichiurus lepturus*) and I. Buntal (Tetraodontidae)

Table 2. Protein profile of nine species of marine fishes from Java Sea of West Java, Indonesia

Species	Total nitrogen (%)	Soluble protein (mg/g)	α-amino nitrogen (mM)	Degree hydrolysis (%)
Leiognathus equulus	11.03	286.68	53	16.82
Mystacoleucus padangensis	11.85	252.67	69	20.39
Nemipterus hexodon	11.63	134.29	31	9.33
Oxyeleotris marmorata	12.57	169.30	52	14.49
Pampus argenteus	9.95	203.03	54	19.00
Selaroides leptolepis	11.69	168.34	47	14.98
Terapon jarbua	10.39	196.53	53	17.86
Tetraodontidae	13.89	163.22	42	10.59
Trichiurus lepturus	13.34	191.74	38	9.97

Table 3. Fatty acid profiles of nine species of marine fishes from Java Sea of West Java, Indonesia (µg/g)

Species	(C14:0)	(C16:0)	(C18:0)	Σ SFA	(C18:1)	(C18:2)
Leiognathus equulus	113.51	700.15	280.37	1094.03	257.91	nd
Mystacoleucus padangensis	534.69	1376.91	451.58	2363.18	362.73	nd
Nemipterus hexodon	61.16	812.16	469.70	1343.02	423.26	nd
Oxyeleotris marmorata	295.50	1750.22	679.86	2725.58	621.21	249.40
Pampus argenteus	37.33	737.13	572.15	1346.60	nd	nd
Selaroides leptolepis	250.68	2320.88	1661.52	4233.07	1216.06	171.36
Terapon jarbua	97.67	959.92	364.47	1422.05	628.82	nd
Tetraodontidae	65.69	1564.68	1131.00	2761.37	749.49	140.35
Trichiurus lepturus	122.31	652.63	338.51	1113.45	313.14	nd

Discussions

According to the statistic data from Noegroho et al. (2013), Indramayu was the highest fish producer in West Java, Indonesia. The total production was around 128,548 ton per year, in which the highest production ± 16,664 ton was peperek fish (*L. equulus*), followed by selar fish (*S. leptolepis*) ± 3,367 ton and layur fish (*Trichiurus lepturus*) ± 2,601 ton. Nugroho et al. (2016) reported that the catching composition in inshore water was also predominantly composed of small size Leiognathida, included *L. equulus.* The sampling location was at Tegal City, the western part of the north coast of Central Java, Indonesia.

The results on Table1 showed that Tetraodontidae contained the highest protein content. Nevertheless, Tetraodontidae was not recommended for human consumption. Hashiguchi et al. (2015) reported that family Tetraodontidae or pufferfish contained tetrodotoxin and saxitoxin in their organs and the degree of toxicity was highly variable even within each toxic species. Tetrodotoxin in the pufferfish localized in the ovary of its larvae as protection against predators (Itoi et al. 2014). From all samples study, *S. leptolepis* and *O. marmorata* were recommended as good edible fish due to its high protein content, no toxin and its taste.

Protein profile of nine fish samples from Java Sea of West Java Indonesia (Table 2) can represent the protein profile of fishes in this area. Diniz et al. (2013) reported that the total nitrogen of fish samples from coastal water of Brazil ranging from 11.6% (*M. argetinae*) to 14.9% (*R. porosus*) of the dry weight. Taheri et al. (2016) also reported that the biochemical composition of *Sardinella gibbosa, Clupeonella engrauliformis* and *Stolephorus indicus* bones from the Oman Sea and Caspian Sea became an alternative of fish meal production with the adding value and high quality to fulfill the market demand. The concentration of α-amino nitrogen (α-AN) and degree hydrolysis (% DH) are often used as the quality indicator for fish protein hydrolysate product. This product can be used as condiments with unique flavors and aromas. The amount of amino acids formation is very important key to the flavor of sauces or flavoring. Wang et al. (2012) reported that fish sauce can be graded by α-AN concentration and the higher the α-AN concentration is the better of quality. Analysis of protein hydrolysate from the mixed marine fishes showed that the DH was in the range of 20-24% (Wisuthiphaet and Kongruang 2015). This product was obtained by hydrolysis the marine fish sample with 2-6% papain at 40^{o}C for 15 hours. The condition of enzymatic hydrolysis is important for improvement the quality of protein hydrolysate product. The hydrolysis of protein is a conversion process from big molecule of protein to low molecular weight products, hydrolysis proteins yield proteomes, peptones, polypeptides, and finally the simpler amino acids (Al Bahri et al. 2009). During enzymatic hydrolysis, the exposures of peptide bonds lead to the increase of DH. However, the activity of enzyme can be reduced by high temperature and decreasing the hydrolysis rate (Salwanee et al. 2013). In our study, *M. padangensis* has the highest% of DH. This fish species can be recommended for peptone or protein hydrolysate production.

The molecular weight of protein fish samples had a similar profile primarily for *S. leptolepis, P. argenteus, O. marmorata, L. equulus, T. lepturus* and Tetraodontidae with the molecule weight of typical band around 49 kDa. This data was indicated that marine fish has typical protein profile and could be explored as protein character of each fish species. Species identification, primarily using sarcoplasmic proteins as target proteins, has been studied using SDS-PAGE method. However, the quantification of fish myofibrillar protein (surimi) using SDS-PAGE has not been studied, primarily for the application of species identification (Reed and Park 2008).

The results on Table 3 show that *S. leptolepis* and *O. marmorata* contained high Oleic acid (MUFA) and also linoleic acid (PUFA). Fish oils and other marine animal oils are identified as a big group of fatty acids. These fatty acids are classified as saturated, monounsaturated and polyunsaturated groups (Immanuel and Palavesam 2010). Marine fishes had higher levels of PUFAs compared to freshwater fish. The differences of fatty acids content in marine and freshwater fish were influenced by its species and their natural diet (Dhaneesh et al. 2012). Fatty acids content of fishes were based on their feed and affected by its size, age, reproductive and environmental conditions, especially the temperature of water which could influence the lipid content and fatty acid composition. Marine fish and shellfish from warm water area contain a good composition of fatty acids and provided the health benefit if they were consumed regularly (Aziz et al. 2013). Fatty acid profiles in marine and freshwater fish were also studied by Łuczyńska et al. (2014). In this study, the most abundant n-6 polyunsaturated fatty acids were linoleic (C18:2 n-6) and arachidonic (C20:4 n-6). Fatty acids in freshwater fish were on approximately similar to some of the marine fish examination.

We concluded that marine fishes are a source of high protein with low lipid content. Some of fish samples are potential as raw material for fish protein hydrolysate production due to its high% of DH. *S. leptolepis* and *O. marmorata* are recommended as potential sources of PUFA.

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TRFLP analysis for revealing the diversity of rice phyllosphere bacteria

SRI MARTINA WIRASWATI[1], ARIS TRI WAHYUDI[1,♥], IMAN RUSMANA[1], ABDJAD ASIH NAWANGSIH[2]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Pertanian Bogor. Jl. Agatis, Dramaga, Bogor 16680, West Java, Indonesia. Tel./fax. +62-251-8622833, ♥email: aristri2011@gmail.com

[2]Department of Plant Protection, Faculty of Agriculture, Institut Pertanian Bogor. Jl. Agatis, Dramaga, Bogor 16680, West Java, Indonesia

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Abstract. *Wiraswati SM, Wahyudi AT, Rusmana I, Nawangsih AA. 2018. TRFLP analysis for revealing the diversity of rice phyllosphere bacteria. Biodiversitas 19: 1743-1749.* Phyllosphere environment of rice plant is usually inhabited by diverse bacteria which mostly contribute beneficial effects to the plant fitness. TRFLP method is a rapid and straightforward method to determine the bacterial diversity of many environments, including rice phyllosphere environment. This study aimed to analyze rice phyllosphere bacterial diversity of healthy rice plant cultivar Ciherang obtained from Sukabumi, Jasinga, and Situgede. The bacterial genomes were amplified and digested with two restriction enzymes, i.e., *Msp*I and *Bst*UI. The bacterial diversity (H' index) and evenness (E index) were calculated from the peak value. From TRFs analysis, Betaproteobacteria and Pseudomonadales were dominantly found in nearly all samples with different relative abundance. In addition, Alphaproteobacteria and Gammaproteobacteria were also dominant in the several samples. The unique bacteria groups were inhabited in the sample from specific regions with certain growth phase. This finding informs us that the geographical factors might be more influent than the growth phase factor. Furthermore, the bacterial diversity and evenness of the metagenomic approach are higher than cultivation-dependent approach.

Keywords: Bacteria, phyllosphere, rice, TRFLP

INTRODUCTION

Phyllosphere environment is commonly occupied by diverse microbes such as bacteria, filamentous fungi, and yeast where bacteria are the most predominant (Lindow and Leveau 2002). The phyllosphere microbes mostly live as commensals on their host plant through their prosperity to increase plant fitness and function. They play an important role in decomposing natural substances as saprophytes, remediating of remnant pesticides and air pollutant, inducing plants health and development as biofertilizer, phytostimulator, and biopesticides against plant pathogen (Muller and Ruppel 2014). Studying the phyllosphere microbe diversity and behavior could facilitate biotechnology applications for combating plant diseases, increasing plant growth, preventing infection of the human pathogen in crop food, and handling volatile pollutant from the air (Vorholt 2012). The phyllosphere represents a habitat with great agricultural and environmental significance. Several evidence showed the importance of phyllosphere microbes for the fitness of natural plant populations and the quality as well as productivity of crops (Whips et al. 2008).

Rice is the most important crop in the world and staple food for 90% of the Indonesian population. However, rice can be attacked by several pathogens such as *Pyricularia oryzae* and *Xanthomonas oryzae* pv. *oryzae* as the major pathogens (Costa et al. 2006). The rice plant is habitat for diverse microorganisms that colonize aerial parts, tissues plant, root surface and area around the root (Knief et al. 2011). These various microorganisms have a significant role in increasing the health and growth of rice plant. The rice phyllosphere bacterial community is playing an important role in influencing the disease resistance of rice plant. Phyllosphere bacteria can promote plant growth, and both suppress and stimulate the colonization and infection of tissues by plant pathogens (Rasche et al. 2006). Several rice phyllosphere bacteria with antifungal and antibacterial activity against plant pathogen were successfully isolated from rice leaves. These become evidence that commensal bacteria dominantly inhabit phyllosphere environment. However, research on the diversity of rice phyllosphere bacteria is still rare, especially in Indonesia. Thus, it is essential to fill this gap by applying metagenomic analysis to reveal the diversity of the phyllosphere environment.

Terminal restriction fragment length polymorphism (T-RFLP) is one of high throughput microbial community analysis methods based on the use of 16S rDNA gene directed PCR process. There is no DNA sequencing process in TRLFP analysis. The combination of direct PCR from an environmental sample and restriction enzymes were the basic method of TRFLP. Direct PCR used fluorescently labeled primers on the 5'-end that would label 16S rDNA genes amplicon subsequently tracked. The labeled 16S rDNA gene amplicon is digested with one or more restriction enzymes that have four base-pairs recognition sites and the resulting labeled terminal restriction fragments (TRFs) are analyzed using an automatic DNA sequencer to determine the size and relative abundance of each TRF (Chauhan et al. 2011). The T-RFLP method is commonly used to analyze the microbial community because it is relatively simple, rapid and high reproducible. It can also be used to analyze large samples from several environments and microbial

community changing due to the current condition. Therefore, in this study, we use the T-RFLP method to determine the phyllosphere bacterial diversity of rice cultivar Ciherang from West Java, Indonesia.

MATERIALS AND METHODS

Sample collection

Healthy rice plants cultivar Ciherang were collected from rice field with blast symptoms at Jasinga, Situgede, and Sukabumi, West Java. The rice plants were collected at the vegetative and generative growth phase. The aerial parts of the plants were cut and immediately transferred for bacterial isolation.

Isolation of rice phyllosphere bacteria

The rice phyllosphere bacteria were isolated with serial dilutions method from Yadav et al. (2010). Ten grams of rice leaf from each region were transferred to 90 mL of saline buffer solution (NaCl 0.85%) and dislodged by shaking at 150 rpm for 1 hour at room temperature. The solution was diluted by the factor of 10^{-3}-10^{-7} and 100 µL solution aseptically transferred to Luria Bertani (LB) agar medium (1% NaCl, 1% Tryptone, 0.5% Yeast extract, 2% agar in 1 L aquadest). The rice phyllosphere bacteria were observed after three days of incubation for DNA extraction.

DNA extraction

The isolated rice phyllosphere bacteria were harvested by transferring 1 mL of distilled water to cultured bacteria. The bacteria solution from each sample was scraped and transferred to falcon tube and dried at 60°C of temperature. For DNA extraction, 0.25 gram of dried bacteria colonies were transferred to a microtube and extracted with Power Soil DNA Isolation Kit (MO BIO Laboratories, Inc.) based on the manufacturer's procedures. For metagenomic approach, the total bacterial genomes were directly extracted from rice leaf samples; 10 grams of leaf samples were transferred to a saline buffer solution (NaCl 0.85%) and dislodged by shaking at 150 rpm for 1 hours at room temperature. Bacteria on the leaf solution were pelleted by centrifugation at 10,000 rpm at 4°C for 15 minutes. The total DNA genomes from the bacteria pellet were also extracted with Power Soil DNA Isolation Kit (MO BIO Laboratories, Inc.). The bacterial genomes were then quantified with Nanodrop 1000 (*Thermo Scientific*, Wilmington, DE, USA).

Amplification and digestion of 16S rRNA gene

Fluorescein dye-labeled primer (5'-6 FAM) 27F (5'-AGAGTTTGATCCTGGCTCAG-‘3) and 1429R (5'-TACGGTTACCTTGTTACGACT-‘3) were constructed to amplify 16S rRNA gene from rice phyllosphere bacterial genome. PCR mixture contained 25 µL Go Taq Green Master Mix 1x (Promega®, USA), 1 µL (10 pmol. μL^{-1}) of each primer, 1 µL DNA template (~ 100 ng. μL^{-1}) and adjusted with 22 µl nuclease free water. The 16S rRNA gene amplification was conducted using the following reaction: predenaturation (94°C, 5 min), 30 cycles of denaturation (94°C, 45 sec), annealing (55°C, 45 sec), elongation (72°C, 45 sec) and post-elongation (72°C, 7 min). The PCR product was then visualized on 1% agarose under UV light and purified with Gel/PCR DNA Fragments Kit (Geneaid Biotech Ltd.) based on the manufacturer's procedures. The DNA purity was quantified before digested using restriction enzyme. Purified DNA was digested with *Msp*I and *Bst*UI (BioLabs, UK) restriction enzymes based on the manufacturing procedures. Afterward, DNA fragments digestion product was analyzed using Applied Biosystem Genetic Analyzer and interpreted using the GeneMapper® v 4.0 analysis software.

Data analysis

T-RFLPs and diversity analysis for each sample, the TRFs (terminal restriction fragments) peak size between 50 bp and 500 bp and peak high more than 1% from total peak high were further analyzed to determine the phylogenetic relationship. The TRFs from the same sample with differences size less than 0.5 bp digested with one restriction enzymes were grouped as one TRF (Zhang et al. 2008). The observed TRFs were identified by comparing each TRF with the Ribosomal Database Project (RDP) (R10 U27) 700,829 Good Quality (>1200 Bacteria) in MiCA III (Microbial Community Analysis) website (http://mica.ibest.uidaho.edu/digest.php) using Virtual digest (IsPaR) program. The TRFs with same size was assumed as same bacterial group. Furthermore, the richness of TRFs was analyzed by comparing the peak area of each TRF to total TRFs. The bacterial community diversity and evenness were analyzed using Shannon's index (H') and Pielou's evenness index (E) respectively, using the following formulation:

$H' = -\Sigma (Pi \times \log Pi)$, where $Pi = niN^{-1}$, ni is the peak area, and N is the sum of the total peak areas.

$E = H'/\ln (S)$, where S is the total number of TRFs.

RESULTS AND DISCUSSION

Digestion of 16S rRNA gene by restriction enzymes

TRFLP method was used to determine the bacterial diversity of rice phyllosphere environment from different regions and growth phase in West Java. One TRF represents one or several bacteria species from the samples. The amplified 16S rRNA genes were digested with restriction enzymes *Msp*I and *Bst*UI to obtain terminal restriction fragments. Digestion with restriction enzyme *Msp*I produced more TRFs than *Bst*UI, except the sample SKBV from Sukabumi (Table 1). A total of 29 TRFs were obtained from digestion with *Msp*I restriction enzyme, while 17 TRFs were obtained from digestion with *Bst*UI restriction enzyme.

A total of 29 TRFs were digested with *Msp*I restriction enzyme; 8 TRFs were found on metagenomic approach as well as the cultivation-dependent approach (Figure 1). Also, metagenomic approach analysis resulted in 12 TRFs, while cultivation-dependent approach resulted in 9 TRFs.

The number of TRFs from *Bst*UI digestion is less than that from *Msp*I digestion. A total of 7 of TRFs were found on both metagenomic and cultivation-dependent approach, while the metagenomic approach resulted in fewer TRFs than cultivation-dependent (Figure 1). Generally, the number of TRFs from metagenomic approach digested with *Msp*I restriction enzyme is higher than *Bst*UI. This finding indicates that the *Bst*UI restriction enzyme is less significant in the analysis of rice phyllosphere bacteria with metagenomic as well as cultivation-dependent approach.

Relative abundance analysis

The relative abundance represents the proportion of each TRF from each sample calculated based on the ratio between the peak area of each TRF to the total peak area. Histogram of relative abundance is constructed based on TRF data from *Msp*I and *Bst*UI digestion which had been analyzed using MiCA III website (Figure 2 and 3). The MiCA web-based tools were used to determine the affiliation between each TRF and bacteria group in MiCA database. One TRF usually represents one or more bacteria species or one bacteria group. The relative abundance histogram shows that *Msp*I restriction enzyme resulted in more diverse bacteria group than *Bst*UI restriction enzyme. Several bacteria groups such as Sphingobacteriia, Clostridia, and Deltaproteobacteria were not detected in the samples digested with *Bst*UI (Figure 3). In addition, the digestion with *Bst*UI resulted in only one TRF in several samples, which indicates that this restriction enzyme is less significant in the analyzing of the rice phyllosphere bacteria.

A total of 29 different TRFs were obtained from all samples digested with *Msp*I restriction enzyme. Afterward, the MiCA III analysis resulted in 15 different bacteria groups from the RDP database. This finding is more significant than *Bst*UI digestion, i.e., eight bacteria groups. From all bacteria groups, Betaproteobacteria is commonly found in all samples with diverse relative abundance. Also, the SKBV sample (cultivation-dependent) and STGG sample (metagenomic) were inhabited by the same bacteria group with different relative abundance (Figure 2). The Actinobacteria group is only found from JSNV and STGV samples with the metagenomic approach, while the Bacteroidetes group is only found from JSNG samples with the cultivation-dependent approach. This finding also indicates that the metagenomic approach generally shows more diverse bacteria groups than the cultivation-dependent approach.

Analysis of bacteria TRFs digested with MspI

The TRFs digested with *Msp*I restriction enzyme were used in the further analysis because it resulted in higher resolution than *Bst*UI. TRFLP analysis is approached by the metagenomic and cultivation-dependent method of DNA genome isolation. Bacterial diversity is analyzed by H' index whereas bacteria evenness is analyzed by E index. The TRFs obtained from metagenomic approach are higher and more diverse than those obtained from the cultivation-dependent approach. In addition, the sample SKB from vegetative growth phase showed the highest bacteria diversity of all samples. Furthermore, the bacteria evenness are very diverse among all samples (Table 2).

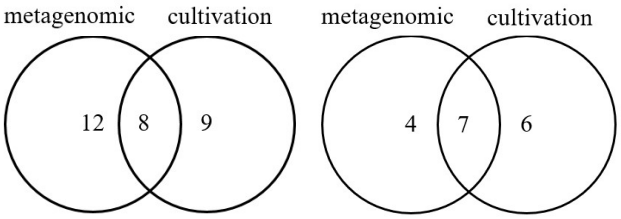

Figure 1. Comparison of total TRFs among metagenomic and cultivation-dependent approach from *Msp*I (left) and *Bst*UI digestion (right)

Table 1. The number of TRFs from rice phyllosphere bacteria communities that digested with two restriction enzymes, *Msp*I and *BstU*I

Rice plant Samples	Growth phase	Number of TRFs digested with restriction enzymes			
		*Msp*I		*Bst*UI	
		Metagenomic	Cultivation-dependent	Metagenomic	Cultivation-dependent
Sukabumi	Vegetative	7	2	1	5
	Generative	3	5	1	5
Jasinga	Vegetative	5	3	4	3
	Generative	6	4	6	3
Situgede	Vegetative	7	4	4	2
	Generative	2	3	1	1

Table 2. Comparison of the bacterial diversity (H') and evenness (E) from rice phyllosphere environment digested with *Msp*I restriction enzyme

Rice plant sample	Growth phase	Analysis approach			
		Metagenomic		Cultivation-dependent	
		H' index	E index	H' index	E index
Sukabumi	Vegetative	1.73	0.89	0.32	0.45
(SKB)	Generative	1.08	0.78	1.04	0.65
Jasinga	Vegetative	1.32	0.83	1.04	0.95
(JSN)	Generative	1.72	0.96	0.89	0.56
Situgede	Vegetative	1.65	0.85	1.23	0.77
(STG)	Generative	0.32	0.47	0.95	0.69

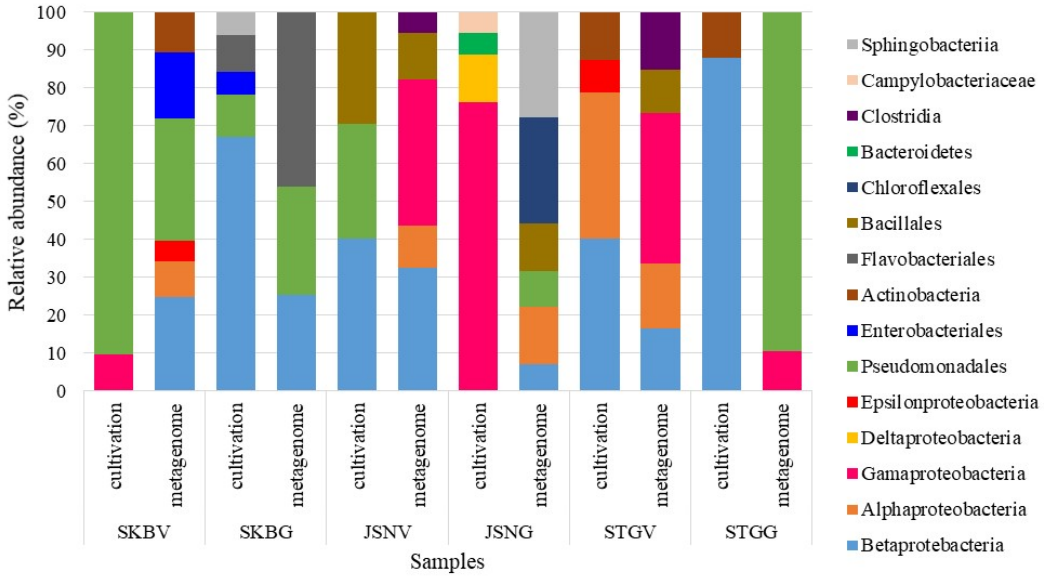

Figure 2. Relative abundance of rice phyllosphere bacteria communities with the metagenomic and cultivation-dependent approach. The 16S rRNA genes were digested with *Msp*I restriction enzyme

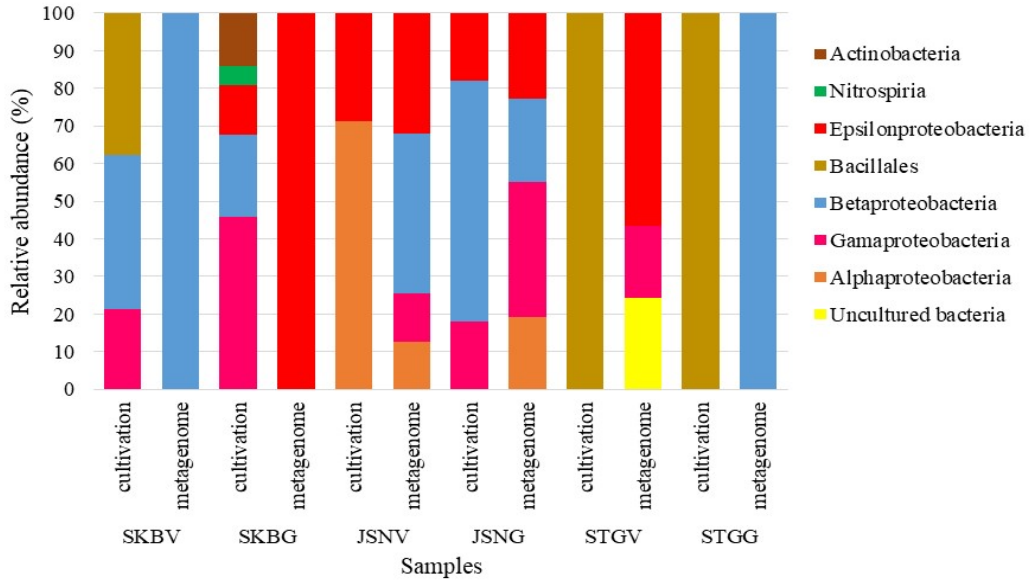

Figure 3. Relative abundance of rice phyllosphere bacteria communities with the metagenomic and cultivation-dependent approach. The 16S rRNA genes were digested with *Bst*UI restriction enzyme

Discussion

The variety of Ciherang has been known as cultivar susceptible to rice blast disease. This variety is commonly cultivated by several farmers in West Java, such as Sukabumi, Jasinga, and Situgede. These three regions are also known as blast disease-endemic areas in West Java. Healthy rice plants among infected rice plants from those three regions become an interesting phenomenon to explore. Therefore, rice phyllosphere bacterial diversity was analyzed from the rice leaves from Sukabumi, Jasinga, and Situgede, because this bacterial community is predicted as having an essential role in rice plants fitness. Knowledge of composition and diversity of rice phyllosphere bacteria is necessary for explaining the microbial mechanism inducing sustainable rice cultivation. The phyllosphere microbiology could be applied to the field of microbial ecology and contribute to more effective and environmentally friendly means of plant protection (Chaudhary et al. 2017). To describe the structure of rice phyllosphere bacteria taxa, the culture-independent method has often been used by researchers. In this study, rice phyllosphere bacterial diversity was analyzed using TRFLP

method with two restriction enzymes, i.e., *Msp*I and *Bst*UI. The type and number of restriction endonucleases are essential factors when an accurate representation of the microbial diversity is desired (Engebretson and Moyer 2003). Besides that, the use of more than one restriction enzymes can facilitate the resolution of a bacterial community (Liu et al. 1994). Engebretson and Moyer (2003) assessed 18 restriction enzymes and revealed that *Bst*UI, *Dde*I, *Sau96*I, and *Msp*I most often determined individual populations in their communities. Also, the *Bst*UI and *Msp*I restriction enzymes produced the highest number of TRFs and OTU in the range of 50-500 bp in length.

In this present work, the digestion with *Msp*I restriction enzyme produced more TRFs and OTU than digestion with *Bst*UI, i.e., 29 TRFs (15 OTU) and 17 TRFs (8 OTU), respectively (Table 1). Among 29 TRFs from *Msp*I digestion, there are 8 TRFs found from both metagenomic and cultivation-dependent approach (Figure 1). These TRFs have affiliations with Pseudomonadales, Betaproteobacteria, Bacillales, Actinobacteria, Epsilonproteobacteria, Gammaproteobacteria, and Sphingobacteriia. Besides that, a total of 7 TRFs from *Bst*UI digestion having associations with Alphaproteobacteria, Gammaproteobacteria, Betaproteobacteria, and Epsilonproteobacteria were also found from the metagenomic and cultivation-dependent approach. Those bacteria groups were obtained from both *Msp*I and *Bst*UI digestion process. On the other hand, at least seven bacteria groups resulted from the digestion with *Msp*I, but not *Bst*UI, i.e., Sphingobacteriia, Campylobacteriaceae, Flavobacateriales, Bacteroidetes, Enterobacteriales, Pseudomonadales and Deltaproteobacteria (Figure 2). This finding indicates that the digestion with *Msp*I offers better resolution than *Bst*UI to determine the rice phyllosphere bacteria diversity. Therefore, the TRFs produced from *Msp*I restriction enzyme will be used for further analysis in this study.

The profiles of rice phyllosphere bacteria diversity from each sample were described in the histogram of relative abundance (Figure 2), in which each sample generally has a different profile of bacteria groups. Bacterial diversity profiles of rice plants from Sukabumi are different in the vegetative and generative growth phase. The Pseudomonadales group were found on vegetative and generative growth phase of rice plants. The relative abundance of Pseudomandales from the vegetative sample is higher than that from the generative sample. On the other hand, the relative abundance of Betaproteobacteria group from a generative sample is higher than that from the vegetative sample. This finding indicates that vegetative growth phase of rice plants from Sukabumi is dominantly inhabited by Pseudomanadales groups, while generative growth phase of rice plants is dominantly inhabited by Betaproteobacteria group. Also, Actinobacteria, Alphaproteobacteria, Gamaproteobacteria, and Epsilonproteobacteria were unique groups with low relative abundance only found in vegetative rice plants, while Flavobacteria and Sphingobacteriia group just found in generative rice plants (Figure 2).

The bacterial diversity from Jasinga also showed different profiles where the relative abundance of Betaproteobacteria on vegetative rice plants is higher than that of the generative rice plants. Meanwhile, the relative abundance of Gammaproteobacteria group on vegetative rice plants is lower than that of the generative rice plants. Different from Sukabumi, Pseudomonadales group was found in vegetative and generative rice plants from Jasinga with low relative abundance. Clostridia and Bacillales are unique groups just found on vegetative rice plants, while Campylobacteriaceae and Bacteroidetes are found in generative rice plants from Jasinga. Similar to Sukabumi, the generative rice plant from Situgede is dominantly inhabited by Betaproteobacteria. In this region, Pseudomandales group is only found from generative rice plants with high relative abundance. In addition, Epsilonproteobacteria, Clostridia, and Bacillales groups were found on vegetative rice plants with low relative abundance (Figure 2). Although those three regions have a different profile of phyllosphere bacterial diversity, Betaproteobacteria and Pseudomonadales were found from all areas. This finding indicates that the phyllosphere environment of rice variety Ciherang is generally harbored by Betaproteobacteria and Psedomonadales group with different abundance depending on the regions and growth phase of rice plants. In the previous study, Knief et al. (2011) revealed that several bacteria groups such as Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, and Actinobacteria mainly contributed to the bacterial community in the phyllosphere environment of rice variety IR-72. Bodenhausen et al. (2013) and Kembel et al. (2014) also showed that the phyllosphere environment is mainly inhabited by Alphaproteobacteria (e.g., *Methylobacterium* and *Sphingomonas*) and Gammaproteobacteria (e.g., *Pseudomonas*).

Several species from Betaproteobacteria and Pseudomonadales groups are known as biological control agents and plant growth promoters. *Nitrosomonas* sp. and *Burkholderia* sp. are two species from the Betaproteobacteria class having a beneficial effect on agriculture. *Nitrosomonas* sp. is known as nitrogen fixation bacteria that provide a nitrogen source for the host plant. Meanwhile, several species from *Burkholderia* sp. like *Burkholderia rhizoxinica* and *Burkholderia phytofirmans* PsJN have an important role in controlling *Rhizopus microsporus* (Martinez and Hertweck 2005) and increasing plants resistance to environmental stress (Compant et al. 2005). Also, *Pseudomonas fluorescens* has also been developed and commercially used by farmers to manage several plant pathogens such as *Pyricularia oryzae* and *Rhizoctonia solani* (Reddy and Reddy 2009). Based on the explanation above, this finding confirms that commensal bacteria inhabit the phyllosphere environment. This finding also confirms that healthy rice plants might be harbored by commensal phyllosphere bacteria increasing the rice plants fitness.

The unique bacteria groups were only found on certain samples such as Flavobacteriales and Enterobacteriales groups from SKBV and SKBG samples,

Campylobactericeae, Bacteroidetes, Deltaproteobacteria and Chloroflexales groups from JSNG sample. The relative abundance of those unique bacteria groups is interestingly lower than that of other groups, such as Betaproteobacteria and Pseudomonadales. Accordance to Knief et al. (2011), the relative abundance of Deltaproteobacteria and Chloroflexales groups on phyllosphere environment of rice variety IR-72 are 1.6% and 0.6%, respectively. Moreover, this result also confirms that geographical factors influence the bacteria community of rice phyllosphere environment. The rice variety of Ciherang that was planted in three different regions (Sukabumi, Jasinga, and Situgede) showed very different profiles of rice phyllosphere bacteria community. This had been proved by Finkel et al. (2011) who revealed that geographic factors are the factor significantly influencing epiphytic bacteria of *Tamarix* trees than plant species factors. Meanwhile, Knief et al. (2011) also declared that geographic factors play a more critical role than host species to determine epiphytic microbial composition.

In addition, the Epsilonproteobacteria and Clostridiales groups inhabit rice phyllosphere environment on the vegetative growth phase from three regions, while Sphingobacteria group only inhabits rice phyllosphere environment on the generative growth phase from Sukabumi and Jasinga. This result confirms that different growth phases of rice plant derived the shape of bacteria community in the phyllosphere environment. Lindow and Brandl (2003) state that bacterial population in young leaves comprises a higher number of taxa than old leaves. There are different morphological and physiological characters between vegetative and generative leaves of rice plants which also influence the bacteria community profiles. Costa et al. (2006) revealed that anatomical and physiological characteristics of rice leaf surface and its physiochemical environment properly affect the diversity and density of rice phyllosphere bacteria.

Species richness (the number of species within a community) and species evenness (the sizes of species populations within a community) are two essential parameters for defining community structure and diversity. In this present study, bacteria diversity of metagenomic approach is generally higher than cultivation-dependent approach. As explained before, the number of bacteria that could be cultured is only 1% of the total bacteria in the environment. Therefore, the metagenomic approach is an appropriate method for revealing the bacteria community in many environments. The highest bacteria diversity was found on sample SKB vegetative and JSN generative with a metagenomic approach, i.e., 1.73 and 1.72. The bacteria diversity and evenness are very fluctuating between metagenomic approach as well as cultivation-dependent approach. This finding also explains that the influence of geographical and environmental factors are more significant than the growth phase factor to the bacteria diversity and evenness. This present study provided information about the rice phyllosphere bacteria diversity from Indonesia and several factors that influence the diversity. Diverse bacteria that were found from rice phyllosphere of cultivar Ciherang might contribute to the plant fitness. As previously explained, the samples of healthy rice plant were obtained from rice field infected by blast disease.

We concluded that terminal restriction fragment length polymorphism is proved to be an effective method for revealing the rice phyllosphere bacteria from three regions of West Java, i.e., Sukabumi, Jasinga, and Situgede. The use of *the Msp*I restriction enzyme is more significant than *Bst*UI because *Msp*I generates more TRFs and OTU (operational taxonomical unit). This study has successfully revealed that the rice phyllosphere environment cultivar of Ciherang is dominantly inhabited by Betaproteobacteria, Pseudomonadales, Alphaproteobacteria and Gammaproteobacteria groups with diverse relative abundance. Furthermore, geographical factors are identified as having more influence than plant growth phase to the phyllosphere bacteria diversity from those three regions.

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Effect of the CDC light trap on control of nocturnal mosquitoes in coastal Samut Songkhram Province, Thailand

TANAWAT CHAIPHONGPACHARA[1,♥], SEDTHAPONG LAOJUN[2], CHAEKKI KUNPHICHAYADECHA[2]

[1]College of Allied Health Science, Suan Sunandha Rajabhat University, Samut Songkhram 75000, Thailand. Tel./fax. +66-835-865775,
♥email: tanawat.ch@ssru.ac.th

[2] Bachelor of Public Health, College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram 75000, Thailand.

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Abstract. *Chaiphongpachara T, Laojun S, Kunphichayadecha C. 2018. Effect of the CDC light trap on control of nocturnal mosquitoes in coastal Samut Songkhram Province, Thailand. Biodiversitas 19: 1750-1754.* This study aimed to investigate the effect of CDC light trap on mosquito control and to study the relationship between this effect and weather factors in coastal areas (2 and 4 km from the sea) of Samut Songkhram Province, Thailand. We conducted a field test by trapping for 30 consecutive days from September to October 2017. The trap was hung at a height of 1.5 m and was 50 m away from a house. A total of 2963 adult female mosquitoes of 4 species belonging to 2 genera were trapped, including *Anopheles epiroticus* Linton & Harbach, *Culex quinquefasciatus* Say, *Cx. sitiens* Wiedmann and *Cx. gelidus* Theobald. The trapping rate of the CDC light trap set up 2 km from the sea was 85.70±73.81 adult mosquitoes per night. Meanwhile, at the location 4 km from the sea, the trap collected 13.07±11.40 adult mosquitoes per night. Comparing the numbers of mosquitoes captured by the CDC light trap between these two sites, there was a significant difference at $p < 0.05$. This study shows that the CDC light trap can be used for effective control of mosquitoes in coastal areas of Samut Songkhram Province, Thailand, especially *Cx. sitiens*, a filariasis vector.

Keywords: CDC light trap, coastal area, effect, nocturnal mosquitoes

INTRODUCTION

Mosquitoes are small insects with infectious effects on public health (Mint Mohamed Lemine et al. 2017; Chaiphongpachara et al. 2017). Currently, there are approximately 3500 species of mosquito around the world; each species of mosquito can carry different diseases (Diniz et al. 2017). According to a report from the World Health Organization (WHO), patients with mosquito-borne diseases make up 17% of all infectious disease patients worldwide (World Health Organization 2014). Therefore, the WHO has focused on the control of communicable diseases caused by mosquitoes, especially in tropical and subtropical countries (Mint Mohamed Lemine et al. 2017). Most mosquito-borne human diseases, such as malaria, Japanese encephalitis, and filariasis, are caused by nocturnal mosquitoes (Li et al. 2016).

At least 412 species of mosquitoes are found in Thailand (Pengsakul et al. 2017), including 3 genera that are medically important nocturnal vectors: *Anopheles*, a malaria vector; *Culex,* a Japanese encephalitis, and filariasis vector; and *Mansonia,* a filariasis vector (Baxter et al. 2017). In 2017, the Bureau of Epidemiology, Thailand reported 5273 malaria cases, 14 Japanese encephalitis cases and 4 filariasis cases (Ministry of Public Health 2017). Each species of mosquito has different behavior and breeding sites (Irish 2014). Different methods for the control of mosquito vectors are suitable for different areas depending on the mosquito species (Benelli et al. 2016).

Samut Songkhram is a province with abundant natural resources located on the Gulf of Thailand in central Thailand. In this province, there are coastal areas that differ from other provinces, with distinct plants and animals including species of mosquito. Coastal areas in Thailand are often inhabited by *Anopheles epiroticus* Linton & Harbach and *Culex sitiens* Weidemann because of their tolerance of saline areas (Chaiphongpachara and Sumruayphol 2017). There has been little research on control of mosquitoes in coastal areas of Thailand, which may be a major obstacle in controlling mosquito-borne disease in Samut Songkhram province.

The mosquito trap is a popular tool for vector control and is highly effective in reducing the number of nocturnal mosquitoes (Poulin et al. 2017). Currently, there are many traps available for use in catching mosquitoes, which can be used to break the mosquito life cycle by focusing on the adult mosquitoes (Beck-Johnson et al. 2017). The Centers for Disease Control and Prevention light trap, or CDC light trap, is one popular model of mosquito trap (Li et al. 2016). The operation of the CDC light trap is based on dry ice, which evaporates into carbon dioxide, an attractant for female mosquitoes and a fan sucks the attracted mosquitoes into a mesh bag (Aak et al. 2017). The advantages of the CDC light trap include its small size, portability, ease of use, and previous findings that it is highly effective at attracting mosquitos (Li et al. 2016). However, the effectiveness of the CDC light trap depends on the climate in the area. Weather conditions affect almost every organism in the environment. Mosquitoes can adapt their behavior during unfavorable weather conditions by

reducing activities such as aviation (Ramasamy and Surendran 2012).

Thus, this study aims to investigate the effect of the CDC light trap and the relationship between this effect and weather factors in coastal areas (2 and 4 km from the sea) of Samut Songkhram Province, Thailand. The result of this research provides important guidance for the control and surveillance of mosquito vectors in coastal areas in Thailand.

MATERIALS AND METHODS

Study area

This research consisted of a field experiment to study the effect of the CDC light trap on nocturnal mosquito vectors in coastal areas of Samut Songkhram Province, Thailand. The coastal areas of this study consisted of 2 locations with different environments according to the distance from the sea, being 2 and 4 km. The coastal area 2 km from the sea (13° 25' 11.7" N, 100° 02' 21.0" E) is characterized by low population density. The surrounding area is a mangrove area, and there are saline water sources and salt ponds scattered in the area. Therefore, mosquitoes in this area are species with habitat in coastal areas. In contrast, the coastal area 4 km from the sea (13° 24' 33.6" N, 100° 00' 53.0" E) is characterized by high population density, semi-urban, with both saline water sources and wastewater sources (Figure. 1). Average weather conditions (climate) in our study area including coastal areas of Samut Songkhram province during September to October 2017 were 0.94 mm ± 2.05 of rainfall, 27.50°C ± 0.93 of temperature, 3.79 km/hr ± 1.62 of wind speed and 84.72% ± 5.81 of relative humidity.

Study on effect of CDC light trap on mosquito vectors in coastal areas

In this study, we used the Centers for Disease Control, and Prevention miniature light traps (CDC-LT) baited with CO_2 (John W. Hock Co., Gainesville, Florida) with 1 trap per location to test the effect of the trap on reducing the number of mosquito vectors in the coastal areas. We conducted a field test by trapping from 6: 00 p.m. to 6: 00 a.m. for 30 consecutive days from September to October 2017. The trap was hung at a height of 1.5 m and 50 m away from the nearest houses. Every morning, we collected the trapped samples, recorded details and sent the samples to the laboratory at the College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram Education Center. After that, the nocturnal mosquito samples were identified under a Nikon AZ 100 M stereo-microscope (Nikon Corp., Tokyo, Japan) with the aid of the Illustrated Keys to the Mosquitoes of Thailand (Rattanarithikul et al. 2010).

Study of the relationships among the effect of the CDC light trap, mosquito species and weather factors.

To study the relationship between the effect of the CDC light trap and weather factors, we received the weather information for Samut Songkhram province from September to October 2017 from the Samut Songkhram Provincial Meteorological Department, including rainfall data, temperature, wind speed and relative humidity. After receiving the data, we statistically analyzed the relationships between the total number of mosquitoes trapped and weather factors in both locations comprising of 2 km and 4 km.

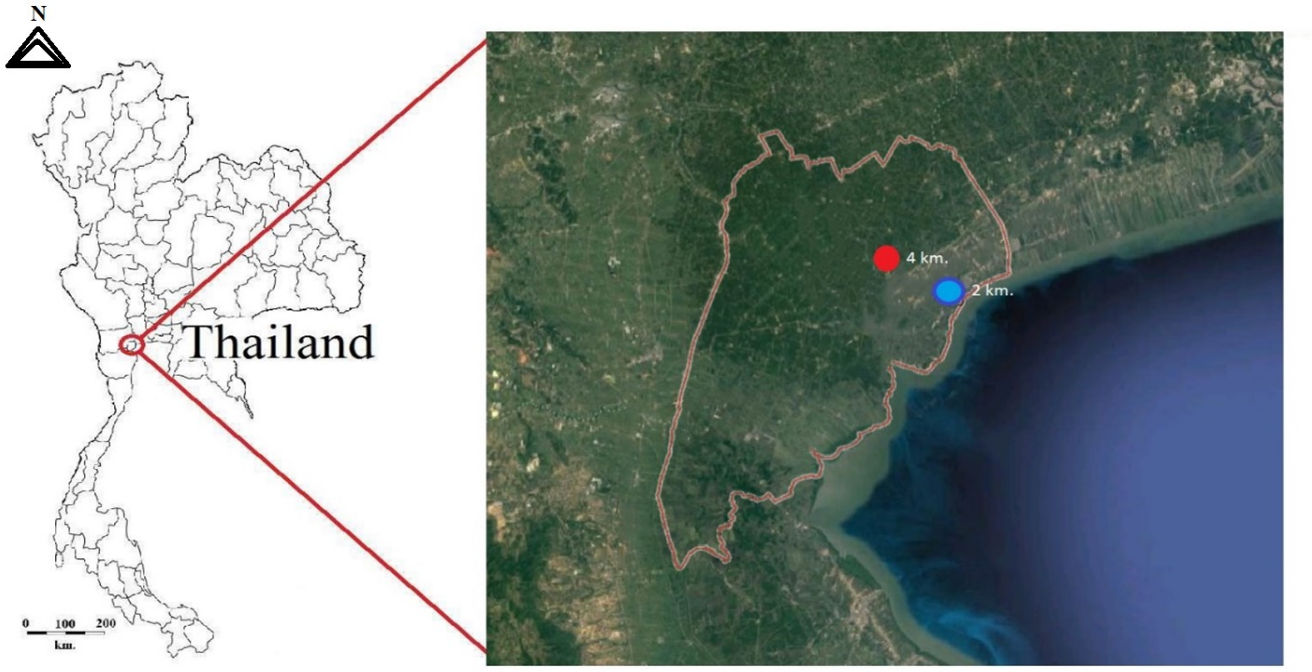

Figure 1. Study areas in coastal areas of Samut Songkhram Province, Thailand. Blue color = area 2 km from the sea, and red color = area 4 km from the sea.

Data analysis

The results of the field testing of the CDC light trap in both coastal locations (2 and 4 km from the sea) are presented as mean values with standard deviations. A t-test was used to compare the effects of the CDC light trap between the two locations. Meanwhile, the relationships between the number of collected mosquitoes, species of vectors in each area and weather factors were analyzed by Pearson correlation.

RESULTS AND DISCUSSION

Effect of CDC light trap on mosquito vectors in coastal areas

Mosquito trapping for 30 consecutive days resulted in a total 2963 trapped adult female mosquitoes within 4 species belonging to 2 genera, including *Anopheles epiroticus* Linton & Harbach, *Culex quinquefasciatus* Say, *Cx. sitiens* Wiedmann and *Cx. gelidus* Theobald (Table 1). The CDC light trap in the location 2 km from the sea captured 2571 adult mosquitoes (85.70 ± 73.81 per night), of which the largest proportion was *Cx. sitiens* with 2499 mosquitoes (83.3 ± 73.96 per night) and the lowest was *Cx. quinquefasciatus* with 6 mosquitoes (0.2 ± 0.41 per night). At the location 4 km from the sea, 392 adult mosquitoes were collected (13.07 ± 11.40 per night). *Cx. sitien*s again represented the highest proportion, with 259 mosquitoes (8.63 ± 10.08 mosquitoes per night), and the lowest was *Cx. gelidus* with 22 mosquitoes (0.73 ± 3.37 mosquitoes per night). Comparing the number of mosquitoes captured by the CDC light trap between the two locations, there was a significant difference at $p < 0.05$ (Table 1).

Relationship between effect of CDC light trap, mosquito species and weather factors

The analysis of the relationship between the total number of mosquitoes trapped and weather factors in the location 2 km from the sea was significantly correlated with temperature (negative correlation) ($p < 0.05$). In contrast, for the site 4 km from the sea, the effect of the CDC light trap was significantly correlated with rain and relative humidity (positive correlation) ($p < 0.05$) (Table 2).

At the species level, nine significant relationships between the number of individuals captured and weather conditions were identified. At the location 2 km from the sea, capture of *An. epiroticus* was related to individuals captured with rain (negative correlation), wind speed (positive correlation) and relative humidity (negative correlation). *Cx. sitiens* were also positively related to individuals captured with temperature. While at the other location 4 km from the sea, we found that capture rates for *An. epiroticus* and *Cx. sitiens* were also related to weather factors, with *An. epiroticus* capture was associated with rain (negative correlation), wind speed (positive correlation) and relative humidity (negative correlation) and *Cx. sitiens* capture was associated with humidity (positive correlation) and rain (positive correlation) ($p < 0.05$) (Table 3).

Table 1. Effect of CDC light trap in coastal areas

Location	Species of mosquito	n	Mean ± S.D. (mosquitoes/night)
2 km from the sea	*An. epiroticus*	66	2.20 ± 2.44
	Cx. quinquefasciatus	6	0.20 ± 0.41
	Cx. sitiens	2499	83.30 ± 73.96
	Total	2571	85.70 ± 73.81[a]
4 km from the sea	*An. epiroticus*	30	1.00 ± 2.35
	Cx. quinquefasciatus	81	2.70 ± 1.90
	Cx. sitiens	259	8.63 ± 10.08
	Cx. gelidus	22	0.73 ± 3.37
	Total	392	13.07 ± 11.40[b]

Note: * Comparison of the effects of CDC light trap (2 vs. 4 km from the sea): Different letters indicate significantly different at $p < 0.05$ by t-test.

Table 2. Relationship between effect of CDC light traps and weather factors

Locations		Rain	Temperature	Wind speed	Relative humidity
2 km from the sea	r	.306	-4.20*	-213	.306
	p	.100	.021	.257	.100
4 km from the sea.	r	.385*	-.256	-.200	.385*
	p	.036	.172	.290	.036

Note: *: Correlation is significant at the 0.05 level (2-tailed)

Table 3. Relationship between mosquito species and weather factors

Locations	Species of mosquito		Rain	Temp.	Wind speed	Relative humidity
2 km from the sea	*An. epiroticus*	r	-.421*	.308	.362*	-.421*
		p	.0.20	.079	.049	.020
	Cx.quinquefasciatus	r	.273	-.216	-.324	.273
		p	.144	.164	.081	.144
	Cx. sitiens	r	.280	.357*	-.213	-.280
		p	135	.041	.285	.135
4 km from the sea	*An. epiroticus*	r	-.372*	.256	.635**	-.372*
		p	.043	.172	.000	.043
	Cx.quinquefasciatus	r	.188	-.349	-.239	.188
		p	.319	.059	.203	.319
	Cx. sitiens	r	.473**	-.311	-.342	.473**
		p	.008	.095	.064	.008
	Cx. gelidus	r	.133	-.323	-.159	.133
		p	.482	.082	.403	.482

Note: **. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).

Discussion

Effect of CDC light trap on mosquito vectors

The mosquito species included in Table 3 are all disease vectors: *An. epiroticus* is a secondary malaria vector, *Cx. sitiens* is a filariasis vector, *Cx. quinquefasciatus* is a Japanese encephalitis and filariasis vector, and *Cx. gelidus* is a Japanese encephalitis vector. *An. epiroticus* and *Cx.*

sitiens is brackish mosquito. In Rayong province, Thailand, there have been some reports of malaria parasite infections detected in *An. epiroticus* (Sumruayphol et al. 2010). While, *Cx. gelidus* is found in flooding areas, fresh water, and fertile areas and the number of the mosquitoes was high during hot and wet, and hot and dry seasons (Ramesh et al. 2015).

In the area of 2 km from the sea, 3 species of mosquito were found: *An. epiroticus*, *Cx. sitiens* and *Cx. quinquefasciatus*. At the location 4 km from the sea, we found another species called *Cx. gelidus.* We did not see this species of mosquito in the area of 2 km from the sea because the water is mostly brackish and sea water throughout the area. The area is different from the area 4 km from the sea which is mostly fresh water. For *Cx. quinquefasciatus*, we found the lowest number of this *Culex* species in 2 km from the sea and the low number in 4 km from the sea because the species were found in households and their breeding habitat is wastewater from households. In addition, these species were rare in our study area (Pennington, Prager, Walton, & Trumble, 2016). These results indicate that the different environments between the two locations affect the species diversity and density of mosquitoes in each area. This is consistent with the research of Chaiphongpachara and Sumruayphol (2017), which was conducted in the coastal areas of Samut Songkram province and found 3 species of mosquito vector in the area of 2 km from the sea and 4 species in the area of 4 km from the sea. However, it is possible that the CDC light trap is specific to *Cx. sitiens* mosquito because previous research used black light traps in this area and showed that the number of *Cx. quinquefasciatus* was higher than that of *Cx. sitiens* (Chaiphongpachara and Sumruayphol 2017).

In this study, we found that the CDC light trap is effective at trapping *Culex* spp, especially *Cx. sitiens,* at both coastal locations, with 83.3 ± 73.96 mosquitoes trapped per night at the site 2 km from the sea and 8.63 ± 10.08 at the site 4 km from the sea. This is consistent with the study of Sriwichai et al. (2015) on the effectiveness of the CDC light trap at the Thai-Burmese border and found that this trap can catch most *Culex* mosquitoes, representing 46.39 percent of the total number of mosquitoes trapped. The effect of the CDC light trap varied between the 2 studied areas of Samut Songkhram province; the trap at the site 2 km from the sea captured significantly more mosquitoes than the trap 4 km from the sea ($p < 0.05$). The CDC light trap is highly effective in the area of 2 km from the sea, because this area is filled with saline water sources and salt ponds scattered throughout, which results in a high density of *Cx. sitiens* populations (Chaiphongpachara and Sumruayphol 2017). This is consistent with previous studies that CO_2, the attractant used in the CDC light trap, can effectively attract the species. CO_2 comes from exhalation of human and animals and can attract targets of female mosquitoes to take blood of the targets. Currently, there are many traps that CO_2 is used as bait which an excellent and environmentally friendly odor for controlling mosquito vectors and other insects (McMeniman et al. 2014).

Relationship between CDC light trap and weather factors.

In this analysis, we found a significant positive correlation between total number of mosquitoes trapped and relative humidity and rain in the area of 4 km from the sea ($p < 0.05$). High humidity, especially rain, could increase the mosquito survival (Chuang et al. 2011). The relationship may come from oviposition behavior of female mosquitoes. In fact, previous research has reported the positive relationship between *Cx. nigripalpus* and high relative humidity in Indian River County, Florida (Day et al. 1990).

After analyzing the relationship at the species level, we found that both areas including 2 and 4 km from the sea were very similar. *An. epiroticus* captures were associated with rain (negative correlation), wind speed (positive correlation) and relative humidity (negative correlation). Rain is a major factor in the flight of mosquitoes, so the negative relationship that was found is not surprising. Previous research has reported that the slow flight-speed of mosquitoes is associated with rain (Dickerson et al. 2012). However, it is surprising that wind speed is positively correlated with the number of *An. epiroticus* because wind speed affects host-seeking activities and flight direction of mosquito (Chuang et al. 2011). The reason for this positive correlation may be associated with trap placement. In this study, to avoid damaging the trap, we installed traps under a house's roof. Meanwhile, the result of negative correlation of relative humidity was consistent with Bashar and Tuno (2014) which showed strong negative correlation between relative humidity and abundance of *An. karwari*, *An. minimus* s.l., *An. annularis*, and *An. jeyporiensis* in Bangladesh.

Cx. sitiens captures in 2 km from the sea were associated with temperature (positive correlation) and in the captures, in the area, 4 km from the sea were associated with rain (positive correlation)and relative humidity (positive correlation). Previous studies have reported that rain, wind speed and relative humidity affect the flight behavior of female mosquitoes (Rowley and Graham 1968; Tran et al. 2013). Normally, blood feeding of female mosquitoes is associated with weather (Chuang et al. 2011). However, these results have revealed a positive correlation with the rain effect including rainfall, temperature and relative humidity on *Cx. sitiens*, which may be the specific stimulus on this species of mosquito. Another possible reason is the oviposition behavior of female mosquito, which such a relationship has been reported.

In conclusion, this study showed that the CDC light trap can be an effective tool to control mosquitoes, especially *Cx. sitiens* as a filariasis vector, in coastal areas of Samut Songkhram Province, Thailand. The mosquito is predominant species in coastal areas. Therefore, the use of this trap to control the vectors, especially near the sea which is *Cx. sitiens* habitat, is appropriate.

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Short Communication: Biological aspects of *Charybdis anisodon* (De Haan, 1850) in Lasongko Bay, Central Buton, Southeast Sulawesi, Indonesia

ABDUL HAMID[1,♥], YUSLI WARDIATNO[2,3♥♥]

[1]Department of Aquatic Resources Management, Faculty of Fisheries and Marine Sciences, Halu Oleo University, Kampus Hijau Bumi Tridharma Anduonohu, Kendari 93232, Indonesia, ♥email: abdhamid_lamun@yahoo.com

[2]Department of Aquatic Resources Management, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University (IPB), Kampus IPB Darmaga, Bogor 16680, Indonesia, ♥♥email: yusli@ipb.ac.id

[3]Center for Coastal and Marine Resources Studies, Bogor Agricultural University (IPB), Kampus IPB Baranangsiang, Bogor 16143, Indonesia

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Abstract. *Hamid A, Wardiatno Y. 2018. Short Communication*: *Biological aspects of* Charybdis anisodon (*De Haan, 1850) in Lasongko Bay, Central Buton, Southeast Sulawesi, Indonesia*. *Biodiversitas 19*: *1755-1762.* Data on the biological aspects of *Charybdis anisodon* (De Haan, 1850) were still very limited. This study was aimed to determine the distribution of size, growth type, sex ratio and spawning season of *C. anisodon* in Lasongko Bay, Central Buton, Southeast Sulawesi. Crab collection was conducted from May 2013 to March 2014 using a crab gillnet. The carapace width of males and females *C. anisodon* ranged between 3.05-7.61 cm and 3.45-7.98 cm, respectively. Mann-Whitney test showed that width and length of carapace of males and females were significantly different ($p<0.05$). Type of growth of carapace width-body weight male and female were both allometric negative. Carapace length-body weight relationships of the males was isometric, but it was allometric negative for females. Spatially and temporally, the sex ratio of *C. anisodon* showed a variation, and the total sex ratio was 1: 0.38. The spawning season of *C. anisodon* tend to occur throughout the year.

Keywords: Allometric relationship, crustacea, growth type, sex ratio, spawning season, two-spined arm swimming crab

INTRODUCTION

Charybdis anisodon (De Haan, 1850) spreads across the Indo-West Pacific to Africa including Hawaii, Australia, New Zealand, Papua New Guinea, Indonesia, Philippines, Malaysia, Singapore, Thailand, Vietnam, Japan, India, China, Red Sea, Madagascar, Mayotte and Tanzania (Ng 1998; Chung 2002; Chande and Mgaya 2003; Kunsook and Dumrongrojwatthana 2017; GBIF 2018). The crab prefers waters with muddy substrate and can still be found to a depth of 25 m (Ng 1998, Chung 2002). This crab is edible and has economic value (Ng 1998, Santhanam 2018), but the value is lower than *Portunus pelagicus*.

From biological point of view, information on the size distribution, the relationships between carapace sizes and body weight, sex ratio, first-sex maturity and spawning season are needed in sustainable crab fisheries management (Kamrani et al. 2010; Ikhwanuddin et al. 2012, Hamid et al 2016b, 2017). Researches on biological aspect of the genus *Charybdis* have been carried out on *C. natator* (Sumpton 1990; Sallam and Gab-Alla 2010; Kannathasan and Rajendran 2011; Vidhya 2016), *C. affinis* (Chu 1999), *C. hellerii* (Mantelatto and Garcia 2001, Sant'Anna et al., 2012, Ferry et al., 2017), *C. bimaculata* (Doi et al. 2010), *C. feriatus* (Dineshbabu 2011; Nieves et al., 2015) and *C. japonica* (Fowler 2011; Wong. 2013), but it is lacking in *C. anisodon*.

The research on crustacea in Lasongko Bay regarding to the stock of shrimp and crab in general has been conducted by Supardan (2006). More researches in the bay were focused on the blue swimming crab (*P. pelagicus*) biology, i.e. reproductive biology (Hamid et al. 2015, 2016a, b, c), population dynamics, stock and management of the blue swimming crab (Hamid and Wardiatno 2015; Hamid et al. 2016d, 2017). The two-spined arm swimming crab, *C. anisodon* in Lasongko Bay is one of the bycatch species of blue swimming crab fishery with local economic value. This study was aimed to determine some biological aspects of *C. anisodon* including size distribution, growth type, sex ratio, and spawning season.

MATERIALS AND METHODS

Location and study period

The study was conducted from May 2013 to March 2014 at Lasongko Bay of Central Buton District, Indonesia with position 05°15'-05°27' S and 122°27'-122° 33'E. The sampling location of *C. anisodon* covered the head of the bay to the center of the bay and consisted of six stations (Figure 1).

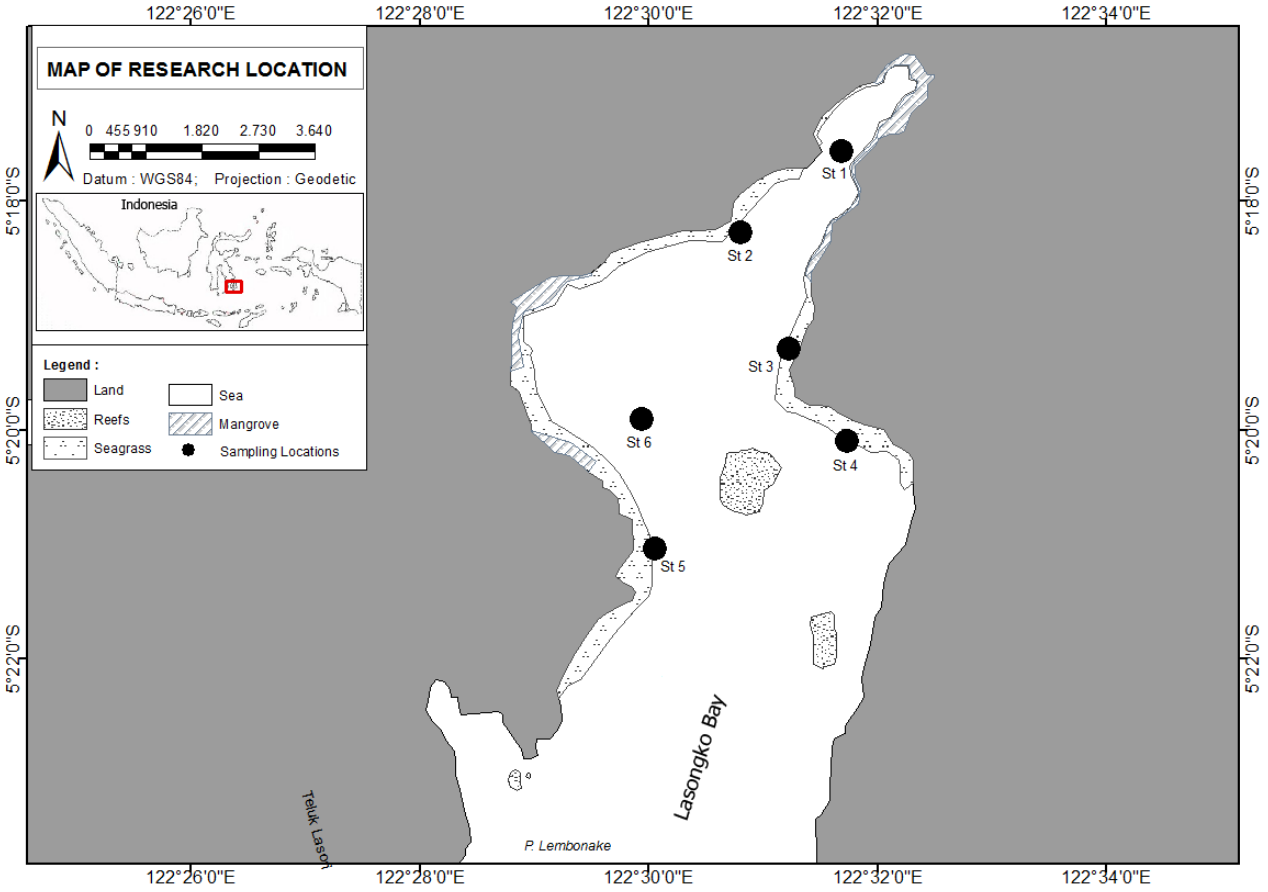

Figure 1. Location map and research station in Lasongko Bay (adapted from Hamid and Wardiatno 2015)

Sampling

Sampling of *C. anisodon* was conducted once a month on each station using gillnet. Gillnets were set at each station in the afternoon and hauled in the morning of the next day. The collected crabs were separated according to station, sex and counted. The carapace width and length were measured with calipers (Vernier Caliper with precision 0.05 mm), and the weight was weighed with a digital scale (Xon Med Digital Scale) with a precision of 0.01 g.

Determination of Size Class Distribution

The data of carapace width of male and female *C. anisodon* were tabulated and presented with the size class distribution with the width of the class of 0.5 cm. The carapace width and length between males and females totally were tested with Mann-Whitney-test at p = 0.05 (Steel and Torrie 1992) to determine the size difference between sexes.

Determination of Carapace Width-Body Weight and Carapace Length-Body Weight Relationships

The carapace width or carapace length with body weight relationship of *C. anisodon* were determined by the power and linear equations (Hartnoll 1978; Josileen 2011; Hamid 2015), i.e. by the equation as follows:

$$Bw = aCW^b \quad \text{..... (1)}$$

Bw = body weight (g), CW = carapace width (cm) or carapace length (cm), a = intercept and b = growth coefficient. To simplify calculation, the equation (1) was transformed to $\log_{10}$ to obtain linear equations as follows:

$$\text{Log } Bw = \log a + b \log CW \quad \text{..... (2)}$$

To determine the growth type of allometric relationships between carapace width/length and body weight of *C. anisodon*, the test of b value equal to 3 was performed with t-test at p = 0.05 (Steel and Torrie 1992). If the b = 3 it is isometric, if the value of b <3 it is negative allometric, and if the b> 3 is positive allometric (Hartnoll 1978; Josileen 2011).

Determination of sex ratio and spawning season

The sex ratio of *C. anisodon* is the ratio of the number of females to the number of males caught on each station and sampling period. Sex ratio of *C. anisodon* was calculated by the following equation:

$$\text{Sex ratio} = \frac{\sum \text{Female}}{\sum \text{Male}} \quad \text{..... (3)}$$

The total sex ratios of *C. anisodon* was tested by chi-square test ($\chi 2$) at p = 0.05 (Steel and Torrie 1992) to determine of sex ratio 1: 1. The spawning season of *C. anisodon* was determined based on the presence of ovigerous female (Sukumaran 1995; Karmani et al. 2010; Hamid et al. 2015; Ernawati et al. 2017).

RESULTS AND DISCUSSION

The number and carapace size

The number of male *C. anisodon* ranged between 6 and 35 individual while the female ranged from 1 to 16 individuals per sampling period. The total number of male caught at each station ranged between 3 and 120 individuals and the female ranged between 1 and 51 individuals. The samples were mostly caught in stations 1 and 2, but it was rarely found in stations 3, 4, 5 and 6. In terms of sampling period, the crabs were mostly caught in January 2014, but was hardly found in September and June 2013 (Table 1).

The carapace width and length of the male ranged between 3.05 and 7.61 cm and between 2.81 and 4.61 cm, respectively. While the carapace width and length of the female ranged between 3.45 and 7.98 cm and between 1.96 and 3.93, respectively. The average of carapace width and length of both sexes of *C. anisodan* was listed in Table 2. Mann-Whitney test of carapace width and length of *C. anisodon* showed a significantly different ($p<0.05$) between males and females (Table 2). Body size (carapace width and length) of male was larger than females, and this is identical to those found in some other species of *Charybdaris*, such as *C. feriatus* (Dineshbabu 2011; Dash et al. 2014), *C. natator* (Sumpton 1998; Sallam and Gab-Alla 2010; Vidhya 2016), *C. bimaculata* (Doi et al. 2008), *C. hellerii* (Ozcan et al. 2010; Sant'Anna et al. 2012; Ferry et al. 2017) and *C. japonica* (Fowler 2011; Wong 2013).

Among the species of the genus *Charybdis*, *C. anisodon*, *C. hellerii* and *C. japonica* are categorized as small-sized species while *C. natator*, *C. bimaculta* and *C. feriatus* are the large-sized ones. The carapace width of male *C. hellerii* ranged between 2.5 and 7.8 cm and female ranged between 1.73 and 6.50 cm (Ozcan et al. 2010; Bolanos et al. 2012; Sant'Anna et al. 2012; Ferry et al. 2017), carapace width of male *C. japonica* ranged between 2.8 and 10.37cm and female ranged between 2.8 and 9.9 cm (Fowler 2011; Wong 2013). The maximum carapace width of *C. feriatus* and *C. natator* reached 20 cm and 17 cm, respectively (Ng 1998), and carapace width of the male *C. bimaculata* ranged between 5.35 and 24.14 cm and the female ranged between 4.94 and 20.75 cm (Doi et al. 2008).

In Lasongko Bay, the male *C. anisodon* was dominated by the 5.09-5.59 cm carapace width class size, while the female was dominated by the 4.07-4.57 cm carapace width class size (Figure 2). The carapace width class size distribution of male and female *C. hellerii* in Martinique, French was dominated by the 3.70-3.95 cm class size (Ferry et al. 2017), while Ozcan et al. (2010) found the dominant class size was 6.1-7.0 cm in males and 5.1-6.0 cm in female for the same species in Iskenderun Bay, Turkish.

Table 1. The number of *C. anisodon* caught on each station during the study in Lasongko Bay, Central Buton, Southeast Sulawesi

Collection date	Number of crab on each station and sex (individual)											
	1		2		3		4		5		6	
	M	F	M	F	M	F	M	F	M	F	M	F
May 2013	3		7	2			2					1
June 2013	4	4					1		1			
July 2013	16	2	2									
Augusts 2013	13	3							1			
September 2013			6	1								
October 2013	15	1	1		2	1		3				
November 2013	13	15	1	1								
December 2013	16	3										
January 2014	18	9	11		2		1		2		1	
February 2014	13	10	1		1		1			6	3	
March 2014	9	4	6				2				2	

Note: M = Male F = Female

Table 2. The Carapace size of male and female *C. anisodon* in Lasongko Bay, Central Buton, Southeast Sulawesi

Station	Carapace width (cm)		Carapace length (cm)	
	Male	Female	Male	Female
1	5.18±0.56	4.53±0.66	2.97±0.30	3.49±0.32
2	5.57±0.78	4.57±0.45	3.17±0.49	2.52±0.23
3	5.63±1.02	4.66	3.18±0.56	2.52
4	5.65±1.01	4.61±0.18	3.39±0.41	2.55±0.14
5	6.10±0.69	4.91±0.32	3.50±0.50	2.71±0.15
6	6.24±1.19	3.45	3.62±0.70	1.97
Average	5.34±0.71a	4.56±0.62b	3.06±0.40a	2.51±0.30b

Note: a and b are significantly different at $p<0.05$.

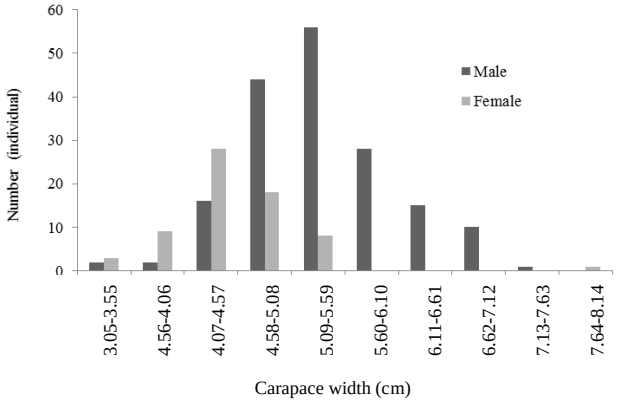

Figure 2. The carapace width class size distribution of *C. anisodon* in Lasongko Bay, Central Buton, Southeast Sulawesi

Carapace width/length-body weight relationships

The analysis results of the relationships of carapace width and carapace length with the body weight of male and female *C. anisodies* can be seen in Table 3 and Figure 3. The r-values of the relationships between the carapace width and the carapace length with body weight the male were 0.887 and 0.949, respectively; while in the female they were of 0.835 and 0.858, respectively. All relationships were very strong and positive. The carapace width-body weight relationship of male and female *C.*

anisodies showed allometric negative (t-test, p<0.05) with b-value of 2.642 and 1.996, respectively. The carapace length-body weight of male *C. anisodon* relationships showed b value of 3.021, and the result of t-test of the value was not significantly different (p> 0.05) indicating isometric growth. Meanwhile, in the female *C. anisodon* the b value was 2.244 and t-test result of the value was significantly different (p <0.05) indicating negative allometric growth (Table 3).

Table 3. Carapare width/length-body weight relationship, correlation coefficient (r), t-test of b value and growth type of *C. anisodon* with linear equations in Lasongko Bay, Central Buton, Southeast Sulawesi

Relationship	Linear equations	r	t-test for b=3	Growth type
Carapace width- Body weight				
Male	LogBw = - 0.561 + 2.642logCW	0.887	9.396*	Negative Allometric
Female	LogBw = -0.244 + 1.996 logCW	0.835	5.522*	Negative Allometric
Carapace length- Body weight				
Male	LogBw = -0,111 + 3,021logCL	0.949	0.905^{ns}	Isometric
Female	LogBw = 0,162 + 2,244logCL	0.858	62.858*	Negative Allometric

Note: *significantly different (p<0.05) ns = not significantly different (p>0.05)

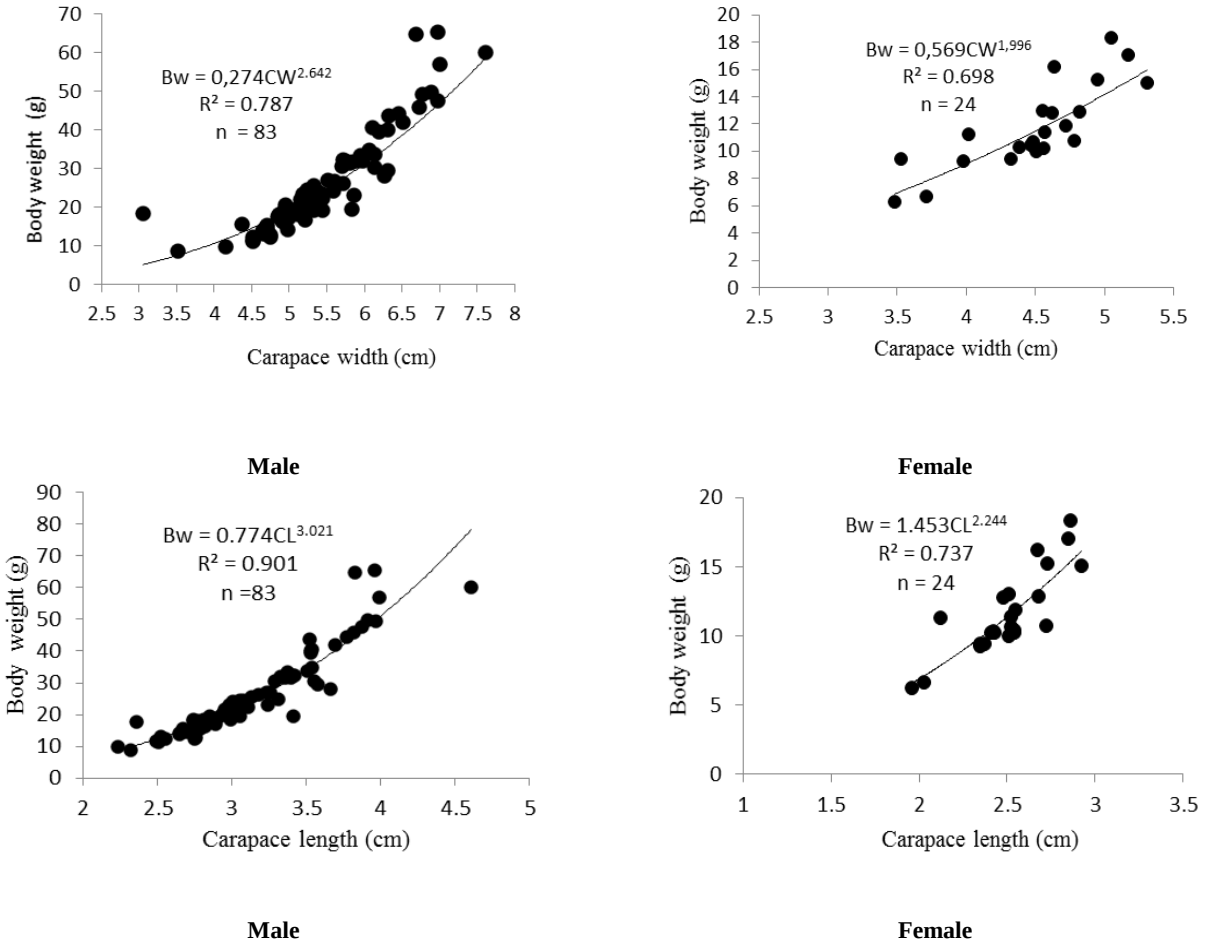

Figure 3. Carapace width/length-body weight relationship of male and female *C. anisodon* in Lasongko Bay, Central Buton, Southeast Sulawesi

Table 4. The b value and the growth type of some *Charybdis* species in different locations

Location	Spesies (Sex)	Relation	b value	Growth type	Source
Tokyo Bay, Japan	*C. bimaculata* (M)	CL-Bw	3.312	Positive Allometric	Doi et al. 2008
	C. bimaculata (M)	CL-Bw	3.038	Positive Allometric	
Suez Bay, Egypt	*C. natator* (M)	CW-Bw	2.9771	Negative Allometric	Sallam and Gab-Alla
	C. natator (F)	CW- Bw	3.064	Positive Allometric	2010
Mannar Bay, India	*C. natator* (M)	CW- Bw	3.387	Positive Allometric	Vidhya 2016
	C. natator (F)	CW- Bw	2.958	Isometric	
	C. natator (M)	CL- Bw	3.428	Positive Allometric	
	C. natator (F)	CL- Bw	2.939	Isometric	
Northeastern New Zealand	*C. japonica* (M	CW- Bw	3.1413	Positive Allometric	Fowler 2011
	C. japonica (F)	CW- Bw	3.2015	Isometric	
San Miguel Bay, Philippines	C. *feriatus* (M)	CW- Bw	2.83	Negative Allometric	Nieves et al. 2015a
	C. *feriatus* (F)	CW- Bw	2.73	Negative Allometric	
Karnataka, India	*C. feriatus* (M)	CW- Bw	3.078	Positive Allometric	Dineshbabu 2011
	C. feriatus (F)	CW- Bw	3.005	Positive Allometric	
Veraval, India	*C. feriatus* (M)	CW- Bw	2.94	Isometric	Dash et al. 2014
	C. feriatus (F)	CW- Bw	2.97	Isometric	
Lasongko Bay, Central Buton,	*C. anisodon* (M)	CW- Bw	2.642	Negative Allometric	This study
Southest Sulawesi,	*C. anisodon* (F)	CW- Bw	1.996	Negative Allometric	
Indonesia	*C. anisodon* (M)	CL- Bw	3.021	Isometric	
	C. anisodon (F)	CL- Bw	2.244	Negative Allometric	

Note: M = Male F = Female CW = Carapace width CL = Carapace length Bw = Weigth body

The value of b relationship of carapace width/length-body weight of male *C. anisodon* in this study was larger than that of females. This is identical to those of *C. natator* in Manar Bay, India (Vidhya 2016), and carapace width-body weigth relationships of *C. feriatus* in San Miguel Bay, Philippines (Nieves et al. 2015a) and in the coast of Karnataka, India (Dineshbabu et al. 2011), as well as the carapace length-body weight relationship of *C. bimaculata* in Tokyo Bay, Japan (Doi et al. 2008). The b-values the carapace width-body weight relationship in male and female *C. anisodon* were smaller than those in *C. natator*, *C. feriatus*, *C. hellerii* and *C. japonica* (see Table 4). The b-values of carapace length-body weight of male and female *C. anisodon* were also smaller than the b values of carapace length-weight *C. bimaculata* in Tokyo Bay, Japan (Doi et al. 2008) and *C. natator* in Manar Bay, India (Vidhya 2016). The growth type of carapace width-body weight relationship of male and female *C. anisodon* in this study was identical to that found at *C. feriatus* in San Miguel Bay, Philippines (Nieves et al. 2015a). The growth type of the carapace length-body weigth relationship of male and female in *C. bimaculata* of Tokyo Bay, Japan are both positive allometrics (Doi et al. 2008), and male *C. natator* in Manar Bay, India is positive allometric whereas in females is isometric (Vidhya 2016).

Sex ratio

The number of *C. anisodon* caught during the study was 243 individuals consisting of 176 males and 67 females. The sex ratio of *C. anisodon* at each station was varied, with the highest value was found at station 5 and lowest at station 2 (Table 5). The total sex ratio of *C. anisodon* was 1: 0.38, and the results of $\chi 2$-test was significantly different ($p<0.05$) with a ratio of 1: 1 or unbalanced between males and females.

Based on the sampling period, the number of male and female of *C. anisodon* was also varied (Table 6). The sex ratio of *C. anisodon* based on the sampling period ranged 1: 0.11 - 1: 1.14 with the highest was found in November 2013, and the lowest was in May 2013 (Table 6). Generally, the number of male *C. anisodon* was higher than the female, except for November 2013. Based on the total sex ratios, it seems that one male *C. anisodon* can fertilize as much as two to three females.

The total sex ratio of *C. anisodon* was generally smaller than those of some other species of *Charybdis* found in some waters in the world (Table 7), except for the sex ratio of *C. hellerii* found in Iskenderun Bay, Turkey (Ozcan et al. 2010) and in São Vicente, Brazil (Sant'Anna et al. 2015) were larger. Variation in sex ratio occurred also spatially and temporally in this study. Similar patterns were found in population of *P. pelagicus* in the Lasongko Bay (Hamid et al. 2016a) and in *C. natator* in Bengal Bay, India (Kannathasan and Rajendran 2011). The variation in sex ratio is probably due to spawning and feeding migration in the portunid groups, the different type of fishing gear used as well as the season (Kannathasan and Rajendran 2011; Hamid et al. 2016b), or due to the differences in age and growth rate (Doi et al. 2008).

Ovigerous females and spawning season

The number of ovigerous female of *C. anisodon* was 43 individuals or 64.18% of the total females found during the study. Ovigerous females *C. anisodon* were collected from stations 1, 2, 4 and 5 (Table 8), but majority were found at station 1 (32 individuals) in February 2014 (Figure 4). Ovigerous females *C. anisodon* generally could be found every month during the study, except for July. Carapace width of ovigerous females *C. anisodon* in Lasongko Bay ranged from 3.48 to 7.98 cm; whilst carapace lengths

ranged from 1.96 to 3.06 cm. The presence of ovigerous females indicates that the spawning season of *C. anisodon* in Lasongko Bay tend to to occur all year-round with three peaks, i.e. in February (highest), November and June (Figure 4).

The carapace width of ovigerous female *C. anisodon* found in this study was larger than that of ovigerous female *C. hellerii*, i.e. 3.82 cm (Ferry et al. 2017), but smaller than that of *C. japonica*, i.e. 6.25±0.24 cm (Wong 2013) and *C. natator*, i.e. 9.4 cm (Sumpton 1998).

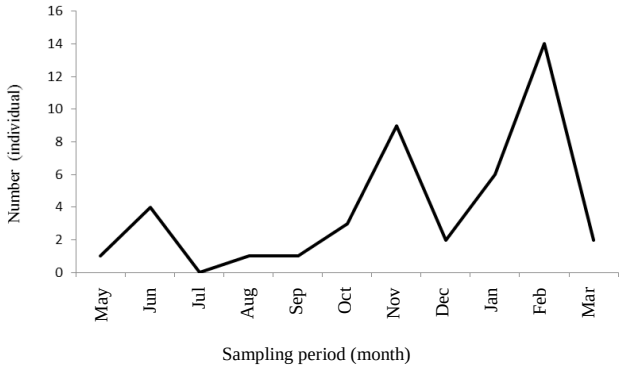

Figure 4. The presence of ovigerous females *C. anisodon* at each month in Lasongko Bay, Central Buton, Southeast Sulawesi

Table 5. Number and sex ratio of *C. anisodon* at each of station in Lasongko Bay, Central Buton, Southeast Sulawesi

Station	Number (individual)		Proportion (%)		Sex ratio Male: Female
	Male	Female	Male	Female	
1	120	51	70.2	29.8	1: 0.43
2	35	4	89.7	10.3	1: 0.11
3	5	1	83.3	16.7	1: 0.20
4	7	3	70.0	30.0	1: 0.43
5	3	7	30.0	70.0	1: 2.33
6	6	1	85.7	14.3	1: 0.17
Total	176	67	72.4	27.6	1: 0.38*

Note: * significantly different ($p<0.05$)

Table 6. Number and sex ratio of *C. anisodon* on every month in Lasongko Bay, Central Buton, Southeast Sulawesi

Month	Number of sample (individual)		Proportion (%)		Sex Ratio
	Male	Female	Male	Female	Male: Female
May 2013	12	3	80.0	20.0	1: 0.25
June 2013	6	4	60.0	40.0	1: 0.67
July 2013	18	2	90.0	10.0	1: 0.11
August 2013	13	4	76.5	23.5	1: 0.31
September 2013	6	1	85.7	14.3	1: 0.17
October 2013	18	5	78.3	21.7	1: 0.28
November 2013	14	16	46.7	53.3	1: 1.14
December 2013	16	3	84.2	15.8	1: 0.19
January 2014	35	9	79.5	20.5	1: 0.26
February 2014	19	16	54.3	45.7	1: 0.84
March 2014	19	4	82.6	17.4	1: 0.21
Total	176	67	72.4	27.6	1: 0.38*

Note: * significantly different ($p<0.05$)

Table 8. Time, caught location and carapace width of ovigerous female *C. anisodon* in Lasongko Bay, Central Buton, Southeast Sulawesi

Month	Caught location (station)	Carapace width (cm)
May 2013	2	4.25
June 2013	1	3.93-5.41
July 2013	-	-
August 2013	5	5.24
September 2013	1	4.32
October 2013	4	4.51-4.82
November 2013	1 dan 2	3.80-4.82
December 2013	1	4.62-4.88
January 2014	1	3.71-7.98
February 2014	1 dan 5	3.48-5.31
March 2014	1	3.96-4.02
Mean carapace width (cm)		4.62±0.70

Note: - = not found

Table 7. Sex ratio and spawning season of several species of *Charybdis* at different locations

Location	Species	Sex Ratio	Spawning Season	Source
Tokyo Bay, Jepang	*C. bimaculata*	1: 2.07	Not year-round	Doi et al. 2008
Mangalore, India	C. *feriatus*	1: 1	-	Babu et al. 2006
Karnataka, India	C. *feriatus*	1: 1	Year-round	Dineshbabu 2011
San Miguel Bay, Philippines	C. *feriatus*	1: 0.50	Year-round	Nieves et al. 2015b
Iskenderun Bay, Turkey	*C. hellerii*	1: 0.24	-	Ozcan et al. 2010
São Vicente, Brazil	*C. hellerii*	1: 0.32	Year-round	Sant'Anna et al. 2012
Caribia Sea, Venezuela	*C. hellerii*	1: 0.46	Year-round	Bolanos et al 2012
Martinique, French	*C. hellerii*	1: 0.42	-	Ferry et al. 2017
Northeastern New Zealand	*C. japonica*	1: 0.56	Not year-round	Fowler 2011
Northeastern New Zealand	*C. japonica*	1: 0.65	Not year-round	Wong 2013
Moreton Bay, Queensland	*C. natator*	1: 0.55	Year-round	Sumpton 1998
Suez Bay, Egypt	*C. natator*	1: 1.10	Year-round	Sallam & Gab-Alla 2010
Nagapattinam, India	*C. natator*	1: 1.01	-	Kannathasan & Rajendran 2011
Mannar Bay, India	*C. natator*	1: 0.62	Year-round	Vidhya 2016
Lasongko Bay, Indonesia	*C. anisodon*	1: 0.38	Year-round	This study

Note: - = no data

The peak spawning season of *C. natator* in Manar Bay, India takes place from December to February (Vidhya 2016), whereas the low spawning season occur in winter in Moreton Bay, Australia (Sumpton 1998). Spawning season of other *Charybdis* species occur throughout the year and some was only in certain months (Table 8). The *Charybdis* that spawn throughout the year are *C. natator* (Sumpton 1998, Sallam and Gab-Alla 2010; Vidhya 2016), *C. feriatus* (Dineshbabu 2011; Nieves et al. 2015b) and *C. hellerii* (Bolanos et al. 2012; Sant'Anna et al. 2012). While those which did not spawn throughout the year are *C. japonica* (Fowler 2011; Wong 2013) and *C. bimaculata* (Doi et al. 2008). The spawning season of *C. bimaculata* in Tokyo Bay, Japan is only occurred from March to October (Doi et al. 2008).

In conclusion, the carapace width distribution of male and female *C. anisodon* in Lasongko Bay ranged from 3.05 to 7.61 cm and 3.45 to 7.98 cm, respectively, with an unbalanced sex ratio and the spawning season is likely occur the whole year-round. The growth type of carapace width-body weight of male and female *C. anisodon* are negative allometric, whereas the growth type of carapace length-body weight of male is isometric and females is negative allometric. Negative allomertic growth is carapace width or carapace length growth more than body weight growth, whereas isometric growth is carapace length growth balanced with body weight growth of *C. anisodan*. Growth of *C. anisodan* of males is faster than females. This research is the first report to inform the biological aspects of *C. anisodon* in Indonesia.

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Management of coastal biodiversity based on social-cultural values in constructing conservation character education

ABUBAKAR SIDIK KATILI[1,2,♥], RAMLI UTINA[1,2,♥♥], YOWAN TAMU[3], ELYA NUSANTARI[1]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Gorontalo. Jl. Jenderal Sudirman No. 6, Kota Gorontalo 96128, Gorontalo Province, Indonesia. Tel.: +62-435-821125; Fax.: +62-435-821752. ♥email: dikykatili@gmail.com, ♥♥ ramli.utina@ung.ac.id
[2]Coastal Ecology Based on Local Wisdom Research Center, Universitas Negeri Gorontalo. Kota Gorontalo 96128, Gorontalo Province, Indonesia
[3]Department of Sociology, Faculty of Social Sciences. Universitas Negeri Gorontalo. Kota Gorontalo 96128, Gorontalo Province, Indonesia

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Abstract. *Katili AS, Utina R, Tamu Y, Nusantari E. 2018. Management of coastal biodiversity based on social-cultural values in constructing conservation character education. Biodiversitas 19: 1763-1768.* Coastal biodiversity is quite high including coral reef, mangrove, seagrass, and fishery resources. Management of coastal biodiversity can be conducted interdisciplinary covering various aspects. Four main aspects can be integrated, i.e., physical-biodiversity, social-cultural, character education, and conservation. This present study aimed to describe: coastal biodiversity in Gorontalo Province, Indonesia community's social-cultural value and local wisdom embodying conservation character of the coastal ecosystem, and character education of coastal ecosystem biodiversity in primary school by learning with a prototype of conservation character-based materials. Specifically, the present study aimed to construct the conservation character education based on social-cultural values. Data were analyzed with descriptive qualitative method by comparing and referring to findings from the previous studies. The procedure used in this research was *four*-D, i.e., (i) Define stage; by doing the identification and exploration of the coastal biodiversity potential. The methods used in this stage was exploration survey method. Focused group discussions were conducted with coastal communities to identify social-cultural values and local wisdom and to analyze the core and basic competence of learners by examining the tools of the lesson and determining the competence. (ii) Design stage; by designing a prototype of learning material to construct the conservation character for learners. (iii) Development stage; by validating the prototype of learning material for constructing the conservation character for the learner. (iv) Dissemination stage, by doing seminars and information dissemination on a prototype of learning material to construct the conservation character. The results showed that in Gorontalo, there were three components of the coastal ecosystem which included mangrove, seagrass, and coral reef. The communities in the coastal area of Gorontalo were prominent in their strengthened social-cultural roots taking the form of ecological awareness. The community in coastal area possessed local knowledge of the natural resources, e.g., plants and animals, and local attribution of such resources in the local language. The conservation character-education based on social-cultural values, specifically local wisdom, is the most appropriate education model to encourage the pattern of biodiversity coastal ecosystem management. Conservation character education was highly relevant to life-enhancing skills, based on the empowerment of skills and coastal biodiversity potential in each region.

Keywords: Biodiversity, coastal ecosystem, social-cultural, conservation character

INTRODUCTION

The increasing demands of the community for goods to fulfill their needs put pressure on the ecosystem of coastal and marine areas in Indonesia. The most significant threat comes from the land conversion of mangrove area into fishponds and coastal reclamation to fulfill the demands of settlement infrastructure, by which the projects profoundly damage the ecosystems of seagrass and mangrove. Coral reefs were destroyed when fish and cyanide bombs were used for catching reef fish. Such damages have further destructed the ecological function of coastal areas that support the life of the locals. Deforestation has always been associated with poverty, especially in the villages located around the forest (Golar et al. 2017).

Damage to the ecosystem and coastal environment depicts the carelessness of human being to the order of ecological system in the environment. Human beings position themselves at the outside of the order of nature and not as a part of the ecosystem and the environment, thus, they claim to be able to exploit the environment to fulfill their needs without realizing that their characteristics and behavior are gradually damaging the nature. By that, it is essential to construct conducive character-building based on social-cultural values to the community starting from the primary education phase, for it is an investment for the future generation. Learning materials discussing ecosystem in Natural Sciences subject in primary school is considered crucial not only to build the students' comprehension of the concept of the ecosystem but also to shape the student's character-building and behavior towards the ecosystem and its environment. An ecosystem is an ecological order consisted of living things and non-living elements within a system that influence each other. One of the teaching methods about ecological system is to involve students directly in discovering the components of ecosystem within their school environments, like inquiry learning process (Glynn et al. 2004). By this method, it is expected that the students will have a more comprehensive understanding and responsibility of their behavior towards the environment. The notion is in line with Piaget who argues that the cognitive development of primary school students experiences operational

and concrete phase, in other words, the logical processing depends on what they see and experience (Utina et al. 2017).

The ecological system in the coastal area is an excellent learning apparatus for primary schools in the coastal area. The schools can benefit from coastal biodiversity, such as mangrove, seagrass, and coral reef. By optimization of the ecosystem and its constituent components into learning material, it is expected that students will not only be able to understand and consider the ecosystems as part of themselves, but also to build intimacy between themselves and biophysical components in the coastal environment. The intimacy developed between the community and nature can lead to the development of social, cultural, aesthetic, and religious values within themselves; this environment awareness is actualized in behavior and local wisdom of the coastal community (Nusantari et al. 2017).

Hence, it is essential to implement social-cultural values in coastal ecosystem education to communities in the coastal area; this is to develop the understanding of the ecosystem and its components and the students' character and awareness towards coastal ecosystem. Moreover, it is significant to put into consideration the contextual of education, in which the learning process of coastal ecosystem needs to relate to the local coastal area (Zeidler 2005; Nuangchalerm 2010). On the other hand, Subiantoro (2011) indicated that in the local coastal area have some values including values of social, cultural, and aesthetics that are developing within the community in the form of local wisdom. The formulation of coastal biodiversity education also needs to involve learning source and media (Navarro et al. 2012).

This present study aimed to describe coastal ecosystem in Gorontalo Province, to describe the community's social-cultural values and local wisdom embodying conservation character of the coastal ecosystem and also to describe character education of biodiversity of coastal ecosystem in primary school by learning with the prototype of learning material to construct the conservation character. With this learning method, the students will have a firm understanding of the concept of ecosystem science and have a character caring for the coastal environment.

MATERIALS AND METHODS

Study area

This study was the coastal area of Northern Gorontalo District, Pohuwato District, and Boalemo District of Gorontalo Province, Indonesia (Figure 1). The potency of the coastal region of Gorontalo is depicted in the map of coastal area and distribution of primary schools in Northern Gorontalo, Pohuwato, and Boalemo. The period of this study was conducted within six months, i.e., from February to July 2018.

Procedures

The study is classified as development research. Development research is a form of research-oriented towards product development. There were four stages carried out in this study called the four-D, namely; definition, design, development, and dissemination (Thiagarajan et al. 1974). The four-D procedures in this study included (i) Define stage; by doing the identification and exploration of the coastal biodiversity potential. The methods used in this stage was exploration survey method. Besides, focus group discussions were carried out involving coastal communities to identify social-cultural values and local wisdom. The social-cultural values and local wisdom will become the basis for constructing conservation characters. Other research activities included analysis of the core and basic competence of learners by examining the tools of the lesson and determining the competence. The competence in question is the ability to generate conservation characters. (ii) Design stage; by designing a prototype of learning material to construct the conservation character for learners. The content of the material and the questions were related to the coastal biodiversity and social-cultural potentials in the research sites. (iii) Development stage; by validating the prototype of learning material to construct the conservation character. This stage involved two validations by experts, i.e., validation of subject material and validation of education and learning. (iv) Dissemination stage, by doing seminars and information dissemination on the prototype of learning material to construct the conservation character (Mappalotteng et al. 2015).

Data analysis

The data were analyzed descriptively employing a qualitative method. The analysis was performed by comparing and referring to findings from three previous studies, i.e., (i) Study about utilization of mangrove ecosystem as media and learning resources biology science in primary school. This is a development of learning material and media of Biology subject in primary schools, particularly in the competence of coastal ecosystem. This study applies the learning of mangrove ecosystems in primary schools with a contextual approach. Mangrove area is used as a material and learning media (Katili et al. 2015); (ii) Study about the management of coastal ecosystem and preservation of local wisdom values of Bajo tribe through the development of environmentally-aware groups. This is an analysis and description about scientific meaning of various local wisdom and ecological intelligence of community in the coastal area (Utina 2017); (iii) Research about education strategy of natural resources conservation in the coastal area of Tomini Bay in Gorontalo. The research explored the varieties of games that contain values of environment preservation within children in the coastal area (Utina 2016).

RESULTS AND DISCUSSION

Description of coastal ecosystem in Gorontalo Province

Among the varieties of the local potential of Gorontalo is mangrove forest. Within the province, there are several regions with vast mangrove forest area, i.e., Pohuwato, Boalemo, and Northern Gorontalo districts. A report of the region's living environment status by the Office of Environmental Issues in 2012 indicated that relatively well-preserved mangrove forest area was found only in Northern Gorontalo District (Balihristi 2012).

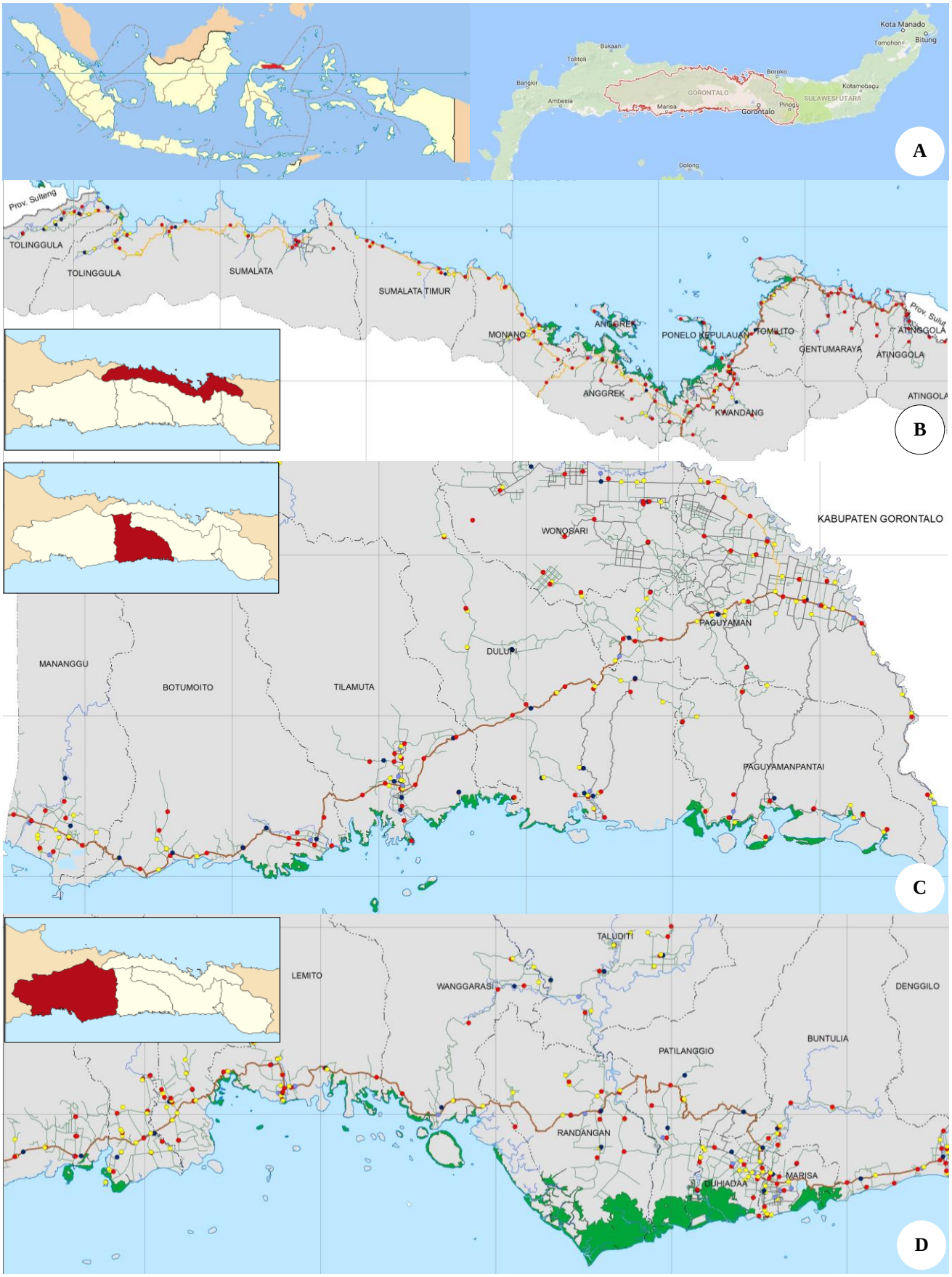

Figure 1. Study area: the map of the coastal area in (A) Indonesia and Gorontalo Province, (B) Northern Gorontalo District, (C) Boalemo District, (D) Pohuwato District. The red areas indicate the region of each district within the province, the green areas show the coastal area of each district, and the red dots indicate the school distribution of each district

Among the diverse natural resource potencies of the coastal environment in Gorontalo are mangrove, seagrass, and coral reef; which are the main components of the coastal ecosystem. On top of that, the communities in the coastal area of Gorontalo are prominent in their strengthened social-cultural roots taking the form of ecological awareness. A study in Bajo tribes in Torosiaje revealed that there was a significant correlation between the availability of natural resources in the coastal environment and the communities' ecological awareness (Utina 2017). The research discovered that the natural resources and ecosystems of mangrove, seagrass, and coral reef nearby Bajo community in Torosiaje Village, Gorontalo Province were well-preserved. The ecological awareness of the Bajo community is in contrast with the condition of the ecosystem within other regions; the community has been building emotional intimacy and awareness of the nature that supports their living, which results in the wise management of natural resources. Local wisdom such as traditions, norms, and prohibition cascaded from generations to generations within the community has developed into the community's legacy of norms.

As extracted from an interview conducted with the school teachers, the research highlighted that the primary schools did not perform an optimal implementation of the potential values of coastal ecosystem as learning material and media in the classroom. On top of that, the learning activity still involved conventional style, not maximizing the potentials of coastal biodiversity as a study object.

Description of the community's social-cultural values and local wisdom embodying conservation character of the coastal ecosystem

The research conducted exploration of potential values of the coastal ecosystem and Focused Group Discussion discussing values of social-cultural and local wisdom, in which the activities provided a significant elaboration of the background of the research problems. This is to say that the potential values of coastal ecosystem conceal diverse possibilities for human beings to harness, e.g., as primary sources of the coastal community's living, as a balance of ecological system within coastal area, and as sources of education innovation.

The community in coastal area possesses local knowledge of the natural resources, e.g., plants and animals, and local attribution of such resources in the local language. The Bajo tribe in Pohuwato District calls different types of mangroves in their local language, i.e., *apapi* (*Avicennia*), *bangkao* (*Rhizophora*), *munto* (*Bruguiera*). This signifies that the community has developed intimacy and awareness of nature, particularly mangrove environment; by which they perform conservation of mangrove area. Another proof is that within the Bajo tribe, it is prohibited to consume sea turtles due to the belief that the sea turtles are their savior during incidents in the ocean. The prohibition reflects that the community possesses high sensitivity of nature conservation. These conservation values need to be implemented in learning activities in schools, for the students to develop intimacy and awareness towards the nature in daily life.

Moreover, the community in the coastal area of Dulupi, Boalemo District possesses hereditary knowledge of fishing seasons, i.e., *tahulo, ewela, munggiyango,* and *pahi*. *Tahulo* season begins when smaller fish like *duwo* (smaller anchovies that only appear during the end of the month) and anchovy appear. *Ewela* season starts when medium-sized fish begin to appear. *Munggiyango* in local terms is a kind of shark and predator fish, while *pahi* is a term for fish who has venomous poison at the tip of its tail. The seasons of fish appearance depicts the food chain in the marine ecosystem. Smaller fish like *duwo* and anchovy are the prey of medium-sized fish (the second level predators in the food chain), while bigger predators like the shark are on top of the food chain; this clarifies that shark and other predators only appear after medium-sized fish. Such local knowledge of the community in Dulupi illustrates that the hereditary knowledge and awareness of marine conservation is recommended to be applied in learning activities in schools.

Description of conservation character education of biodiversity of coastal ecosystem in primary school

It is believed that by developing a lesson plan that involves coastal ecosystem as the material learning, one can provide an alternative to preserve the ecosystem. This is to embed sensitivity and awareness to the students of changing phenomena in the coastal area. At further phase, development of local content-based learning materials encourages the students to perform preservation and maintenance of the coastal ecosystem. It is crucial to implement such innovation to shape the students' critical thinking, considering that an environmentally-aware community is significant to the ecosystem. There are four core elements of education, formal or informal, i.e., learn to know, learn to do, learn to understand one self's identity, and learn to live together and get along with the community based on principles of equality and tolerance.

The optimization of potential values of coastal ecosystem as learning materials in primary school is categorized as an effort to build the students' critical thinking and character of awareness of nature conservation and an effort of instilling integrative values of ethics and norms of interaction between human and nature within the students. Character education is expected to be actualized in the students' behavior towards the environment and performing conservation of natural resources. Learning source from nature, e.g., coastal biodiversity, can be implemented as an alternative to support learning activities since it provides direct interaction between the students and nature as the learning object. Further, the interaction is capable of fostering the students' knowledge in identifying, analyzing, and formulating conclusions of the learning object; this is to encourage the students to perform scientific research from their early ages. The previous notion elaborated that the students are expected to be able to express opinions based on truth and to formulate solutions based on observed problems or phenomena; in

another word, the students can perform scientific approach in learning activities.

Learning activities that implement the scientific approach, that is a process of learning designed to actively foster the students' construction of concepts and principles of the learning materials by certain scientific processes towards a phenomenon or an event (Utina 2016). The approach is included in the core elements of learning strategies in the 2013 curriculum employed to enhance the students' competence. The Regulation of the Ministry of Education number 65 in 2013 about standards of the learning process in primary and secondary education highlights the significance of the application of scientific approach principles in learning activities. Moreover, the learning activities are recommended to involve not only books as the primary learning source but also environment exploration as the learning source. By implementation of the coastal ecosystem as a learning source, this research expected that the students are capable of conducting scientific observations and explorations to enrich their knowledge by experiencing direct involvement with nature. Direct instruction could result in direct knowledge and skill acquisition, also known as the instructional effect (Ruutmann et al. 2011). As a branch within Natural Sciences subject that studies about interactions between living beings and the environment, studying Biology does not always engage traditional learning by reading and memorizing only; it also does not only involve one-way communication between teachers and students. Learning activities of Biology subject should also include direct interaction between the students and the learning objects, such as the coastal ecosystem.

Discussion

Two marine waters surround Gorontalo Province with a notable potential of natural resources and biodiversity, i.e., Tomini Bay in the south and Sulawesi Sea in the north. The marine waters are included in mega-marine biodiversity and the center of the world's marine biodiversity also known as the Earth's Coral Triangle. Gorontalo Province consists of five districts and one capital city, i.e., Boalemo District, Bone Bolango District, Gorontalo District, Pohuwato District, Northern Gorontalo District, and Gorontalo City as its capital. Based on the report of regional environment status Gorontalo Province in 2012 (Balihristi 2012), Pohuwato District is included in the province's protected forest area, which also includes mangrove forest and marine aquaculture. The activities of aquaculture ponds tended to result negatively to the coastal ecosystem; the converted mangrove forests were highly unproductive to provide sustainable support for the community's living in the coastal area in this district.

Further, this research finding depicted that the damage to the coastal ecosystem is the after-effect of inefficient management of coastal and marine ecosystem. The inefficient decision could result from the lag of policy, in which the existence of a coastal ecosystem is only considered as a minor variable compared to the exploitation engaged in squeezing economic benefits from the ecosystem. As the key stakeholder that plays a vital role in policy formulation and implementation, the local government faces various problems, e.g., the ineffective coordination between offices related to coastal biodiversity conservation, thus producing policy that lacks synergy. The government lacks institutions that specify its focus on coastal biodiversity management and conservation, which leads to not optimal management of coastal ecosystem employed by the government and the community.

Moreover, another factor that contributed to the damage of coastal biodiversity is lag of community, as a result of the community's poor competence in addressing environmental problems and poor capabilities and capacity to put pressure on the sides responsible for environment preservation. The local community's participation in design and implementation of coastal biodiversity management policies is less optimal; thus they cannot be accounted responsible for their economic orientation of the ecosystem's exploitation without being aware of its sustainability. The management of coastal biodiversity preservation should emphasize the balance between aspects of biophysical, socioeconomic, cultural, and administration to engage in optimal management. The balance is only possible if the government implements regulatory devices of coastal biodiversity management as a part of governance formulated integrative between the government, the community, and related sectors.

The result of this study can give an employs comprehensive approach from the three previous aspects to produce formal regulations that lighten the ecological burden of coastal ecosystem in Gorontalo Province. Development of governance model of coastal biodiversity management based on the local wisdom of the coastal community is crucial in implementing comprehensive and optimal resource management of coastal biodiversity; this is to formulate regulations of coastal biodiversity in Gorontalo. The diversity of ethnic within a region provides multicultural local wisdom to the community, as is the case in Pohuwato featured with various inter-ethnic interactions. The local wisdom progresses through time and is passed on generations thus it roots within the community's way of life. By reconstructing cultural values and local wisdom and implementing in daily life, the community is able to preserve their culture from the interference of massive modernization (Utina et al. 2017).

Thus, the efforts undertaken by the coastal community in Pohuwato District is to re-actualize the culture, since the community has the potency and wealth of local wisdom; the wisdom is adopted and maintained within the value systems. The local wisdom is essential to be developed in school to foster the value of the character conservation, especially since early childhood, to encourage children to love the nature. This is parallel with Katili et al. (2017) that the strategy applied would be able to overcome the social-economics problems of society as well as planning the development of regional spatial. One of the things that can be done for example is maintaining the natural conditions of mangroves in the coastal area and making its ecosystem as a buffer zone, while still involving the people around the areas. Other ways are introducing the use of learning material activities in schools around the coastal area; and

also hatcheries management while considering the suitability of environmental factors such as the type of substrate and salinity.

The synergy of perception between government and society in conducting conservation character education will encourage the learners to take care of their local wisdom and continue the legacy of the community's way of life. For instance, the learners are introduced and promoted to take care of the environment. Consequently, the learners will develop the sense of belonging and care towards the nature based on local wisdom within the community. Hence, the implementation of local wisdom in character education in school is actualized by providing contents that emphasize mutual interaction between human and nature. Furthermore, Katili et al. (2015) said that educational approach by coastal biodiversity as learning media and resources in learning and incorporating learning material of coastal biodiversity in the national curriculum of primary education and the society management activities through reforestation, training, and extension, and non-formal education, can make the conservation character sustainable.

The government's capability of managing local development to address the environmental problems contributed significantly to the acceleration of regional development and the trickle down effect for the community's state of welfare. The study considers that by implementing local wisdom in character education from the students' early ages, it provides the correct solution to the environmental problems. Moreover, this research argues that character education employed within primary schools will result in future generations of Indonesia that embody Conservation character. The utilization of coastal biodiversity based on social-cultural values in the learning activities at the most basic level of education will result in a comprehensive scientific understanding and conservation character. This is due to the model of conservation character education that involves the learners to study and provide the solution for the surrounding environment, in this case, the coastal area. By implementing coastal ecosystem learning in primary schools with a social-cultural and local wisdom approach, it is hoped that the character of conservation and environmental friendly will be formed in the students. This is a character capital that contributes to the management of coastal biodiversity.

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Short Communication: Categorization models as a powerful tool in paleontological data analyses – the Phanerozoic bivalves

AHMED AWAD ABDELHADY[1,♥], MOHAMMED MASOUD ABDALLA[2]

[1]Department of Geology, Faculty of Science, Minia University. 61519 El-Minia, Egypt. ♥email: alhady2003@yahoo.com
[2]Department of Computer Science, Faculty of Science, Minia University. 61519 El-Minia, Egypt

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Abstract. *Abdelhady AA, Abdalla MM. 2018. Short Communication: Categorization models as a powerful tool in paleontological data analyses – the Phanerozoic bivalves. Biodiversitas 19: 1769-1776.* Predicting biotic responses to current and future global change can be acquired through understanding how biological and environmental traits shaped the past origination, dispersion and extinction patterns. A global dataset encompasses 161,357 taxon occurrences belonging to 2,378 bivalve genera from past and recent environments were analyzed based on the categorization model, a widely-used machine-learning analysis, using MS-SQL and Excel PowerView. The occurrence data was standardized using square-root transformation to downplay the effect of sampling effort. Thus, the examined traits are resulting from reliable ecological interactions. The results indicate that the biotic traits of the bivalve can be determined by the abiotic ones. Moreover, ecological traits such as life habit (i.e., infaunal vs. epifaunal), diet (suspension vs. deposit feeders, herbivores vs. carnivores), composition (aragonite vs. calcite), and locomotion (stationary vs. mobile) all exhibit significant relation to a specific environment. The results demonstrated that decision tree and association rules are primary powerful tools in analyzing huge biological data and in testing many useful bio-ecological hypotheses.

Keywords: Association rules, biotic traits. bivalvia, biodiversity, decision tree

INTRODUCTION

The efforts of the paleontologists in the last centuries have generating ultra-scale data sets of very high spatial resolution. These data were stored in many databases such as the Paleobiology Database (PBDB; http://paleobiodb.org/#/). However, the web-based system of the PBDB has not yet sufficient analytical functions for spatiotemporal analysis (http://paleobiodb.org/#/). Ecologists and climatologists are focusing their research now on understanding climate changes over broader range of time and space scales via the paleontological data from the fossil record (Alroy 2008; Nürnberg and Aberhan 2013; Foote 2014; Abdelhady and Fürsich 2015). According to Groth et al. (2012), insightful analysis of the life evolution on the earth is depending on applying different software tools to explore, manipulate, and visualize huge data sets. In addition, closer integration of geographic visualization and/or geo-computation is essential to address many environmental concerns (Varela et al. 2009; 2015). However, exploration tasks are usually complex and consume much time (Groth et al. 2012; Varela et al. 2015).

Advances in the field of information visualization offer a number of innovative and promising approaches (Kehrer et al. 2010). The applications of the visualization techniques has grown rapidly for different geologic purposes (Best and Lewis 2010; Gorricha and Lobo 2012; Romañach et al. 2012; Du et al. 2015). Effective computer tools, incorporate the Microsoft office package can provide intelligence from raw paleontological data by creating visual graphs, thus new concepts will be constructed to understand the paleo-ecosystems easily and efficiently. The set of utilities in MS-SQL and Excel PowerView enable preprocessing and visualization of large data in addition to plenty of statistical and numerical analysis. In addition, although categorization models are fundamental in decision-making and all kinds of environmental interaction, they are rarely used by paleontologists (e.g., Boyer 2010; Finnegan et al. 2012). In this paper, we implement the stratigraphic and geographic occurrence data of the bivalves, which is one of the best-preserved and documented fossil group through the Phanerozoic to illustrate how interactive exploration and visualization tools such as MS-PowerView and MS-SQL can successfully analyze the paleontological data. Therefore, we proposed to analyze the bivalve occurrence data and to test the ability of the categorization models (decision tree and association rules) to generate meaningful ecological results. Moreover, we aim to determine the main environmental factors that controlling the bivalve diversification (temporal) and distribution (spatial).

DATA AND METHODS

Dataset

The bivalve occurrence data is retrieved essentially from the PBDB. The PBDB was organized and operated by a multi-disciplinary international group of paleontological

researchers (Alroy et al. 2001). It provides a global, collection-based occurrence and taxonomic data for organisms of all ages. The PBDB encourages and enables addressing large-scale paleobiological and biological questions (Varela et al. 2015). Occurrence and range data of bivalve through the Phanerozoic as a whole were downloaded from the PANGAEA (for details see https://issues.pangaea.de/secure/attachment/97680/Appendix%20C.pdf.). The occurrence matrix includes 161,357 records belonging to 2,378 genera. The data were compiled into two tables, the range table, includes genera and their First Appearance Datum (FAD) and Last Appearance Datum (LAD) in addition to mean abundance longitude. While occurrence table include taxonomy (order, family, genus), life habit (infaunal, endobysate, epifaunal, boring, etc.), diet (suspension feeders, deposit feeders, and carnivores, etc.), locomotion (mobile, sessile, etc.), and shell composition (aragonite and calcite), in addition to age data (epoch, 2 MY bin, and 10 MY bin). Spatial data include country, paleo-latitude, and plaeo-longitude (Abdelhady 2015).

Data preparation and standardization

We introduce herein an easy, office-based approach that integrates exploration and visualization tools to different biologic and ecologic data sets. The implemented approach include data extraction, standardization, classification, and visualization of the results (Figure 1). Although the PBDB store a high number of paleontological data, from which many questions regarding the history of earth can be answered, uncertainties about these data clogged such interpretations. The results may be a sampling artifact. Increasing biodiversity from the Cambrian (540 MY) may represent the sampling efforts applied to the younger strata (the pull of the recent, Alroy et al. 2001). Therefore and to reduce potential errors, pre-processing of fossil data is often required before advanced analyses.

There are many standardization methods such as subsampling and residual methods. Although subsampling is one of the most frequently used for data standardization, its implementation has resulted always in reduction of the examined data, which is unfavorable for statistical analysis. Subsampling and residual methods were used to test reproducibility and consistency of the results of diversity calculations. In addition and according to Tomašových and Kidwell (2009), square-root transformation of the abundance data, downplay the impact of numerically abundant species and increase the effect of rare species (Abdelhady and Fürsich 2015).

For measuring sampling effort and for evaluating the latitudinal diversity gradient of the bivalve throughout the Phanerozoic, the occurrence date was square-root transformed for estimating a reliable diversity, origination, and extinction rates. Measuring the geographic dispersion was done by summing the number of genera in equal grids. Each grid include ten latitudinal degrees. The numbers then were normalized by the maximum number of occurrences (quantifying sampling effort). A computer code was developed in Mathematica Wolfram 10 by which standardizations were carried out.

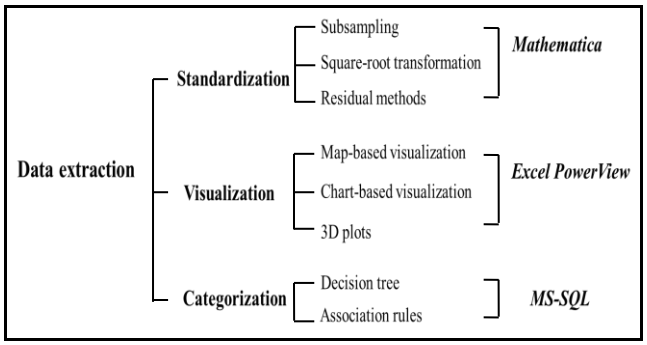

Figure 1. Schematic representation of the procedures applied in this study

Decision tree and association rules

The process in which ideas and objects are recognized, differentiated, and understood is known as 'Categorization' it implies that objects are grouped into categories for specific purpose, and thus, it illuminates a relationship between the subjects and objects of knowledge (Cohen and Lefebvre 2005). We used two different models herein, the association rules and decision tree. Decision tree is data analysis in the shape of extracting a model describing a considerable data classes (Han et al. 2011). For example, we can build a classification model, which predicts whether a taxon will be found in a given environment, or predicts which of five categories a new database item belongs to. Such analysis can assist in providing us with a better recognition of the data at large scale. Many classification methods have been developed regarding pattern recognition, machine learning, and statistical data classification, which is a two-step methodology consisting of a learning step (i.e., a classification model is built) and a classification step (i.e., the model is used to infer class labels for given data). The decision tree is a very popular classification technique characterized by quick training performance (for details see Han et al. 2011). A decision tree is a mathematical model, which help managers make decisions and it is rely on estimates and probabilities to calculate likely outcomes. Thus, it enables individual or organization to tack a decision based on costs and benefits (Quinlan 1987). Therefore, they can be used either to drive informal discussion or to map out an algorithm that predicts the best choice mathematically. For example, suppose that we have a set of N items, which fall into two categories, n have to Label 1 and m = N-n have Label 2. To get our data a bit more ordered, they well grouped by labels and two ratios will be calculated to estimate the Entropy (E):

$$ratio\ p = \frac{n}{N} and\ ratio\ q\frac{m}{N}.\ Entropy\ E = -plog2(p) - qlog2(q)$$

The decision tree graph can be read from left to right as follows: The rectangles, which are referred to as nodes, hold subsets of the data. The title on the node announces the defining characteristics of that subset; the leftmost node, titled 'All', depict the complete data set. All Following nodes represent subsets of the data. A decision tree contains many splits where the data diverges into

multiple sets depending on attributes. As for instance, the first split in the sample model divides the dataset into nine groups by 'Taxon Environment' The split immediately after the 'All' node is most important because it shows the primary condition that divides this dataset. Extra splits occur to the right, thus by analyzing different segments of the tree, we can learn which attributes have the most influence factor.

The Association rules are statements that help uncover relationships between seemingly unrelated data in a relational database or other information repositories. The association Rules find all sets of items (itemsets) that have supported greater than the minimum support and then using the large itemsets to generate the desired rules that have confidence greater than the minimum confidence. The lift of a rule is the ratio of the observed support to that expected if X and Y were independent:

$$Rule\ X \rightarrow Y:\ \text{Support} = \frac{frq(X,Y)}{N}, Confidence = \frac{frq(X,Y)}{frq(X)}, Lift = \frac{Support}{Supp(X) * Supp(Y)}$$

The data set was analyzed by two related software packages, MS-SQL and MS-PowerView. Microsoft SQL Server Data Mining is a collection of machine learning algorithms that explore your data for patterns. Once discovered, these patterns can be browsed for greater insight into your data, or they can be applied to new data to create "predictions" - which allow you to determine unknown facts about data based on data the algorithms have seen before. The MS-SQL analysis service is used for applying the classification and association rules techniques to the data. PowerView and PowerPivot, a feature of Microsoft Excel 2013, are used to perform powerful data analysis and create sophisticated data models. With PowerPivot, one can mash up large volumes of data from various sources, create a Data Model, a collection of tables with relationships, perform information analysis rapidly, and share insights easily. Where the PowerView plug-in within Excel 2013 is used to visualize the geographical information to enable the user to understand and interact with the presented knowledge easily and smoothly. PowerView is an interactive data exploration, visualization, and presentation experience that encourages intuitive ad-hoc reporting. Power Pivot and power view have been described as the most important new feature in Excel in 20 years (Winston 2014).

RESULT AND DISCUSSION

Bivalve occurrence

The final dataset encompasses 161,357 taxon occurrences belonging to 2,378 bivalve genera and 33 orders (Table 1). These taxa have variable ecological traits regarding shell composition, life-habit, diet, and mobility level. The distribution pattern of the bivalves may thus be linked to specific biotic or abiotic factor (see below). The occurrence of the bivalve orders throughout the geologic time scale is given in Table 1. In general, there is a steady increase in the bivalve occurrence from the Cambrian onward (Table 1).

Map-based visualization

Visual representations of the data allow an easy way of constructing knowledge (Varlea et al. 2015). Visualization of taxon distribution during specific ages using PowerView is shown in Figure 2. The PBDB provides a similar visualization technique (http://paleobiodb.org/#/). However, it lacks important filtering tools. Here we have included important auto-ecological filters, namely composition, diet, life habit, and locomotion. The advantage of such filter is to evaluate the influence of ecological parameters on the spatial distribution of the biota. Note that according to Fang et al. (2014) and Abdelhady and Fürsich (2014, 2015) and Abdelhady and Mohamed (2017), ecological aspects such as life-habit influence the geographic dispersion of the invertebrates. In addition, life-habit (e.g., benthic vs. planktic) of the invertebrates is influencing their temporal durations (Abdelhady et al. 2018). Pelagic fauna with planktonic larvae can be drift for longer distances than do benthic and non-planktonic larval taxa. The latter indicates how the filters-based visualization technique are useful in examining and answering very important ecological questions. In addition, the Map-based visualization techniques allow the mapped data to be changed interactively (Dykes 1997). Thus, it permits the users to change the appearance of the objects mapped and consequently define clusters. The occurrences map of the bivalve according to their life-habit shows that the infaunal and epifaunal taxa are the most dominated groups, (Figure 2), while other life habits are less abundant. Moreover, the distribution map indicate a strong latitudinal diversity trend. In general, the map visualization (Figure 2) is neat and the base map is certainly an aesthetic improvement over the base map provided by the PBDB. However, until now, there is no possibility to change the modern world map with paleomaps, which are more informative in paleontological data analyses.

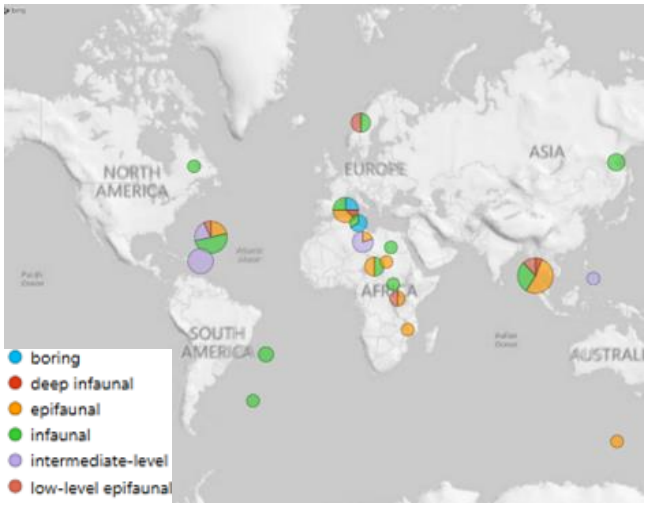

Figure 2. Distribution of the bivalves according to their life-habit

Table 1. Distribution of the bivalve orders throughout the Phanerozoic (Data compiled from the PBDB)

Order	Geologic time										
	Cambrian	Ordovician	Silurian	Devonian	Carboniferous	Permian	Triassic	Jurassic	Cretaceous	Cenozoic	Total
Actinodontida		18		25		1			2		60
Anomalodesmacea										4	4
Arcida		27	6	65		234	168	1270	2353	6530	11295
Cardiida		1	2	49	61	439	587	2143	5606	21263	32208
Carditida		8	27	364	61	285	289	1460	1543	4803	9623
Colpomyida		20			98						22
Cyrtodontida		238	462	168		11					1023
Fordillida					4						11
Hiatellida	11						3	15	358	849	1369
Hippuritida			3				14	72	3685		4520
Lucinida			14	252		18	164	717	782	3451	5699
Megalodontida				5	2	2	215	247			517
Modiomorphida		511	203	532		24	2	4			1441
Myalinida		401	124	248	18	690	444	363	3208	1	6045
Myoida					235			80	44	89	225
Mytilida		5	2	25		140	268	1617	1133	1811	5450
Nuculanida		160	106	1055	46	452	316	546	940	2664	7083
Nuculida		224	45	320	187	235	150	553	670	1426	4146
Nuculiformes					114						61
Ostreida	60	197	288	965		597	2644	4935	5005	3931	19932
Pandorida					233		13	91	186	191	509
Pectinida			15	260		2906	3917	7134	5384	7802	29577
Pholadida					581	302	220	1402	1276	3482	7242
Pholadomyida		60	7	69	4	411	82	1258	412	115	2734
Poromyida					124		3	24	443	349	859
Protobranchia		1									1
Pterioida		19	11	29		134	66	122	71	24	518
Solemyida		192	73	74	6	72	11	37	57	73	725
Solenida		2	12	48	14	9	1	3	176	603	902
Thraciida							11	221	92	447	829
Trigoniida			5	108		557	1371	1108	1209	30	4819
Tuarangiida					93						3
Unionida	3	2				4	198	23	51	90	414
Total	74	2251	1486	5058	1881	7864	11187	25446	34691	60113	161357

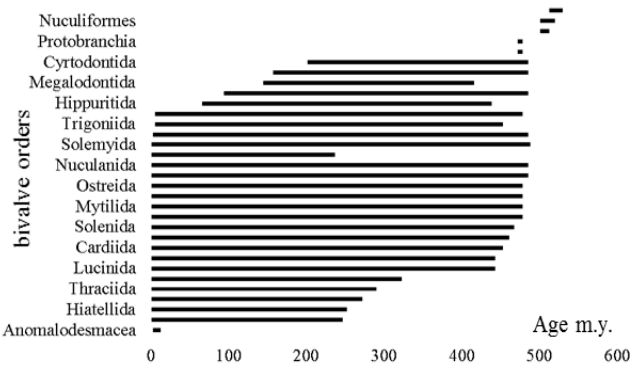

Figure 3. Range chart (temporal range of the taxa: species, genera, families, etc.) of the bivalve orders through the Phanerozoic using the stacked bar charts. Data figured include LAD and the duration of the taxa. The timescale is by millions of years before

Chart-based visualization

There are two forms of data visual representations in PowerView; maps and charts. Maps are frequently used for climate studies. Meanwhile, charts are needed where no country data are examined (i.e. latitudes instead of country). Histograms, bar chart, scatter, and bei-graph are the most used charts. Therefore, both are integrated into the analysis of the bivalve data to explore additional options. For example, fossils can be used for relative dating of rocks. To date rocks, range chart has to build. Almost all of the paleontological articles contain such chart. Using the stacked bar chart, temporal range of the taxa (species, genera, families, etc.) can be easily constructed. The required data are the LADs and the duration of the taxa (Figure 3).

One of the most important charts is that representing diversity across time to show the diversity dynamics (Figure 4) or across latitudes to show the pattern of the

latitudinal diversity gradient (Figure 5). Diversity (richness) can be determined based on different taxonomic ranks (species, genera, families, etc.). Moreover, the user can construct different filtering elements using the PowerPivot chart such as life habit, diet and locomotion exactly as done in map-based visualization. The five big mass-extinctions are clearly shown in Figure 4.

Again, implementing filters enable answering much debated ecological questions. According to Ros et al. (2011), the taxa originated under stress such as global crises event (the end Permian mass extinction) have longer ranges. To test this hypothesis the range of the bivalve taxa at the two biggest earth crises, namely the end of Permian and the end of Cretaceous were analyzed. The ranges of the taxa originated at the beginning of the crises (i.e, Induan ≈ 252.17 Ma, and Danian≈66 Ma) compared to those originated at normal stable conditions (i.e. the Carnian ≈227 Ma and the Maastrichtian≈ 72.1 Ma) using the range chart method described above. Just in few seconds, one can answer the question from a lock to the chart, which agrees to the hypothesis of Ros et al. (2011) in case of Danian and disagree in case of the Induan. Note that taxa originated under stress (Induan) have shorte age ranges (contrasting Ros et. 2011), while in Danian have longer age ranges agree with (Ros et al. 2011).

Similarly, FADs and LADs can be used to show number of originations and extinctions (Figure 5A). Group function of the pivot-chart was used to divide the Phanerozoic into 18-equal intervals (each = 30 MY). In addition, the temporal change of the epifaunal/infaunal proportional was constructed using the 100% stacked chart (Figure 5B). The information obtained from this chart could be used to analyze the diversity patterns at specific age or to follow replacement among taxa and their controlling factors (autoecological or eco-environmental).

The latitudinal distribution of the Phanerozoic bivalves shows greatest concentrations of occurrences between 10 and 50 N (Fig. 6). The steepness of the latitudinal pattern and the high similarity/correlation with the total number of collection may suggest a possible sampling artifact. The square-root transformation downplays the impact of sampling effort (Fig. 6B). Note that areas located in Europe and North America have extensive sampling efforts comparatively to those located in Africa or South America; Figure 6C).

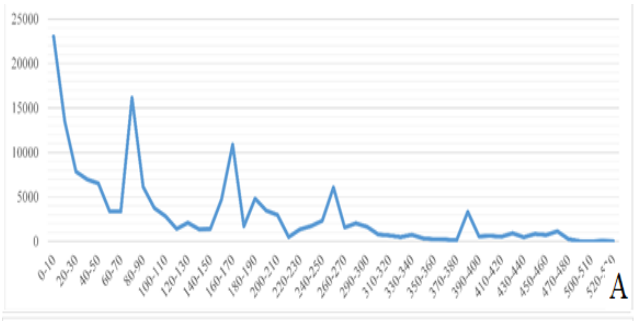

Figure 4. Diversity dynamics (represented number of genera) of the bivalve through the Phanerozoic. The beaks on the curve represent the 5-major mass extinction events

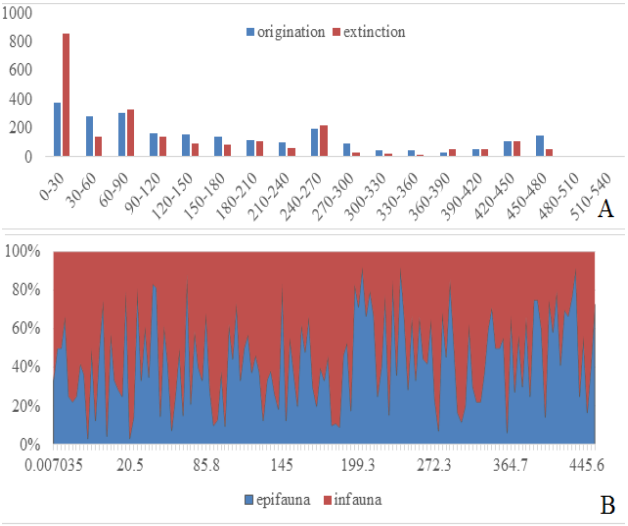

Figure 5. Origination and extinction of the bivalves (A) and the percentage of infaunal vs. epifaunal bivalves through the whole Phanerozoic (B). X-axis represents age in m.y. before present

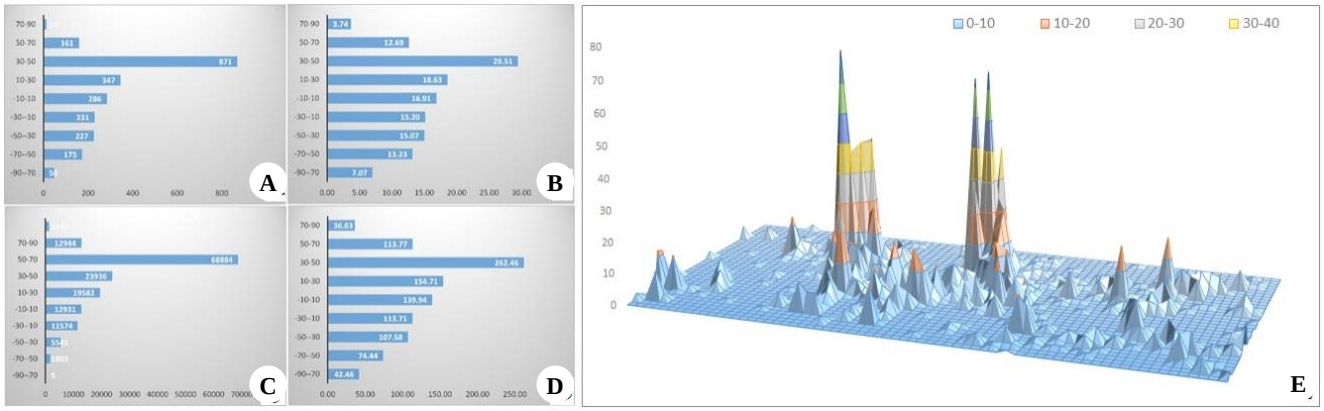

Figure 6. Bar chart shows the Latitudinal (y-axis) Diversity (x-axis, number of genera) Gradient: A. Raw data, B. Square-root data, C. 'Sampling effort' (number of collections) based on the raw data, D. 'Sampling effort' after square-root transformation of the raw data, E. 3D surface plot of the Latitudinal Diversity Gradient

Data model

The Data Model enables combining data that comes from SQL Server (Winston 2014). DISTINCT COUNT, a valuable function is activated when the excel table transformed to a data model. The function enable estimation of number of distinct genera from multiple occurrence record by counting the genus name only once and ignore duplications, hence the real number of the different taxonomic levels (i.e., genus, family, or order) can be estimated together with the normal counts of the records, which represent the density of a taxon (frequency in the rocks). The density of the collections can also be artifact represent the sampling efforts. In addition, relation among variable can be directly estimated quantitatively (Figure 7). The figure correlates between life mode and diet with other biologic or ecologic variables. The score in the figure refers to the importance/strength of each variable. From Figure 7, we can estimate that there is a strong relationship between the bivalve orders and the diet (i.e., for each bivalve order there is a specific diet mode for all genera included within this order (r = 0.76). in addition both environment (abiotic) and life-habit (biotic) can also determine the taxon diet (r = 0.26). In addition, for each bivalve order, there is a given life-habit (r = 0.6). Similarly, environment and shell composition have a considerable relation with the life-habit (r = 0. 34 and 0.22, respectively)

Decision tree

Table 2 summarize two cases of different rules. For each rule, the classification model determines number of cases and the probability of occurrence. Table 2 representing the main findings of the decision tree model elaborating the selected node (i.e. rule).

Association rules

From the itemsets herein, we can conclude that there is a complex pattern and association between biotic factors (such as life-habit, diet, locomotion) and the biotic ones (environment, age, and geographic occurrence (paleo-latitudes). The Rules tab in Figure 7 combines information about the itemsets and their relative value. Probability represents the portion of cases in the dataset that contain the targeted collection of items. Probability gives a hint of how likely the result of a rule is to occur. We can change the value of minimum probability in this pane to filter the rules that are exhibited. The value for minimum probability that we initially see is the threshold value that was used by the association rule algorithm when building the model. After the model is completed, we cannot reduce this value, but we can increase it to show only the higher probability items. Importance column is designed to measure the utility of a rule. A rule that is very common might have little information value. The greater the significance, the more valuable the rule is for predicting the outcome. Herein, we can summarize the following; carnivore bivalve are usually actively mobile taxa. In contrast, herbivore bivalves are usually passively mobile taxa.

In addition, deep infaunal bivalve are chemosymbiotic taxa dominating the reef environments characterized by marl deposition.

Table 2. Decision tree rules and their cases and probabilities

Rule	Diet value	No. of cases	Probability
Taxon Environment = ‘marine' and	Chemo-symbiotic	32	77.07%
Locomotion = 'actively mobile'	Deposit-feeder	9	21.83%
Taxon Environment = ‘inner shelf’ or Taxon Environment = ’outer’ or Taxon Environment = ’shelf’ or Taxon Environment = ’oceanic' and	Chemo-symbiotic	32	77.07%
LAD > = 103.200	Deposit-feeder	9	21.83%

Probability	Importance	Rule
1.000		Locomotion = passively mobile, Life-habit= boring --> Diet = herbivore
1.000		Locomotion = passively mobile, LAD < 64.4 --> Diet = herbivore
0.848		Locomotion = passively mobile,--> Diet = herbivore
0.979		Locomotion = passively mobile, Paleolat. = -7.3- 22..7 --> Diet = herbivore
0.879		Locomotion = passively mobile, Environment = Marine indet. --> Diet = herbivore
0.569		Life-habit = boring, Paleolat. = -7.3- 22..7 --> Diet = herbivore
0.406		Locomotion = actively mobile, Paleolat. >= 41.2 --> Diet = carnivore
0.434		Life-habit = deep infaunal, LAD < 64.4 --> Diet = chemosymbiotic
0.505		Environment = reef, buildup or bioherm, Life-habit = deep infaunal --> Diet = chemosymbiotic
0.417		Life-habit = deep infaunal, Lithology = marl, --> Diet = chemosymbiotic
0.812		Taxon environment = coastal, Life-habit = infaunal --> Diet = deposit feeder
0.763		Taxon environment = coastal, Locomotion = facultatively mobile --> Diet = deposit feeder
0.672		Taxon environment = coastal, --> Diet = deposit feeder
0.990		LAD = 279.91- 39.96, Taxon environment = coastal --> Diet = deposit feeder

Figure 7. The results of the association rules applied to portion of the raw dataset encompassing environment, locomotion, and life habit to predict the taxon diet. The figure shows the item, its probability, and its importance

Finally, we can clonclude that the application of interactive visual methods to analyze paleontological data is still hampered for paleontologists and paleo-climatologists, who are usually non-visualization experts (Groth et al. 2012). The goal here was not to describe in details a new software or the Phanerozoic history of bivalves but to illustrate how to implement the MS-SQL and PowerView software to analyze and visualize the paleontological data quickly and efficiently. Herein, we focused on performing some standard paleobiological analysis. In addition to a less-common machine-learning analysis. In fact, many of the basic analyses such as tabulating diversity, calculating extinction rates can be done with the PBDB using FossilWorks website (http://fossilworks.org). However, one has no chance for filtering such analyses based on bio-ecological traits. Furthermore, there are some specialized statistical analysis scripts such as PAST (Hammer et al. 2001) to achieve particular research purposes. Although PAST is one of the easiest statistics packages for paleontologists and biologists, it lakes exploratory data analysis or machine-learning algorithms (i.e. association rules or decision-making).

Integrating PowerView with MS-SQL here in has enabled the following: (i) Visualizing spatial and temporal data in a similar manner to that of PaleoDB package (Varela et al. 2015), (ii) Transform records into meaningful tables and/or matrices, and (iii) Search and find single piece of information, (iv) analyzing portion of the raw data based on multiple specific criteria as example it determine/draw the geographic range size of taxa that (a) are suspension-feeder, (b) belonging to a specific taxonomic rank such as family, and (c) that became extinct at the end of Cretaceous crises (66 Ma).

Some limitations in PowerView are present and should be highlighted herein. For example, the country map is limited to the modern continental configuration, which makes this visualization tool of limited utility to paleobiologists. Paleobiologists are more interested in where fossils were located in the geologic past than where they are found today. However, the approach implemented herein permits easily map/charts visualization in addition to many community analyses necessary for paleo-/ecological interpretation and ecosystem reconstructions. The results indicated that environmental type are the main factor controlling bivalve taxa (their diet, life-habit, etc.). In addition, there is a cyclic pattern among these attributes (i.e. they affect each other).

We concluded that decision tree and association rules may provide a valuable and advanced information for finding relationships among biological/ecological traits and their environmental parameters.

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Short Communication: Genetic diversity of *Salacca edulis* from West Seram District, Maluku, Indonesia based on morphological characters and RAPD profiles

SOVIAN SESCA ELLY[1,♥], THEOPILUS W. WATUGULY[2,♥♥], DOMINGGUS RUMAHLATU[2]

[1]Biology Education Graduate Program, Universitas Pattimura. Jl. Dr. Tamaela, Ambon 97116, Maluku, Indonesia. Tel./fax. +62-911-311803, ♥email: sovianelly@gmail.com

[2] Department of Biology Education, Faculty of Teacher Training and Education Sciences, Universitas Pattimura. Jl. Ir. M. Putuhena, Ambon 97233, Maluku, Indonesia. Tel./fax. +62-911-3825216, ♥♥email:watugulytheo@gmail.com

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Abstract. *Elly SS, Watuguly TW, Rumahlatu D. 2018. Short Communication: Genetic diversity of* Salacca edulis *from West Seram District, Maluku, Indonesia based on morphological characters and RAPD profiles. Biodiversitas 19: 1777-1782***.** Morphological and RAPD-based genetic diversity analyses of *Salacca edulis* Reinw populations from West Seram District were performed. A survey was conducted in four locations in the village of Riring, Rumahsoal, Taniwel, Neniari, and Soya. Forty-two morphological characters and three RAPD primer were used. Data were analyzed on the NTSys program version 2.0 to perform UPGMA clustering analysis. An UPGMA dendrogram based on morphological characters resulted in two main groups with similarity value varied from 0.46-0.75 for morphology and 0.35-0.89 for RAPD. The result gives us important about cultivar of Salacca edulis in West Seram District Maluku which has high genetic diversity and germplasm. These results and can be used for further research for conservation as native cultivar.

Keywords: Genetic diversity, morphology, RAPD, *Salacca edulis*

INTRODUCTION

Salak (*Salacca edulis* Reinw) or snake fruit has an economic value and potentials both for domestic and export market commodity (Herawati et al. 2012). Salak is a species of palm tree that grows in clusters (Herawati et al. 2018). This plant is predominantly grown in Java and southern Sumatera. The main cultivated varieties of salak in Indonesia, are *Salacca zalacca var. zalacca* from Java and *Salacca amboinensis* (Becc) from Ambon and Bali. Another species related to *Salacca edulis* is *Salacca sumatrana* Becc distributed in Sumatera (Nandariyah, 2010), Sleman, Madura, and Banjarnegara (Murti, 2002).

In Maluku, salak cultivation is centered in the village of Soya, Hatalai, Wakal, Amahusu, and Hative Besar on Ambon island, and Piru, Taniwel, and Riring village on Seram island. Salak grown in West Seram is a species native to Maluku (Pattinama et al. 2007). The fruit has excellent properties, such as red fruit flesh and sweet-sour taste. The fruit is produced by crossing male and female flowers by the farmers. In this region, salak is consumed only when mature while the economic development in the form of processed products does not exist yet. Salak from Seram island and other areas of Maluku have some morphological differences. However, the diversity of the populations, their relationships and genetics have not been studied in details.

Genetic diversity can be assessed based on variation in morphology, protein, and molecules (Govarthanan et al. 2011). Morphological characters are easy to observe, straightforward, affordable, but somehow inconsistent due to subjectivity when evaluating certain characters or character states. Recent studies on genetic diversity mostly involved molecular markers, such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Length Polymorphism (AFLP), microsatellites or Simple Sequence Repeat (SSR), Inter-Simple Sequence Repeat (ISSR), Sequence Characterized Regions (SCARs), Single Nucleotide Polymorphisms (SNPs) (Semagn et al. 2006). RAPD is one of the molecular markers that can be used to study DNA polymorphism based on different sizes of DNA fragment. RAPD is widely used because it is easy, affordable, and quick in producing DNA bands polymorphism (Baig et al. 2009; Gurijala et al. 2015).

Previous studies on genetic diversity of salak from Java and Sumatera have used morphological characters and RAPD profiles (Suskendriyati et al. 2000; Murti 2002; Sudjijo 2009; Nandariyah 2010; Fatimah 2013; Herawati et al. 2012; Ariestin et al. 2015; Herawati et al. 2018). However, no study on the genetic diversity of salak from Maluku islands was reported. This present study was aimed to assess genetic diversity of *Salacca edulis* from West Seram District based on morphological characters and RAPD profiles. The results of the analysis will contribute to the improvement strategy of utilization and conservation salak as a native plant species.

MATERIALS AND METHODS

Study site

Samples were collected from four locations in West Seram District, Maluku (Moluccas), Indonesia, i.e., the villages of Riring, Rumahsoal, Taniwel, Neniari, and a village in Ambon City named Soya as a comparison (Figure 1).

Morphological observation

Morphological characters of the species were selected and scored based on the International Plant Genetic Research Institute guidelines for coconuts genetic resources (IPGRI, 1995). Observation on plant organs included vegetative and generative organs, consisting of three root characters, nine stem characters, 12 leaf characters, ten fruit characters, four seed characters, and four thorn characters.

RAPD analysis

RAPD analysis was conducted in the molecular laboratory of the Biology Department, Faculty of Mathematics and Natural Sciences, Brawijaya University. The leaf samples were cut, packed with aluminium foil and kept in cool box. DNA isolation was carried out using a modified CTAB method (Doyle and Doyle, 1987). DNA concentration and purity was measured quantitatively using Genesys 10 spectrophotometer at the absorbance wavelength of 260/280 nm. PCR amplification of RAPD was using primers OPA-3, OPA-17, and OPA-19 (Nandariyah et al. 2004; Ediwirman and Mansya, 2011; Ayuningrum et al. 2012). The condition of PCR reaction was as follows: pre-denaturation at 94 ^{0}C for 5 minutes followed by 40 cycles consisted of denaturation at 94 ^{0}C for 30 seconds, annealing at 35 ^{0}C for 30 seconds, and extension at 72 ^{0}C for 30 seconds, and terminated by a final extension at 72 ^{0}C for 10 minutes. The results of PCR amplification were run in an electrophoretic tank using 1.5% agarose gel and visualized in a UV transilluminator.

Data analysis

Morphological data consisted of qualitative and quantitative characters. Scoring for each character based on IPGRI (1995) and then was standardized into binary data. Data were arranged in NT-edit (Rohlf, 1998). RAPD profiles were analyzed based on the presence or absence of bands DNA generated by primers at each locus. The scored data were treated as binary data. Coefficient of similarity was determined with Simple Matching (SM) on SIMQUAL (Similarity of Quantitative Data) procedure. Cluster analysis was performed using Sequential Agglomerative Hierarchical and Nested Unweighted Pair-Group Method with Arithmetic (SAHN-UPGMA) on NTSYs program version 2.0 (reference?)

RESULTS AND DISCUSSION

Morphological variations of salak populations

Our observation showed that Maluku salak plant has the height of 2.5-7 m with stem circumference ranging from 20-80cm. The stem is circular and jagged with brown and dark brown color. Leaves are elongated, mostly green or dark green color. Leaves size is 18-80 x 2-7 cm, every petiole has different sizes. Each cluster produces 5-20 fruits, fruit diameter ranges from 2.5-14 cm. The rind is black and brownish black while the flesh can be yellowish white, white, or reddish white. The flesh is 0.5 -1.8 cm thick and it tastes sweet when it is ripe (Figure 2).

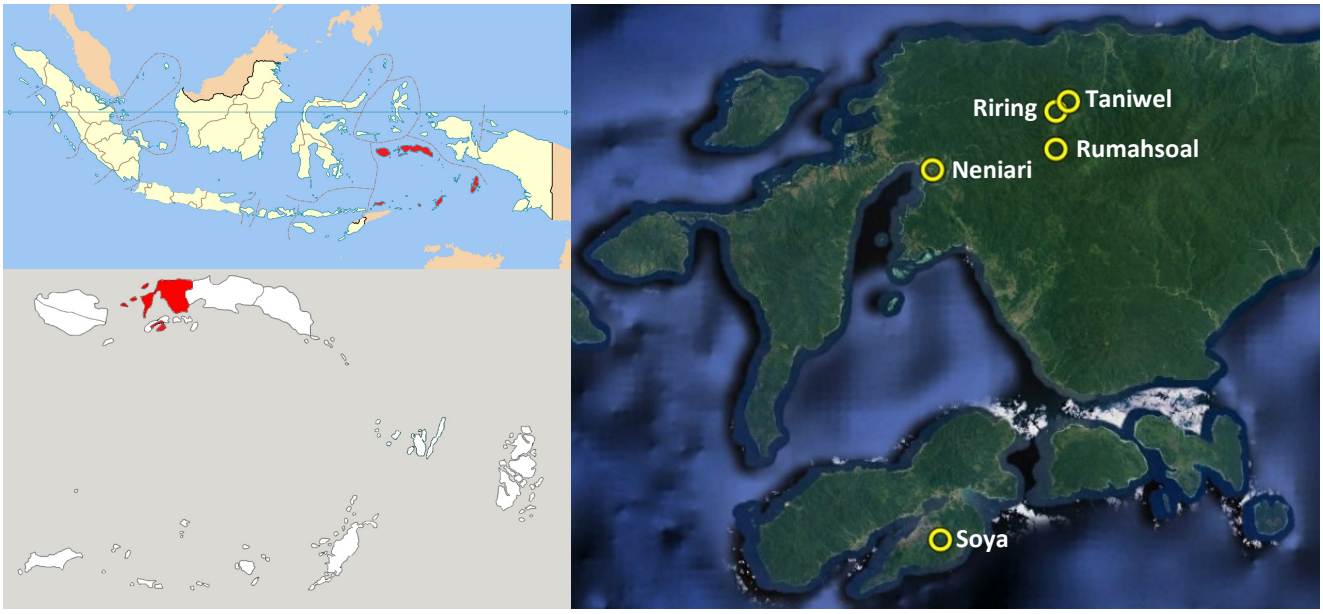

Figure 1. Study sites in West Seram District (i.e., villages of Riring, Rumahsoal, Neniari, Taniwel) and Ambon City (i.e., Village of Soya), Province of Maluku (Moluccas), Indonesia

Figure 2. Morphological variation of *Salacca edulis* from West Seram District, Maluku, Indonesia

The results of the present research are in line with Suskendriyati et al. (2000) who state that the varieties of salak can be differentiated based on the texture of the flesh, the color of the skin, the size of the fruit, the taste, and the habitus.

Traditionally, diversity is estimated by measuring variation in phenotypic or qualitative and quantitative traits. However, this approach is often limited and expression of quantitative traits is subject to strong environmental influence (Kameswara, 2004). Genetic diversity is a prerequisite for the genetic improvement of a plant. But rational use of the genetic diversity present in germplasm collections requires a good knowledge about their characteristics.

The coefficient similarity was ranged from 0.4609-0.7500 (Table 1), with the highest similarity (0.7500) was observed in sample A3 and A4; while the lowest similarity (0.4609) was found in A1-A5 and A6-A9 sample. Based on cluster analysis, the dendrogram showed two main groups (Figure 5). The first group consisted of Riring (A1, A2, A3) and Rumahsoal (A4, A5) and the second group was composed of Taniwel (A6), Neniari (A7, A8) and Soya (A9).

High similarity value suggests high similarity of characters among the OTU. Generally, each location shared the same qualitative characters such as habitus, shape of canopy, appearance of leaf upper and lower surface, shape flowers, and fruit texture. Individual A1 to A8 are samples from the same locations, which is from an island. A9 sample was located on a separate island, but clustered with A6 that was from different island (Figure 5). This proves that island differences cause variations in environmental factors that can cluster in different clusters.

Genetic diversity in salak populations based on RAPD markers

PCR amplification of Salak cultivar from 9 locations using three RAPD primers yielded 87 bands to which 43.68% are polymorphic bands. The length of polymorphic band ranging from 50-1000 bp with number of polymorphic ranging from 1 to 6 for each location and 12-13 band per primer. The highest polymorphism was

observed in primer OPA-3 (50-600) and OPA-19 (50-1000) (Table 2). The DNA polymorphic bands could be found in different sizes and positions of loci for each sample. The number of polymorphic DNA fragments is an essential factor in determining the genetic diversity level of a population. The difference in the number and polymorphism of DNA bands generated by each primer describes the complexity of plant genomes (Nandariyah et al. 2004). High polymorphism level suggests high genetic diversity in plant samples. Polymorphism generated in this study (43.68%) was lowered than that was reported by Nandariyah (2010), who studied 12 cultivars of Salam from with 68.4% polymorphism.

In general, DNA bands produced from the RAPD amplification had different numbers, sizes, and intensity even though they were amplified using the same primers (Figure 6). These variations may often be resulted in polymorphism at individual level. Harkingto in Herawati et al. (2018) explains that DNA polymorphism can be caused by the differences in an individual's genome sequence, this was implied by the presence and absence of bands in each sample. The intensity of the DNA bands appearance might be influenced by the charging weigh of migrated distinct molecules and number of DNA band copies which had been amplified. Lee (1998) reported that large molecules can lead to improperly separated bands so that the DNA bands become much thicker. The fact that some primers could not generate bands indicated that the primers were not complementary with the DNA genome template.

Cluster analysis resulted in similarity values ranging from 0.34-0.88 (Table 3). The highest similarity coefficient was observed between A1 and A2 (0.8888) while the lowest similarity coefficient was found between A6-A9 (0.3472). UPGMA dendrogram two main groups. The first group was composed of a group of samples from A1 and A2, and another individual group of A5, A3, and A4. The second group consisted of A6 and A7, A8, and A9 (Figure 7). Sample A1 and A2 populations were in one group because they were from the same location of Riring. They were grouped in a cluster with A3, A4, and A5.

The results of the current research indicated that salak populations found in West Seram District had moderate genetic diversity based on RAPD profiles but low morphological variations.

Table 1. Similarity coefficients based on morphological characters

Sample locations	A1	A2	A3	A4	A5	A6	A7	A8	A9
A1	1								
A2	0.6875	1							
A3	0.5521	0.5521	1						
A4	0.5521	0.5521	0.75	1					
A5	0.5521	0.5521	0.7188	0.7188	1				
A6	0.4609	0.4609	0.4609	0.4609	0.4609	1			
A7	0.4609	0.4609	0.4609	0.4609	0.4609	0.5469	1		
A8	0.4609	0.4609	0.4609	0.4609	0.4609	0.5469	0.5938	1	
A9	0.4609	0.4609	0.4609	0.4609	0.4609	0.625	0.5469	0.5469	1

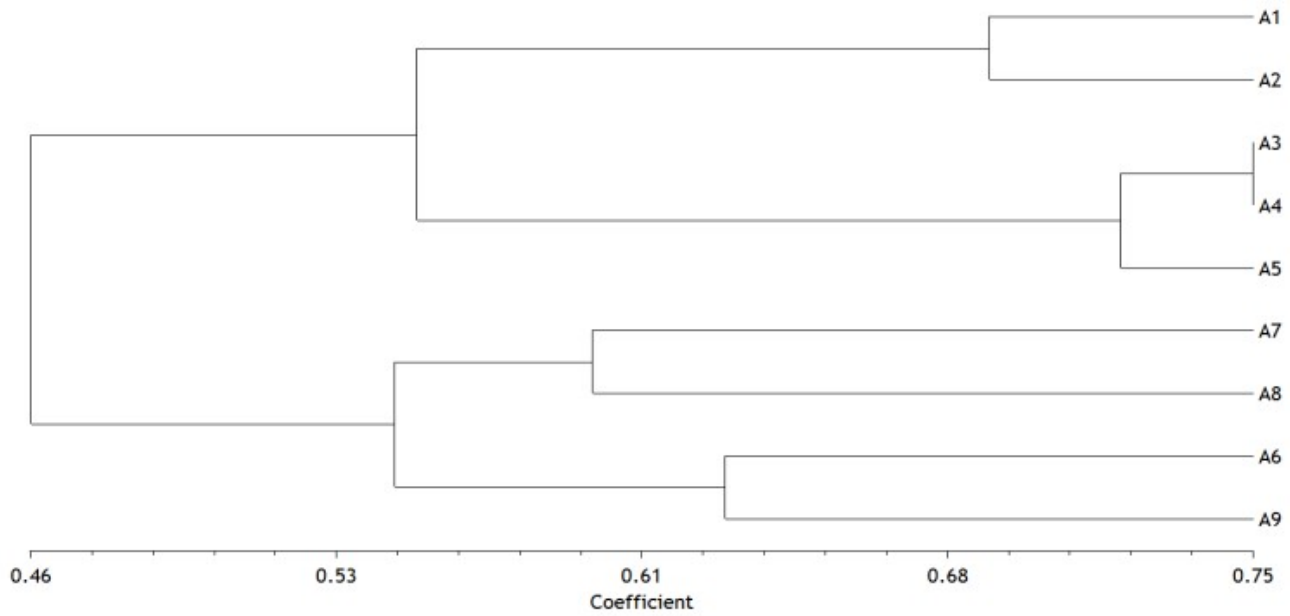

Figure 5. An UPGMA dendrogram of Salak based on morphological characters. Note: A1 = Riring I, A2 = Riring II, A3 = Riring II, A4 = Rumahsoal I, A5 = Rumahsoal II, A6 = Taniwel, A7 = Neniari I, A8 = Neniari II, A9 = Soya

Table 2. The list of primers, sequence, and number of DNA bands in RAPD analysis

Primer	Sequence 5'-3' (Nandariyah et al. 2004; Ayuningrum et al. 2012)	Size	Number of polymorphic bands	Number of monomorphic bands	Total
OPA-3	AGT CAG CCA C	50-600	13	23	36
OPA-17	GAC CGC TTG T	50-900	12	12	24
OPA-19	CAA ACG TCG G	50-1000	13	14	27
Total			38 (43. 68%)	49	87

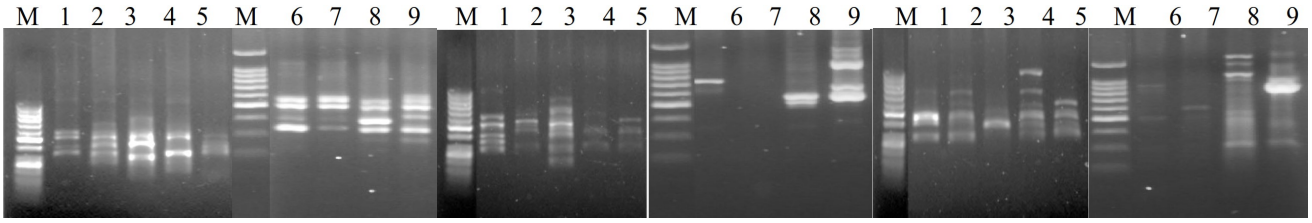

Figure 6. The profiles of DNA bands of Salak from West Seram District, Maluku, Indonesia found in each sample location. Note: A. Primer OPA-3; B. Primer OPA-17; C. Primer OPA-19; M=DNA marker, 1. Riring I, 2. Riring II, 3. Riring II, 4. Rumahsoal I, 5. Rumahsoal II, 6. Taniwel, 7. Neniari I, 8. Neniari II, 9. Soya

Table 3. Similarity index of Salak from West Seram District, Maluku, Indonesia based on RAPD profiles

Location	A1	A2	A3	A4	A5	A6	A7	A8	A9
A1	1								
A2	0.8888	1							
A3	0.7963	0.7963	1						
A4	0.7639	0.7639	0.7639	1					
A5	0.8333	0.8333	0.7963	0.7639	1				
A6	0.3472	0.3472	0.3472	0.3472	0.3472	1			
A7	0.3472	0.3472	0.3472	0.3472	0.3472	0.7222	1		
A8	0.3472	0.3472	0.3472	0.3472	0.3472	0.6389	0.6389	1	
A9	0.3472	0.3472	0.3472	0.3472	0.3472	0.5	0.5	0.5	1

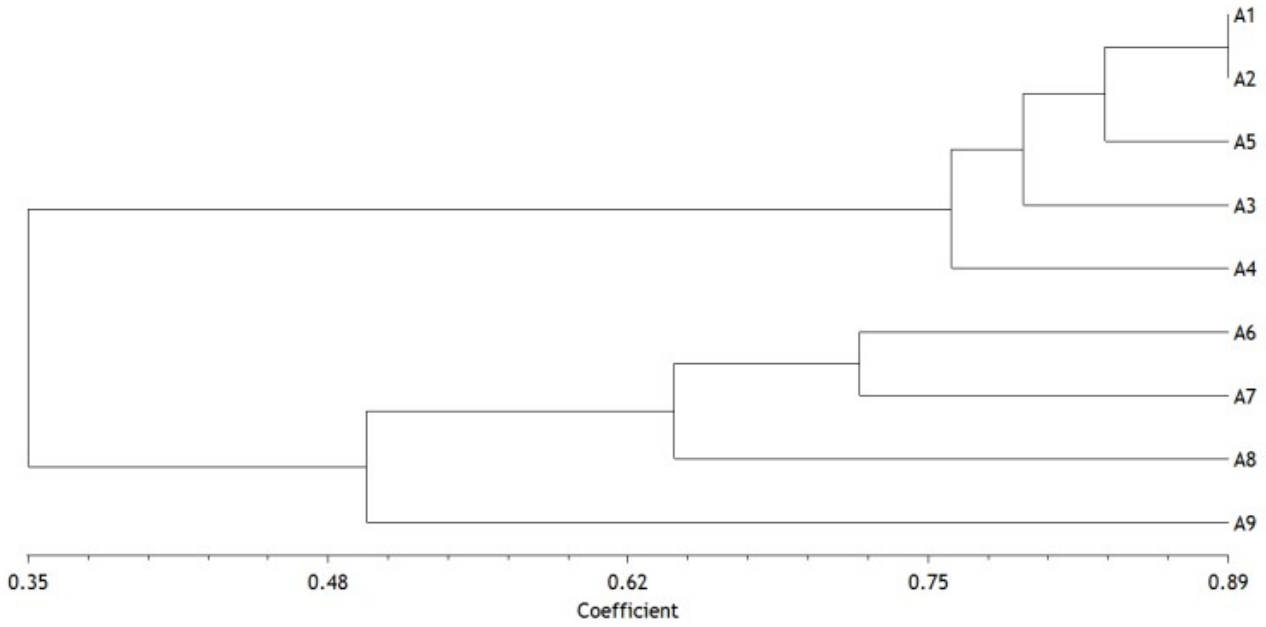

Figure 7. A UPGMA dendrogram of salak of Salak from West Seram District, Maluku, Indonesia revealed by RAPD markers. Note:A1 = Riring I, A2 = Riring II, A3 = Riring II, A4 = Rumahsoal I, A5 = Rumahsoal II, A6 = Taniwel, A7 = Neniari I, A8 = Neniari II, A9 = Soya

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Genetic diversity of *Amorphophallus titanum* in Bengkulu, Indonesia based on RAPD markers

WAHYUDI ARIANTO[1,2,♥], ERVIZAL A.M. ZUHUD[3], AGUS HIKMAT[3], TUTUT SUNARMINTO[2], ISKANDAR Z. SIREGAR[4,♥♥]

[1]Department Forestry, Universitas Bengkulu. Jl. WR. Supratman, Kota Bengkulu 38122, Bengkulu, Indonesia. Tel./fax.: +62-736-21170, [♥]email: bushido1968@yahoo.com

[2]Graduate School, Institut Pertanian Bogor. Jl. Lingkar Akademik, Kampus IPB, Dramaga, Bogor 16680, West Java, Indonesia.

[3]Department of Forest Resource Conservation and Ecotourism, Faculty of Forestry, Bogor Agricultural University (IPB). Jl. Lingkar Akademik Kampus IPB, Dramaga, Bogor 16680, West Java, Indonesia.

[4]Department of Silviculture, Faculty of Forestry, Institut Pertanian Bogor. Jl. Lingkar Akademik, Kampus IPB, Dramaga, Bogor 16680, West Java, Indonesia. Tel./fax.: +62-251-8327768, [♥♥]email: siregar@apps.ipb.ac.id

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Abstract. *Arianto W, Zuhud EAM, Hikmat A, Sunarminto T, Siregar IZ. 2018.* Genetic diversity of *Amorphophallus titanum* in Bengkulu, Indonesia based on RAPD markers. *Biodiversitas 19: 1783-1790.* Titan Arum [*Amorphophallus titanum* (Becc.) Becc. Ex Arcang], a plant species belonging to the family of Araceae is known for its gigantic floral size and elicited rotten fragrance when the flower bloom. Since it remains only found in Sumatran island, many authors categorized the plant as endemic species. The population of the species in the natural habitat has significantly declined because of the conversion of forest land mainly into plantations or other land uses. Considering the importance of conservation attempts to *A. titanum*, a sufficient data on genetic diversity of the species is necessary. The research was aimed to determine the genetic diversity within and among populations of *A. titanum* in some area of protected forests in Bengkulu Province, comprising the population of Palak Siring, Tebat Monok, and Air Selimang. RAPD genetic DNA fingerprinting approach was used to assess the genetic diversity of *A. titanum* using 13 preselected DNA primer: OPA 11, OPA 19, OPC 04, OPN 14, OPN 19, OPU 03, OPU 06, OPU 07, OPB 17, OPC 07, OPO 04, OPU03-1, OPNI 18E. The result revealed that the method has successfully produced several DNA fragments with varied length ranging from 250 bp to 2000 bp with 4-16 variation in polymorphic bands. Based on RAPD marker analysis, the population of Air Selimang was considered as a potential center of diversity of *A. titanum* because of the others two populations had a lower genetic diversity. In general, the genetic diversity among populations was lower than within population. The cluster analysis of the genetic similarity of 22 individuals of the three populations resulted in the separation into two main groups with the first group consisting of 17 individuals (Population Air Selimang and Tebat Monok) and the second group of 5 individuals (Palak Siring population).

Keywords: *Amorphophallus titanum*, genetic diversity, Random Amplified Polymorphic DNA

INTRODUCTION

Bunga bangkai, the local name of Titan Arum (*Amorphophallus titanum* (Becc.) Becc. Ex Arcang) is an important plant species belonging to the family of Araceae. The plant is known by its gigantic floral morphology characterized by a large sheathing bract, a spathe, wrapped the basal portion of the spadix (a racemose inflorescence having a lot of small flowers seating in a fleshy stem axis). The spadix reaches its vertical axis up to 1,6 m - 3 m the reason why it is considered as the plant with the tallest flower in the world (Barthlott and Lobin 1998; Arianto et al. 1999; Giardano 1999). Since 138 years after first discovered, the plant has significantly attracted many researchers in greenhouses or botanical gardens almost all over the world to study many aspects of the plant biology. The studies include plant morphology and anatomy, vegetative (spathe) and generative (spadix) growth and development (Gandawijaja et al. 1983; Barthlott and Lobin 1998; Hejnowicz and Barthlott 2005; Sholihin and Purwantoro 2005; Lobin et al. 2007; Claudel et al. 2012; Purwanto and Latifah 2013), thermogenesis (Barthlott 2009), floral odor analysis (Fujioka at al. 2012), germination (Latifah and Purwantoro 2015), micro-propagation (Irawati 2011), and estimation of genetic diversity in some populations (Poerban and Yuzammi 2008). The Plant have become symbols or flag species in many botanical gardens around the world in an attempt to attract as many visitors to the botanical gardens (Latifah and Purwantoro 2015).

Naturally, *A. titanum* is widespread over the Sumatra rainforest as understory growth in the calcareous soil below the forest canopy. However, the plants also occasionally found in open area, secondary forest, river bank, and in the edge of the road (Hidayat and Yuzammi 2008). Since it remains only found in Sumatran island, many authors categorized the plant as an endemic species (Barthlott and Lobin 1998; Hidayat and Yuzammi 2008). *A. titanum* has three successive phases, i.e., vegetative, dormant, and generative phase. The vegetative phase is an active green photosynthetic stage indicated by the emergence of a single leaf that grows for 6-12 months initiated in early raining season. The vegetative phase has responsibility for producing photosynthate and stored the sugar for

developing tuber. The underground tuber can reach 100 kg in weight. Following the detachment of the leaf, the dormant phase is beginning, and it is entirely underground tuber for 1-4 years before flowering. The generative phase or flower emergence is accidental and cannot be predicted (Bown 1988; Hetterscheid and Ittenbach 1996; Graham and Hadiah 2004).

Indonesia government designated *A. titanum* as a protected species according to Government Regulation No. 7/1999 (Appendix PP No. 7/1999) and Regulation of Ministry of Environment and Forestry Number 20/ MENLHK/SETJEN/KUM.1/6/2018 concerning in protected species of plants and animals. Based on the 1997 IUCN Red List of Threatened plants, *A. titanum* is classified into Vulnerable (VU). However, in 2002 this species was excluded from the IUCN list because of the lack of available data on population and its presence in nature.

Previous surveys indicated that there was a tendency that the population of *A. titanum* plants has become diminished. The conversion of natural forest for other land used has considered as significant contributor threatening their existence. (Hidayat and Yuzammi 2008). Therefore, if land use changes continue, it will threaten the species existence in nature. Real conservation effort is needed to protect the species in their natural habitat.

Genetic diversity is one aspect of biological diversity that is important for the conservation program (Dyke 2003). Conservation activities require sufficient information of the status of genetic diversity of target species (Heywood and Dullo 2005). Research into genetic diversity of *Amorphophallus* genera has been partially carried out, including *A. paenofiifolius* (Sugiyama et al. 2006), *A. muelleri* (Poerba and Martanti 2008), *A. rivieri* (Hu et al. 2011), *A. variabilis* (Santosa et al. 2012), *A. muelleri* (Wahyudi et al.2013), 35 species of *Amorphophallus* from China and Thailand (Mekkerdchoo et al. 2016), *A. paenofiifolius* (Mandal et al.2016), *A. paenofiifolius* (Santosa et al. 2017).

Research on genetic diversity of *A. titanum* is still relatively limited. A previous report on the genetic diversity of *A. titanum* was published by Poerba and Yuzammi (2008) using 22 accessions of *A. titanum* from West Sumatra and Bengkulu. The study only examines the RAPD profile and genetic dissimilarity analysis, but it was still lacking in discussing genetic diversity measures such as diversities within the population and among populations, genetic distances, and genetic population structures.

One approach that is still being used to determine the genetic diversity of *A. titanum* is Random Amplified Polymorphic DNA (RAPD) markers. The RAPD technique is cost-effective, easy and quick to assay, produces polymorphisms of DNA bands in large quantities, requires no knowledge of the genomic background being analyzed and is easy to obtain the random primers needed to analyze the genomes of all organism types (Tingey et al. 1994; Beebee and Rowe 2008). Although this method has many drawbacks, especially the consistency of its product amplification (Jones et al. 1997), optimizing extraction, well-prepared PCR conditions, and appropriate primer selection would overcome this limitation. The recent research was aimed to determine the genetic diversity of *A. titanum* using a genetic marker of Random Amplified Polymorphic DNA (RAPD).

MATERIALS AND METHODS

Study area

The sampling sites situated on three populations of the plants found in protected forest area in Bengkulu Province, Indonesia consisting of Air Selimang population and Tebat Monok population in Kepahiang District, as well as Palak Siring population in North Bengkulu District as shown in Figure 1. The number of individuals, geographical location, and altitude of the *A. titanum* population were shown in Table 1.

Procedures

Collection of leaflets samples of *A. titanum* was conducted in the 3 populations, namely population of Air Selimang (13 individuals), population of Tebat Monok (4 individuals) and population of Kepala Siring (5 individuals). From each plant, we took 2-3 leaflets, then the leaflets were cut in 2 cm x 2cm and then put in plastic clip bag with silica gel with a volume ratio of 1: 5 (Santoso et al. 2003).

Extraction of DNA

Genomic DNA was extracted using modified CTAB (Cetyl Trimethyl Ammonium Bromide) method referring to Weising et al. (2005) and Aritonang et al. (2007).

Test of DNA quality

The DNA quality test was initiated by preparing agarose 1% (0.33 g agarose in 33 mL buffer TAE) being diluted in microwave for 3 minutes. Afterward, GelRed was added as much as 0.5 µL and decanted into gel mold until being viscous (± 10 minutes). In the electrophoresis process, DNA was taken as much as 3 µL. Afterward, 1 µL blue juice was added, mixed and put into gel well. Electrophoresis ran for 20 minutes. After electrophoresis finished, the gel was lifted and the DNA bands were documented under UV transilluminator TPX - 20. LM.

Polymerase Chain Reaction (PCR)

Extraction results DNA were amplified using machine AB Applied Biosystem Veriti TM Thermal Cycler (www.aapliedbiosystem.com). As many as 13 primers (Table 2) from Operon Technology Ltd being used were OPA-11, OPA-19,OPC-04,OPN-14, OPN-19,OPU-03, OPU-06, OPU-07,OPB-17,OPC-07, OPO-04, OPU03-1,OPNI-18E with annealing temperature of 36℃-38℃.

Data analysis

The interpretation of the RAPD profiles used a binary variable based on the presence or absence of amplification products. The value is one if the band present and zero if it absent. The binary data were analyzed using POPGENE 32 version 1.31 software (Yeh and Yang 1999). Ntedits

version 1.07c (Jamshidi and Jamshidi 2011) and NTSys version 2.0 (Rohlf 1997) and Structure version 2.3.4 (Pritchard et al. 2010). The inter and intrapopulation diversity of genetic distance data generated from POPGENE is used for Clustering analysis with Unweighted Pair method. The Group Method with Arithmetic Mean (UPGMA) uses NTsys version 2.0 which will produce a dendrogram. Population structure analysis using STRUCTURE version 2.3.4 software (Pritchard et al. 2010).

Table 1. Number of sample *Amorphophallus titanum*, geographic position, and altitude of the growing site

Population sites	No. of samples	Geographic coordinate		Altitude (m asl.)
		Latitude	Longitude	
Palak Siring	5	3º25'14.05	102º15'48.51	374-406
Tebat Monok	4	3º40'16,98	102º33'26.27	655-661
Air Selimang	13	3º45'43,51	102º37'11,58	773-804

Note: asl: above sea level

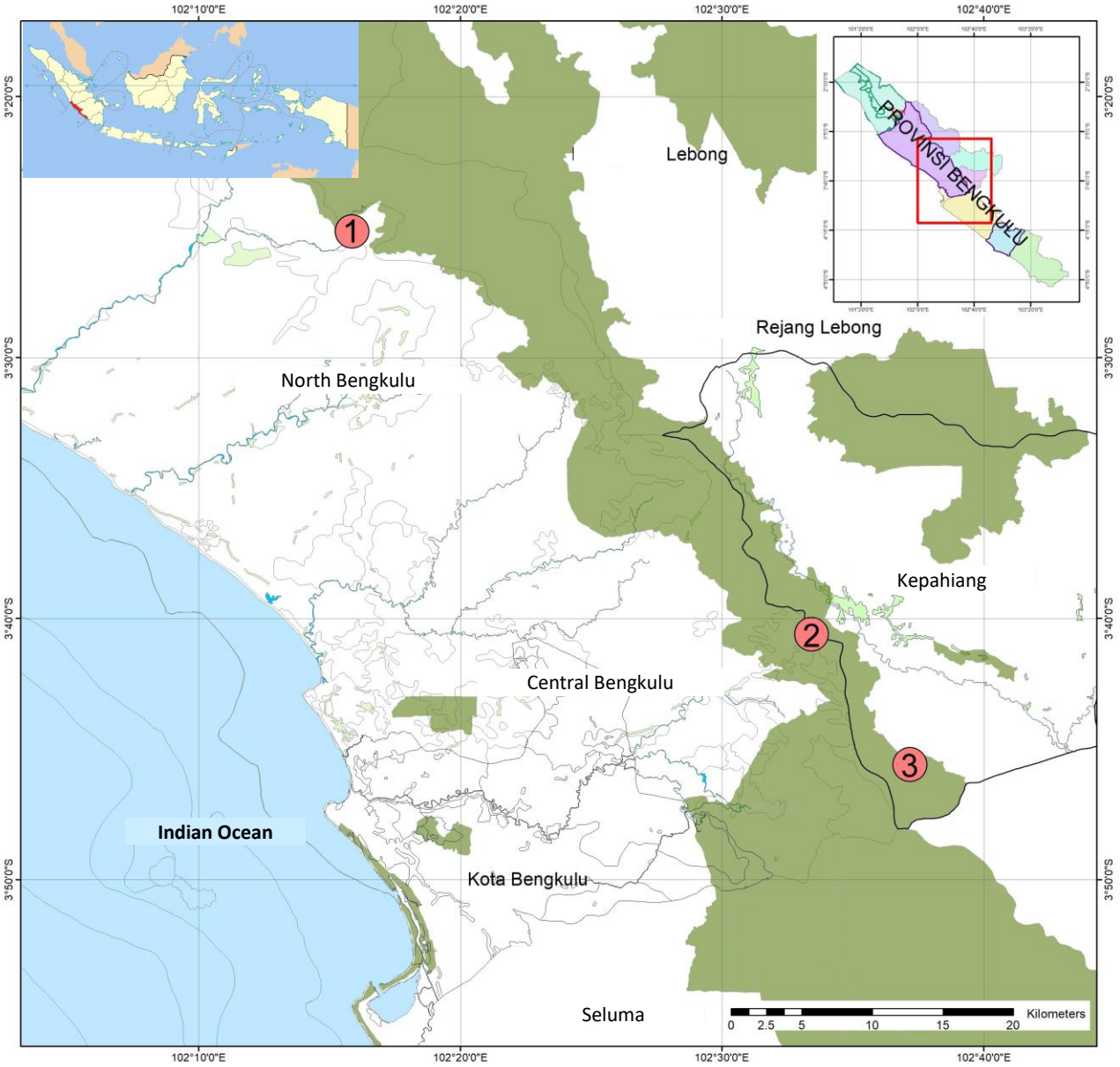

Figure 1. The selected sampling sites of *Amorphophallus titanum* in protected forest area in Bengkulu Province, Indonesia. 1. Palak Siring, 2. Tebat Monok, 3. Air Selimang

RESULTS AND DISCUSSIONS

RAPD profile

Amplification of total DNA genome using 13 RAPD primers in 22 *A. titanum* samples produced clear and reproducible PCR products as presented in Figure 2.

The result revealed that there were 124 DNA fragments with length ranging from 250 bp (base pair) up to 2000 bp with 75-100% polymorphic DNA (Table 3). The appropriate temperature for these 13 primers is 36℃ and 38ºC. The results showed that the RAPD markers used had high levels of polymorphism. On average each primer produces 9.5 bands. The highest number of polymorphic bands (n=16) is found on OPA primer 19, while the lowest band number (n=4) is present in OPU03-1 primer. Based on Poerba and Yuzammi (2008), eight RAPD primers on 25 accessions of *A. titanum*, produced successfully 143 DNA fragments of 100 bp to 1.1 Kb, which has 137 (95.80%) polymorphic bands. Similar results were reported by Poerba and Martanti (2008) in *Amorphophallus muelleri*, 5 RAPDs used had 69.05% polymorphism bands and 30.95% monomorphic bands. Based on research of Mekkerdchoo et al. (2013) on 35 species of *Amorphophallus* spp in China and Thailand obtained 269 bands ranging from 150 to 5000 bp and All amplified fragments were found to have 100% polymorphic bands. In *A. albus* found of a total of 154 bands scored, which ranged from 150bp to 2 kb and averaged 7.3 bands per primer, 32 were polymorphic with 20.8% polymorphism (Hu et al. 2008). In *A. paeoniifolius* using ten microsatellite loci found all loci produced highly polymorphic alleles (Santoso et al. 2003). The existence of a polymorphic gene means that some individuals in the population have heterozygous genes. All levels of genetic variation contributing to the population's ability to adapt to environmental changes (Wise et al. 2002).

Table 2. Preselected RAPD Primer used in this study

Primers	Primer Sequence (5'-3')	Length of primer (bp)	T Annealing (°C)	Number of DNA fragments	Polymorphic DNA fragment (%)
OPA 11	CAA TCG CCG T	300-1500	36	8	100
OPA 19	CAA ACG TCG G	250-1500	36	16	100
OPC 04	CCG CAT CTA C	250-2000	36	15	100
OPN 14	TCG TGC GGG T	300-2000	38	11	100
OPN19	GTC CGT ACT G	300-1500	36	9	100
OPU 03	CTA TGC CGA C	350-1650	36	6	100
OPU06	ACC ITT GCG G	300-2000	36	10	100
OPU07	CCT GCT CAT C	300-2000	36	10	100
OPB17	AGG GAA CGA G	200-1850	36	10	100
OPC07	CAC ACT CCA G	300-1900	36	8	100
OPO 04	TCT GGT GAG G	250-1900	36	9	100
OPU03-1	CTA TGC CGA C	400-1900	36	4	(3) 75
OPN18E	AAG GTG AGG TCA	300-2000	38	8	(6) 75
				124	

Table 3. Comparison of primers for RAPD and their amplification products in several studies of *Amorphophallus titanum*

Primer	Sequence base	Fragment length (bp)	Poerba and Yuzammi (2008)		Recent research	
			Total DNA fragment	Polymorphic DNA (%)	Total DNA fragment	Polymorphic DNA (%)
OPA-11	CAA TCG CCG T	300-1500	21	(20) 95.24	8	100
OPA-19	CAA ACG TCG G	250-1500	20	100	16	100
OPC-04	CCG CAT CTA C	250-2000	14	100	15	100
OPN-14	TCG TGC GGG T	300-2000	15	(13) 86.67	11	100
OPN-19	GTC CGT ACT G	300-1500	16	100	9	100
OPU-03	CTA TGC CGA C	350-1650	13	100	6	100
OPU-06	ACC ITT GCG G	300-2000	21	(20) 95.24	10	100
OPU-07	CCT GCT CAT C	300-2000	23	100	10	100
OPB-17	AGG GAA CGA G	200-1850	-	-	10	100
OPC-07	CAC ACT CCA G	300-1900	-	-	8	100
OPO-04	TCT GGT GAG G	250-1900	-	-	9	100
OPU-03-1	CTA TGC CGA C	400-1900	-	-	4	(3) 75
OPN-18E	AAG GTG AGG TCA	300-2000	-	-	8	(6) 75
Total			143	137 (95.80)	124	

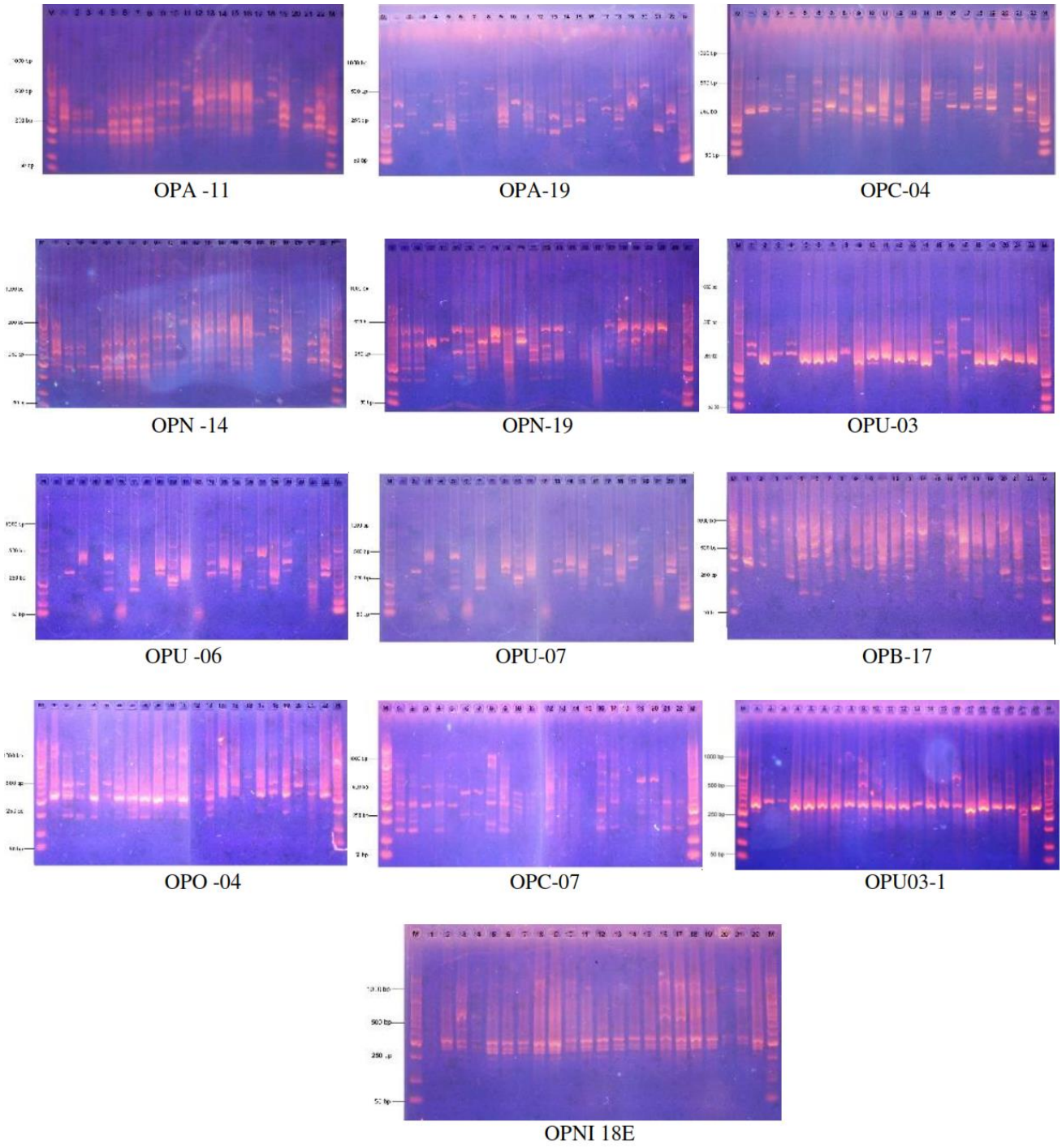

Figure 2. RAPD Amplification product of PCR using 13 primers, line [1-13] Air Selimang, line [14-17] Tebat Monok, line [18-22] Palak Siring. M: Gene Ruler 50 bp DNA Ladder

All preselected thirteen primers has successfully produced 4-16 detectable DNA bands. The highest number of RAPD bands (16 bands) was amplified by primer OPA-19 while the lowest one (4 bands) was resulted by primer OPU03-1 (Table 3). Based on the results of Poerba and Yuzammi's (2008), the number of maximum DNA fragments found in OPU-07 (23 bands), and the minimum number of fragments found in the OPU-03 primer (13 bands). In *A. muelleri* produces 6-11 DNA bands that can be detected and scored, where the maximum number of polymorphic bands 9 is found in primer OPD-04 (Poerba and Martanti 2008). The number and intensity of DNA bands depend on how the primer recognizes its complementary DNA sequence in the DNA of the template used.

Genetic diversity within the population

Genetic diversity of *A. titanum* in Bengkulu varied for each population (Table 4). In Table 4, the population of *A. titanum* in Air Selimang has the highest value for all parameters of genetic diversity (Finkeldey 2005), they were He (0.245), Ne (1.398), PLP (86.29%), Na (1.863) and I (0.381). This condition indicates that the Air Selimang area is probably as one of the centers of *A. titanum* diversity in Bengkulu. A previous report by Poerba and Yuzammi (2008) indicated that value of genetic inequality (dissimilarity) between populations ranges from 0.24-0.52. Genetic diversity in *A. muelleri* ranges from 0.1019 ± 0.1727 to 0.1832 ± 0.2054 (Poerba and Martanti 2008). The amount of genetic diversity in the population is determined by the number of genes that have more than one allele (polymorphic genes).

The lowest genetic diversity was found in the Tebat Monok population with He (0.166), Ne (1,265), PLP (52.42%) and Na (1,524). This is probably due to the Tebat Monok population coming from the same parent. According to Milot et al. 2007, low genetic diversity is predicted to have a negative impact on species viability, and this has become a major concern for conservation.

The high genetic diversity in the Air Selimang population is likely to be influenced by the number of individual per populations that are higher than the other two locations (Palak Siring and Tebat Monok).

Genetic diversity among populations

The total value of genetic diversity in all populations (Ht) (Air Selimang, Tebat Monok, and Palak Siring) is 0.253 with the mean genetic diversity in the population (Hs) is 0.213. The value of genetic diversity between populations (Dst) is 0.040; this value is much lower when compared with the value of Ht and Hs. Genetic differentiation between populations (Gst) is 0.1567 or 15.67%. This means that, in *A. titanum*, a 15.67% differentiation among populations exist. Based on the Gst value, the gene flow level (Nm) is 2,692 (Nm> 1) (Wu et al.2014). These results suggest that gene flow and low differences exist between populations.

The cluster analysis of 22 accessions (individuals) of *A. titanum* in three populations (Figure 3) had the similarity coefficient ranged from 0.02 to 0.5. The accession that has the closest similarities with a coefficient value of 0.022 is found in the Palak Siring collection, i.e. PS 5 with PS 2 and PS3. In the coefficient of similarity 0.452, the 22 individual *A. titanum* from three locations were separated into 3 clusters, the C cluster is the accession sampled from Air Selimang (AS1, AS2, AS3, AS7, AS8, AS9, AS10, AS11, AS12, AS13), the D cluster is filled by accession from Tebat Monok (TM1, TM2, TM3, TM3) and The B cluster is an accessions from Palak Siring (PS1, PS2, PS3, PS4 and PS5). In coefficient 0,476 Air Selimang and Tebat Monok joined in a single cluster, they separated with Palak Siring population. This grouping indicates that Air Selimang and Tebat Monok have a close relationship if compared with Palasiring population.

Genetic distance

Table 6 indicates that the population of Air Selimang and Tebat Monok has the closest genetic distance that is 0.0513 if it is compared to the genetic distance between the population of Tebat Monok with Palasiring, i.e., 0.0932 or Air Selimang to Palak Siring, i.e., 0.0886. If we look at the data of geographical distance shows the same pattern with genetic distance. The population of Air Selimang to Tebat Monok has the closest geographical distance, i.e., 12.20 km than the geographical distance between Air Selimang with Palak Siring, i.e., 54.45 km. Based on these results, it can argue that the value of genetic distance and geographical distance are positively correlated. The genetic distance in *A. muelleri* ranges from 0.0255 to 0.3593 (Poerba and Martanti 2008). This result supports the previous statement by Schnabel and Hamrick (1990) and Alpert et al. (1993), the genetic distance correlates with geographical distance.

Table 4. Parameter value of genetic diversity *Amorphophallus titanum* population

Population	PLP (%)	N	Na	Ne	He	I
Air Selimang	86.29	13	1.863	1.398	0.245	0.381
Palak Siring	70.97	5	1.709	1.376	0.229	0.352
Tebat Monok	52.42	4	1.524	1.265	0.166	0.257
Average	69.893	-	1.699	1.346	0.213	0.330

Note: N: Number of the individual. Na: Observed number of Allele Ne: Effective number of the allele, PLP; Percentage Locus Polymorphic, He: expected heterozygosity, I: Shannon's index

Table 6. Genetic distance based on Nei's Unbiased Measures and geographical distance (Km) among *Amorphophallus titanum* population

Population	Air Selimang	Tebat Monok	Palak Siring
Air Selimang	*	$12,20^{D}$	$54,45^{D}$
Tebat Monok	0.0513^{d}	*	$42,20^{D}$
Palak Siring	0.0886^{d}	0.0932^{d}	*

Note: ^{d} is value of genetic distance and $^{D)}$ is value of geographical distance

Table 5. The mean value of genetic diversity based on analysis of Nei (1978) using RAPD marker

Species	Location	Ht	Hs	Gst	Dst	Nm
Amorphophallus titanum	Air Selimang, Palak Siring, and Tebat Monok	0.253	0.213	0.157	0.040	2.692

Note: Ht: value of genetic diversity in all population; Hs: genetic diversity within population; Dst: genetic diversity among population; Gst: genetic differentiation; Nm: Gene flow

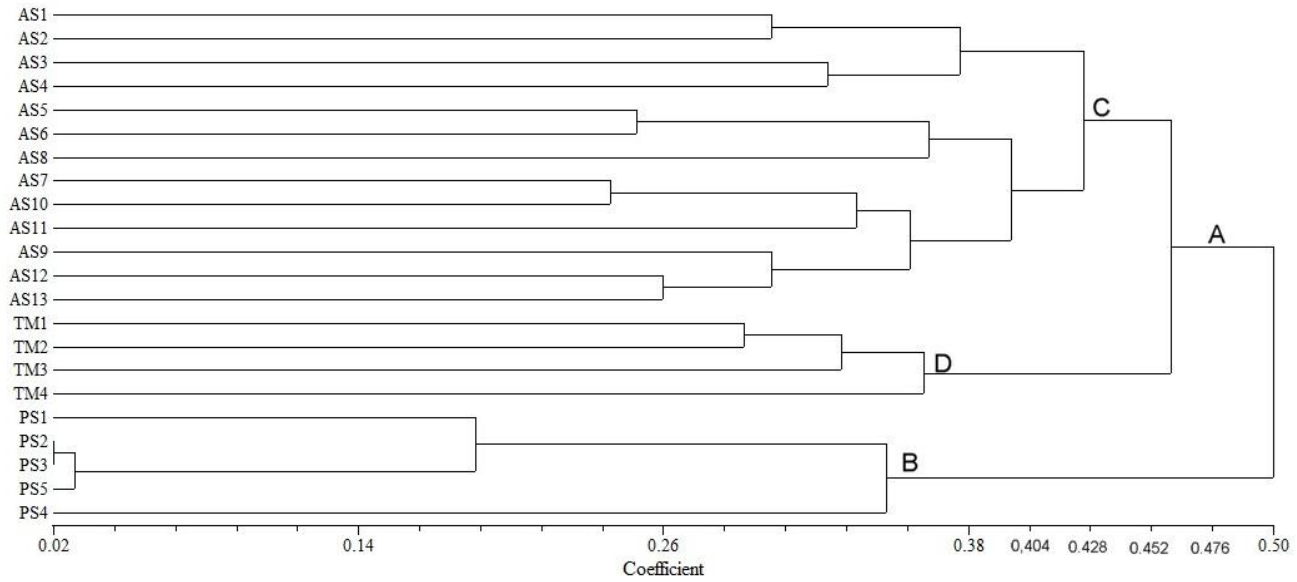

Figure 3. Dendrogram UPGMA of 22 *Amorphophallus titanum* accessions in all location. Note: Accession AS1-AS13: Air Selimang, TM 1-TM4: Tebat Monok, PS1-PS5: Palak Siring

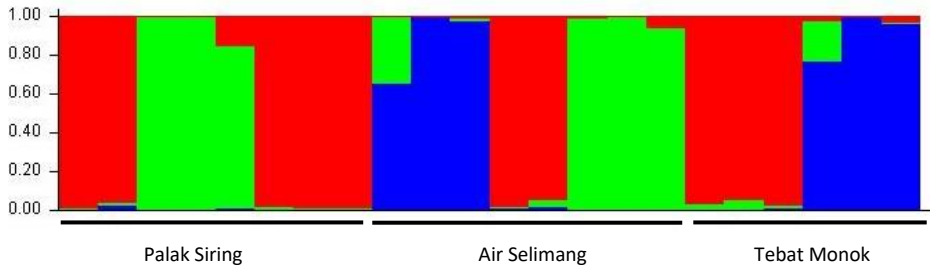

Figure 4. Bayesian clustering Analysis among three populations of *Amorphophallus titanum* using STRUCTURE (K=3)

The structure of population genetics

Structure harvester is used to assess the level of genetic stratification in multi-locus datasets. The result of harvester structure analysis shows that the best dataset number for the three *A. titanum* populations is K = 3 (DeltaK 3 = 29.230). This condition indicates that the three population of *A. titanum* (Air Selimang, Tebat Monok, and Palak Siring) consisting of 22 individuals can be divided into 3 clusters, namely Air Selimang in the first cluster, Tebat Monok in the second cluster, and the remaining third cluster for Palak Siring. The same color pattern in figure 4 illustrates the population has a general genetic structure. The genetic structure of the population is influenced by several factors such as the mating system, genetic drift, population size, seed distribution, gene flow, evolutionary history and natural selection (Hamrick and Godt 1990).

In conclusion, the analysis of genetic diversity of three *A. titanum* populations in Protected Forest Areas in Bengkulu Province revealed that the Air Selimang Population (He = 0.245) was defined as the potential center of genetic diversity of *A. titanum*, because it has the highest diversity value, while the population of Tebat Monok (He = 0.166) has the lowest genetic diversity. The average genetic diversity among populations is lower than the genetic diversity within a population. The results of the clustering analysis of *A. titanum* produced two clusters, where the Palak Siring population is separated from the population of *A. titanum* Tebat Monok and Air Selimang.

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Diversity of ambrosia beetles (Coleoptera: Scolytidae) on teak forest in Malang District, East Java, Indonesia

YOGO SETIAWAN, RINA RACHMAWATI, HAGUS TARNO♥

Department Plant Pest and Disease, Faculty of Agriculture, Universitas Brawijaya. Jl. Veteran, Malang 65145, East Java, Indonesia. Tel.: +62-341-551665, 565845. Fax.: +62-341-560011, ♥email: h_gustarno@ub.ac.id, yogosinaga@gmail.com

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Abstract. *Setiawan Y, Rachmawati R, Tarno H. 2018. Diversity of* ambrosia beetles *(Coleoptera: Scolytidae) on teak forest in Malang District, East Java, Indonesia. Biodiversitas 19: 1791-1797.* Ambrosia beetle plays an important role in the temperate forest. Ambrosia beetle lives symbiotically with microorganism such as fungi, bacterium, and yeast that can cause plant wilt and death. In Java, ambrosia beetle has been reported to attack teak plantations in some regions. This research aimed to investigate the diversity of ambrosia beetles in the teak plant on monoculture and polyculture system in Malang District. This research was conducted in the teak forest in Dampit and Sumbermanjing Wetan, Malang District from March to May 2017. Ambrosia beetles were trapped by using baited bottle trap with 95% ethanol. The diversity of ambrosia beetles trapped was analyzed using Vegan package in R program to calculate the Shannon-Wiener diversity index (H), Species Evenness index (E), and Simpson's dominance index (D). The results showed that ambrosia beetles trapped in monoculture and polyculture teak plants system consist of nine species, i.e., *Xylosandrus crassiusculus, X. morigerus, X. compactus, Xyleborus perforans, Euwallacea simillis, Xyleborinus andrewesi, Premnobius cavipennis, Coccotrypes distinctus,* and *Hypothenemus hampei*. The Shannon Wiener index of Ambrosia beetles in polyculture (H=1.40) was higher than in monoculture (H=1.30), and both locations were categorized in the medium diversity category. The Species Evenness index of ambrosia beetles in polyculture (E=0.67) and monoculture (E=0.66) were also categorized in medium category. The Simpson's dominance index in both locations was categorized in the middle dominance species. *X. crassiusculus* was the dominant species in polyculture and monoculture teak plant system.

Keywords: Ambrosia beetles, diversity, Scolytidae, teak forest

INTRODUCTION

Ambrosia beetles (Coleoptera: Scolytidae) play an important role in the temperate forest ecosystem and also cause substantial economic losses (Lindgren and Raffa 2013). Ambrosia beetles live symbiotically with microorganisms such as fungi, bacterium, and yeast and cause major plant diseases that can cause plant wilt and die (Tarno et al. 2016). They are abundant in the tropics region, and responsible for economic damage to the timber and wood industry (Bumrungsri et al. 2008). Worldwide, the ambrosia beetles contain 7400 species, and over 6000 species belong to Scolytidae (Kirkendall et al. 2015). Ambrosia beetles have been reported to attack many forest plants and teak plantation (Nair 2007).

Tectona grandis L. is native in Indonesia especially in Java and Muna, but teak forest mostly found in Java (Pratiwi and Lust 1994). Teak is one of the most important tropical hardwood tree in the international market as the high-quality timber. This plant has aesthetic values that make it the precious wood for forestry plantation (Bermejo et al. 2004). In Malang District, the plantation teak forests grow in monoculture and polyculture system and carry out by Perum Perhutani.

In several countries, teak plants have been attacked by Scolytidae. In India, teak plants were attacked by five species of ambrosia beetles, i.e. *Trachipholis hisida, Cryphalus tectonae, Xyleborus velatus, Xy. naxius and Xy. hagedorni* (Patil et al. 2016). *Xylosandrus* sp. and *Hypothenemus* sp. were also reported attack teak plant in Ghana and Mexico (Nair 2007). *Xy. Destruens* was reported in the occurrence of the borer of teak plantations growing in South Malang and some regions in Java and now is widely distributed (Kalshoven 1981). They are attacking living teak plant and making branching tunnels that extend into the heartwood (Nair 2007).

Outbreaks of ambrosia beetle population are difficult to detect or forecast, and application of insecticidal treatments perform poorly (Werle et al. 2011). Baited traps with chemical attractants commonly are as an effective control used to capture ambrosia beetles for monitoring, studying population dynamics, predicting outbreaks, and mass trapping to reduce damage (Burbano et al., 2012). Some studies have shown that an ethanol-baited trap can collect a variety of ambrosia beetle species thus it can be facilitated the monitoring of beetle populations (Reding et al. 2011; Werle et al. 2011; Galko 2014).

There is no recent report about ambrosia beetle that attacks teak plant grown in monoculture and polyculture in Malang District. Studies on the diversity of ambrosia beetles in forestry plants especially in the teak plantation are needed because ambrosia beetle is a pest that can damage and increase the economic losses of wood. This research aimed to investigate the diversity of ambrosia beetles in monoculture and polyculture teak plant system in Malang District, East Java, Indonesia.

MATERIALS AND METHODS

Study site

This research was conducted in the teak forests in two Subdistricts in Malang District, East Java, Indonesia (Dampit and Sumbermanjing Wetan) from March to May 2017. The identification of specimens was done in the Laboratory of Plant Pest, Faculty of Agriculture, Brawijaya University. Dampit Subdistrict was monoculture teak plant system, while Sumbermanjing Wetan Subdistrict was polyculture teak plant system with coffee (*Coffea arabica* L.) and mahogany (*Swietenia mahogany* L.). Locations of observations were described in Figure 1 and Table 1.

Procedures: Ambrosia beetles collection and identification

Ambrosia beetles were trapped by using baited bottle trap with 95% ethanol that tied up in teak plants approximately 1 m above the ground (Figure 2) (Oliver et al. 2004; Steininger 2015). At each site, 20 ethanol-bait bottle traps were deployed, and each site was divided into two plots. Each plot was set up as about 10 traps with the distance between the trap was approximately 20 m. Samples were taken eight times with three days interval a month. The trapped ambrosia beetles in bottle trap were collected and fixed on 70% ethanol in small tubes. Identification of ambrosia beetle was based on morphological character, i.e. body size, elytra, and color of the body using the identification keys of Ambrosia beetles (Rabaglia et al. 2006; Wood 2007; Hulcr and Smith 2010).

Table 1. Details of ambrosia beetles sampling in monoculture and polyculture teak plant system, Malang District, East Java, Indonesia

Sites	Plots	Altitude (m asl.)	GPS coordinates
Monoculture	M1	396	06° 87'79.0" S, 90° 90'92.8" W
	M2	392	06° 87'55.3" S, 90° 91'10.6" W
Polyculture	P1	401	06° 85'80.1" S, 90° 89'46.9" W
	P2	403	06° 85'70.6" S, 90° 89'36.1" W

Note: M1: Monoculture plot 1, M2: Monoculture plot 2, P1: Polyculture plot 1, P2: Polyculture plot 2 and Masl: Meters above sea level

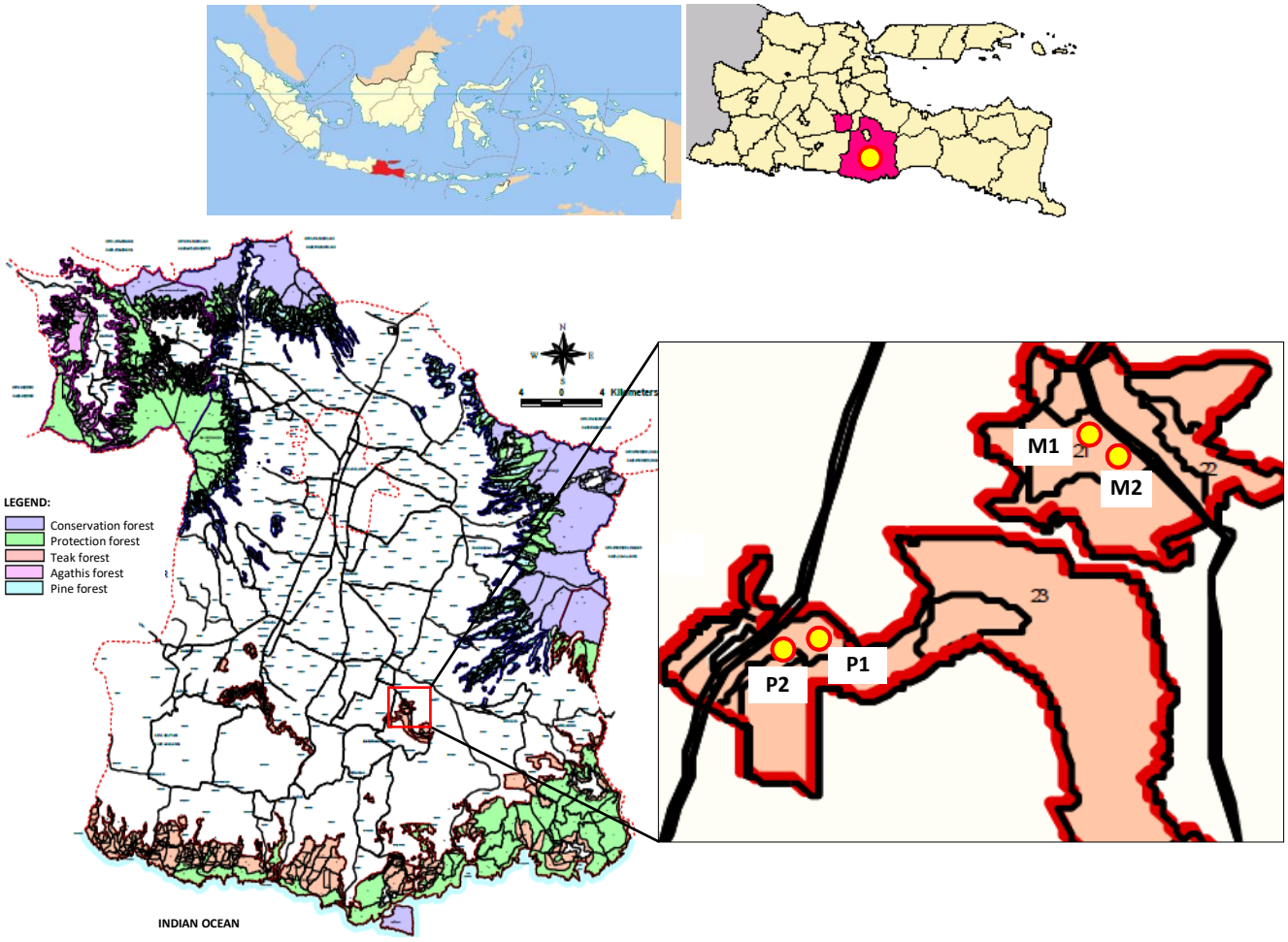

Figure 1. Sampling site in Dampit and Sumbermanjing Wetan Sub-district, Malang District, East Java, Indonesia. M1: Monoculture plot 1, M2: Monoculture plot 2, P1: Polyculture plot 1 and P2: Polyculture plot 2 (Source: Perum Perhutani KPH Malang 2002)

Figure 2. Ethanol bait bottle trap was used to collect ambrosia beetles

Data analysis

Diversity of ambrosia beetles were analyzed using R program with Vegan package to calculate the value of Shannon Wiener diversity index (H), Species Evenness index (E) and Simpson's dominance index (D) (Oksanen 2015; R Core Team 2017).

RESULTS AND DISCUSSION

Diversity and abundance of ambrosia beetles in monoculture and polyculture teak plant system

Species abundance of ambrosia beetles found in the monoculture and polyculture teak plant system was different. Polyculture teak plantations system had higher number of species and individual than monoculture system. The most abundant species in both locations was *X. crassiusculus* (Table 2).

The polyculture teak plantation system had higher Shannon Wiener diversity index than monoculture (Table 3). Both of them were categorized in the medium diversity category because the individual distribution for each species was in the middle level. This showed that the ecosystem of both locations was unstable. According to Tarno et al. (2016), the value of diversity index between 1 and 3 is categorized in the middle level of diversity and middle level of individual distribution for each species. Teak forest in Indonesia is an industrial plantation, and there are human activities such as the cultivation of coffee and seasonal crops so the ecosystem of teak forests is unstable. The Species Evenness index of ambrosia beetles in both locations was also categorized in medium category. Tarno et al. (2016) stated that the value of Evenness index between 0.50 and 0.75, is categorized in the medium level. It means that the community is unstable. The Simpson's dominance index in both locations was categorized in middle dominance of species (Table 3). It showed that there were still dominant species in both locations, although even in the medium category. Dominance species in both locations caused by the ecosystem in teak forests unstable, those the species has not been distributed evenly.

Flight activity of three dominant species of ambrosia beetles (*X. crassiusculus, E. similis,* and *X. morigerus*) in monoculture and polyculture teak plant system

During the collection of ambrosia beetle, flight activity of *X. crassiusculus, E. similis,* and *X. morigerus* fluctuated. Flight activity of *X. crassiusculus* had different pattern with *E. similis* and *X. morigerus*. In both locations, *X. crassiusculus* had the high flight activity on 11 March 2017 (Figure 3), while the highest flight activity of *E. similis* and *X. morigerus* was on 22 March 2017.

Morphological character of ambrosia beetles

Identification of ambrosia beetles was based on morphological character which featured i.e. body size, elytra, and color of the body.

Xylosandrus crassiusculus. Body length was 2.8 mm. Body color was reddish brown. Obsolete was on declivity. Declivity surface was dull, surface with dense, confused small tubercles uniformly distributed from base to apex. Body was less stout. Elytra were longer than pronotum. Declivity was more sloping, and uniformly distributed granules (Figure 4.A).

Table 2. Species diversity and abundance of ambrosia beetles in monoculture and polyculture teak plant system of Malang District, East Java, Indonesia

Species	Abundance	
	Monoculture	Polyculture
Xylosandrus crassiusculus	82	138
Xylosandrus morigerus	18	25
Xylosandrus compactus	4	9
Xyleborinus andrewesi	5	0
Euwallacea similis	22	32
Xyleborus perforans	8	18
Premnobius cavipennis	0	5
Coccotrypes distinctus	0	1
Hypothenemus hampei	2	12
Total	141	240

Table 3. Diversity of ambrosia beetles in monoculture and polyculture teak plant system of Malang District, East Java, Indonesia

Sites	Number of species	Index value		
		H	E	D
Monoculture	7	1.31	0.67	0.61
Polyculture	8	1.40	0.66	0.63

Note: H: Shannon Wiener diversity index, E: Species Evenness index and D: Simpson's dominance index

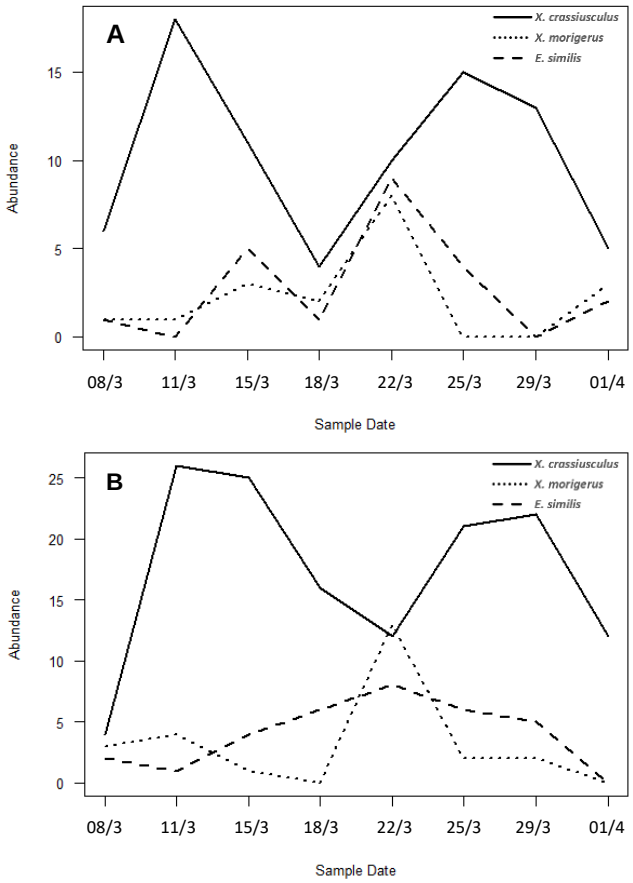

Figure 3. Flight activity of *X. crassiusculus*, *E. similis*, and *X. morigerus* from March to April 2017in Malang District, East Java, Indonesia; A. Monoculture, B. Polyculture

According to Wood (2007), Rabaglia et al. (2006), and Hulcr and Smith (2010), ambrosia beetle with these morphological characters is *X. crassiusculus.* The present distribution of *X. crassiusculus* is in southern Asia, Africa, Indonesia, Australia, Islands of the Pacific, Europe, and the U.S (Horn and Horn 2006; Gomes et al. 2018).

Xylosandrus morigerus. Body length was 1.6 mm. Declivity was commencing one-third elytra length from base. Body color was yellowish (Figure 4.B).

According to Wood (2007) and Hulcr and Smith (2010), ambrosia beetle with these morphological characters is *X. morigerus.* The present distribution of *X. morigerus* is in Tropical Africa, SE Asia to Micronesia, Hawaii, Mexico to Venezuela, Brazil, Madagascar, Indomalaysian Region, Pacific and South America (Beaver and Browne 1978; Wood 2007).

Xylosandrus compactus. Body length was 1.6 mm. Body color was black. Elytra was more slender, evenly arched from middle of disc to apex. Posterior portion of pronotum was shining (Figure 4.C).

According to Wood (2007), Rabaglia et al. (2006), and Hulcr and Smith (2010), ambrosia beetle with these morphological characters was *X. compactus.* The present distribution of *X. compactus* in tropical Africa, Indomalaysian region, Fiji, Samoa, United States (introduced). Its pantropical distribution includes Brazil, Cuba, Indonesia, Japan, and Sri Lanka (Ceylon) and the occurrence of this species in Java was reported by Beaver and Browne (1978), Dixon et al. (2003), Kalshoven (1981), Wood (2007), and Gomes et al. (2018).

Euwallacea similis. The body length was 2.3 mm. Body color was dark brown. Most of postolateral declivital margin were rounded. Declivity face basically was convex and have two pairs of tubercle. Pronotum was commonly subquadrate (Figure 4.D).

According to Wood (2007), Rabaglia et al. (2006), and Hulcr and Smith (2010), ambrosia beetle with these morphological characters was *E. similis.* The present distribution of *E. similis* in Africa; tropical Asia; North America (introduced): Texas; Oceania; South America (introduced): Brazil and North Australia to Micronesia (Wood 2007; Gomes et al. 2018).

Xyleborus perforans. Body length was 2.2 mm. Body was more slender and yellowish brown. Declivity was rather steep and dull, broadly convex. Surface was rarely shining and opaque (not shiny) when dry, with small tubercle in elytra declivity (Figure 4.E).

According to Wood (2007), Hulcr and Smith (2010), and Bateman and Hulcr (2014), ambrosia beetle with these morphological characters is *Xy. perforans.* The present distribution of *Xy. Perforans* is in circum tropical areas such as Southeast Asia and widespread throughout in Java, Indonesia (Beaver and Browne 1975; Beaver and Browne 1978; Kalshoven 1981).

Premnobius cavipennis. Body length was 2.8 mm. Body color was reddish brown.; Frons were broadly convex. Elytra declivity was broadly, rather deeply, concavely excavated on posterior third of elytra length. Antennal club was strongly fattened (Figure 4.F).

According to Rabaglia et al. (2006), Wood (2007), and Hulcr and Smith (2010), ambrosia beetle with these morphological characters is *P. cavipennis.* The distribution of *P. cavipennis* is in Tropical Africa, introduced to South America (Colombia and Venezuela to Bolivia and Brazil). It is possible the species is also introduced to Asia (Wood 2007).

Xyleborinus andrewesi. Body length was 1.9 mm. Body was dark brown, and elongate-cylindrical. Small knob was surrounded by hair between pronotum and abdomen. Scutellum was depressed below the level of the elytra surface (Figure 4.G).

According to Wood (2007), Hulcr and Smith (2010), and Bateman and Hulcr (2014)**,** ambrosia beetle with these morphological characters is *Xyl. andrewesi. Xyl. andrewesi* previously has been recorded in Andaman Islands, Bangladesh, Burma, China, India, Indonesia, Japan, Malaya, Micronesia, Nepal, New Guinea, Philippine Islands, Ryukyu Islands, Seychelles Islands, Sri Lanka, Thailand, Vietnam and New Zealand (Okins and Thomas 2009; Gomes et al. 2018).

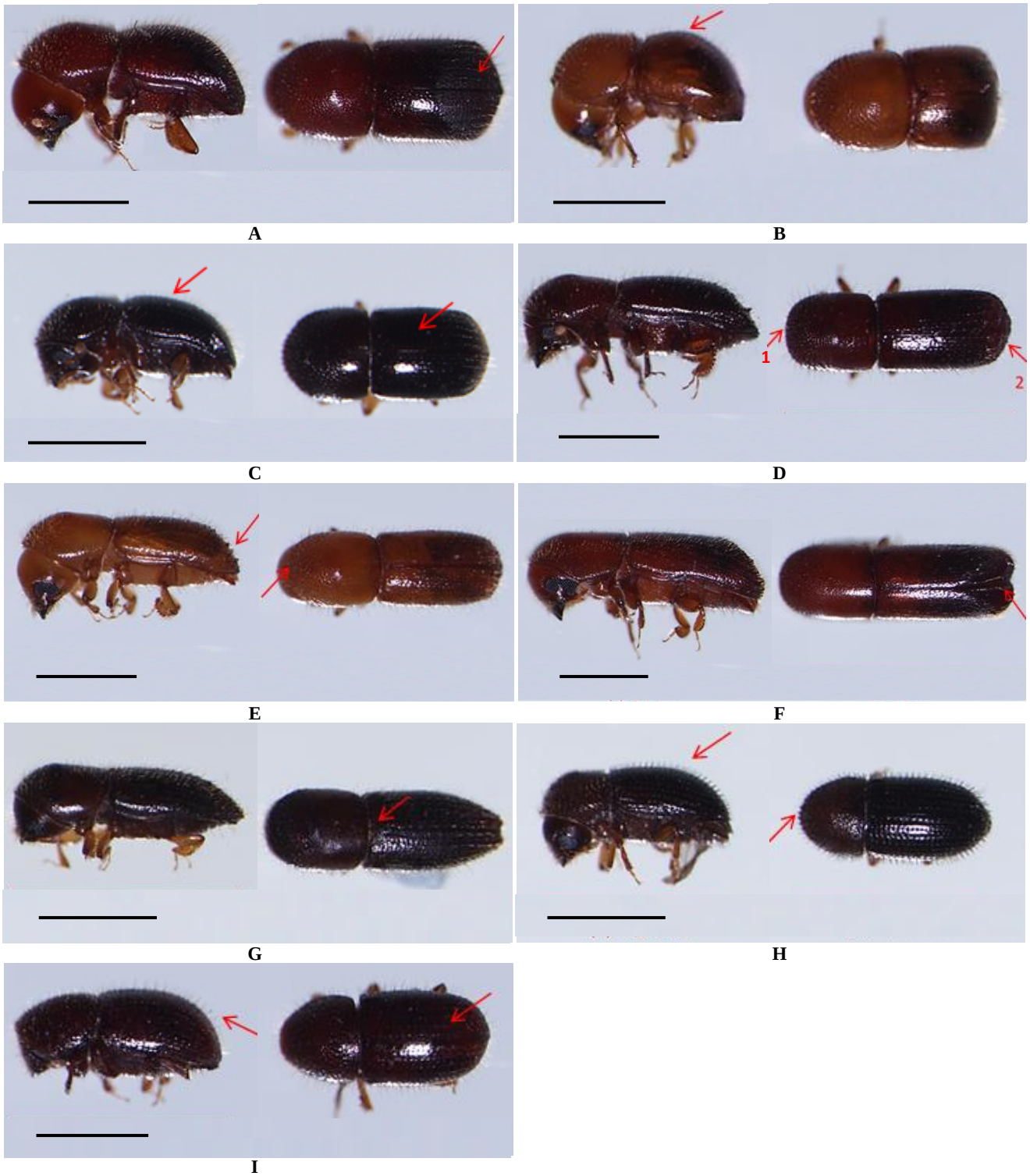

Figure 4. Morphology of ambrosia beetles (Coleoptera: Scolytidae) on teak forest in Malang District, East Java, Indonesia. A. *Xylosandrus crassiusculus*; Lateral side (*left*), and dorsal side, declivity more sloping, declivity surface dull, uniformly distributed granules (*right*). B. *Xylosandrus morigerus*; Lateral side, declivity commencing one-third elytra length from base (*left*), and dorsal side (*right*). C. *Xylosandrus compactus*; Lateral side, elytra more slender, evenly arched from middle of disc to apex (*left*), and dorsal side, surface of elytra shining (*right*). D. *Euwallacea similis*; Lateral side (*left*), and dorsal side, 1. Pronotum commonly subquadrate, 2. Two pairs of tubercle (*right*). E. *Xyleborus perforans*; Lateral side, small tubercle in elytra declivity (*left*) and dorsal side, pronotum commonly convex (*right*). F. *Premnobius cavipennis*; Lateral side (*left*), and dorsal side elytra declivity broadly, rather deeply, concavely excavated on posterior third of elytra length (*right*). G. *Xyleborinus andrewesi*; Lateral side (*left*), and dorsal side scutellum is depressed below the level of the elytra surface (*right*). H. *Hypothenemus hampei*; Lateral side, elytra declivity more gradual, declivity takes up more than half of the length of the elytra (*left*) and dorsal side, very noticeable bumps or teeth on pronotum (*right*). I. *Coccotrypes distinctus*; Lateral side, declivity convex (*left*), and dorsal side, strial punctures shallowly impressed, separated by distances equal to diameter of a puncture (*right*). Bar = 1 mm

Hypothenemus hampei. Body length was 1.1-1.6 mm Body color was black. Anterior margin had a row of serrations, irradiation on the elytra surface, and convex of elytra declivity. Pronotum slope, declivity more gradual, not as steep; declivity takes up more than half of the length of the elytra. Elytra were shiny, hairy or scaly, very noticeable bumps or teeth on pronotum (Figure 4.H). According to Wood (2007), Hulcr and Smith (2010), and Vega et al. (2015), ambrosia beetle with these morphological characters is *H. hampei.* The present distribution of *H. hampei* is in coffee growing areas of Africa, SE Asia, Indonesia, Pacific Islands, etc., Guatemala, Honduras, and Colombia to Brazil. The distribution of this species was also in Java (Kalshoven 1981; Wood 2007; Vega et al. 2015).

Coccotrypes distinctus. Body length was 1.9 mm. Body color was reddish brown (Figure 4.I). Pronotum was weakly to strongly arched, smooth to asperate on anterior half. Elytra were smooth, shining, striate, and convex declivity. Interstrial bristles were pointed, each about twice as long as distance between interstrial rows. Strial punctures were shallowly impressed, separated by distances equal to diameter of a puncture.

According (Wood 2007) and Hulcr and Smith (2010) ambrosia beetle with these morphological characters is *C. distinctus.* The present distribution of *C. distinctus* in Circumtropical areas worldwide such as SE Asia, Sri Lanka, New Guinea to Hawaii South USA, Honduras, Puerto Rico and Jamaica to Suriname, and Guiana (Beaver and Browne 1975; Wood 2007).

Discussion

A total of nine species of ambrosia beetles trapped in both monoculture and polyculture teak plant systems with the highest number of the individual was *X. crassiusculus* (Table 2). Of them, eight species had the higher individual number in the polyculture than in monoculture teak plant system. The higher individual number of those species in polyculture teak plant system was caused by the teaks grow in various vegetation types such as *C. arabica* and *S. mahogany*. While in the monoculture, there were only teak plants and grass as non-host of ambrosia beetle.

Xylosandrus crassiusculus is one of the species of ambrosia beetles that can breed in various hosts. Pennacchio et al. (2003) reported that *X. crassiusculus* had widely polyphagous species which able to colonize at least one hundred species belonging to various genera of plant forest and exhibits the ability to survive on many species of trees shrubs and vines. Horn and Horn (2006) stated that *X. crassiusculus* can breed on the wide variety of hosts, include 124 host and 48 families that mostly in the tropics region including pine, cocoa, coffee, mahogany, rubber, tea, and teak.

The highest individual number of *X. crassiusculus* in both locations was congruent with some research about monitoring of ambrosia beetles population with bottle trap baited with ethanol. Reding et al. (2011) demonstrated that bottle-style traps baited with ethanol captured *X. crassiusculus* and *X. germanus* effectively. Individual number of *H. hampei* in polyculture was also higher than in monoculture (Table 2), because in polyculture also consisted coffee plant. *H. hampei* is a major pest of coffee crop (Vega 2015).

The diversity of woody plants on the land indirectly provides an alternative host for ambrosia beetles. The ambrosia beetle is polyphagous and has no specific host. According to Dinnage et al. (2012), the impact of plant species abundance can positively affect the richness and diversity of herbivorous insects. Hulcr et al. (2007) suggested that ambrosia beetles had widely host variety because 95% of the species of ambrosia beetles did not show the preference for specific host species. Reed and Muzika (2010) also stated that the abundance of ambrosia beetle species was strongly influenced by the abundance and size of the host.

The peak flight activity of each species of ambrosia beetles was different, *X. crassiusculus* on 11 March 2017, while *E. similis* and *X. morigerus* on 22 March 2017 (Figure 3). The flight activity of ambrosia beetles might be related to environmental factors such as temperature. Reding et al. (2010) stated that the flight activity of *Xylosandrus* spp. often had the dip in activity usually coincides with cold temperatures. Another factor that influences the flight activity of ambrosia beetles was the high intensity of rains. Anu et al. (2009) reported that the high intensity of rainfalls had a negative correlation with flight activity of insects. However, the temperature and intensity of rainfall were not observed in this research. Further examination of this relationship between temperature and intensity of rainfall with flight activity of ambrosia beetles is needed.

In conclusion, Ambrosia beetles were trapped in monoculture and polyculture teak plants consist of nine species, i.e. *X. crassiusculus*, *X. morigerus*, *X. compactus*, *Xy. perforans*, *E. simillis*, *Xyl. andrewesi*, *P. cavipennis*, *C. distinctus* and *H. hampei*. The most abundant species in both locations was *X. crassiusculus*. The polyculture teak plant system had higher diversity index than monoculture, and both locations were categorized in the medium diversity category.

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Short Communication: Identification of marine leech and assessment of its prevalence and intensity on cultured hybrid groupers (*Epinephelus* sp.)

MURWANTOKO[1,♥], SRI LAKSONO CONDRO NEGORO[1], ALIM ISNANSETYO[1], ZAFRAN[2]

[1]Department of Fisheries, Faculty of Agriculture Universitas Gadjah Mada, Jl. Flora Bulaksumur, Sleman 55281, Yogyakarta, Indonesia.
♥email: murwantoko@ugm.ac.id

[2]Gondol Institute for Mariculture Research and Fisheries Extention. Penyabangan, Gerokgak, Buleleng 81155, Bali, Indonesia.

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Abstract. *Murwantoko, Negoro SLC, Isnansetyo A, Zafran. 2018. Short Communication: Identification of marine leech and determination of its prevalence and intensity on cultured hybrid groupers (*Epinephelus *sp.). Biodiversitas 19: 1798-1804.* Grouper is an important fish species due to its high price both in domestic and international markets. Several hybrid groupers have been produced and can be accepted in market. A major production constraint in grouper culture is mortality due to diseases. Leech is an ectoparasite for groupers which may cause significant loss. The aims of this study were to identify and to assess the prevalence and intensity of leech on hybrid grouper cultured in sea cages at Buleleng waters. Morphological identification was conducted using fresh and stained specimens while molecular identification was conducted using nucleotides sequence of mitochondrial cytochrome oxidase subunit I (COI). The presence of leech was observed by unaided observation of 14 populations of hybrid grouper. Morphological identification showed that the leech belonged to *Zeylanicobdella arugamensis.* This result was also supported by analysis of COI sequence that showed 100% homology with *Z. arugamensis* (accession number KY 441721.1) and 90% homology with *Aestabdella abditovesicu*lata (accession number DQ414300.1). Hybrid groupers at sea cages were infected by leeches with prevalence and intensity, respectively, of 100% and 21.2 leeches fish^{-1}. The prevalence and intensity were varied depending on the farm and population. Cantik grouper was more susceptible to leech infection than cantang grouper. The bigger fish tended to have higher leech prevalence and intensity.

Keywords: Cytochrome oxidase, hybrid grouper, identification, leech, *Zeylanicobdella arugamensis*

INTRODUCTION

Several species of grouper have been cultured in Indonesia and become important fish commodities due to its high price in both domestic and international markets. Several types of hybrid grouper have been developed to increase the quality of fish. Cantang grouper is produced as a result of crossbreed between female tiger grouper (*Epinephelus fuscoguttatus*) and male giant grouper (*Epinephelus lanceolatus*). The crossbreed between the female tiger grouper and the male brown-marbled grouper (*Epinephelus microdon*) was named cantik grouper. A crossbreed between mouse grouper (*Cromileptes altivelis*) and giant grouper was named kustang grouper (Ismi et al. 2013). Cantang grouper culture has developed well from the rearing of fry to consumption size (Ismi 2012), and fast grows in cages (Sutarmat et al. 2013). Cantik grouper could increase production and showed better quality than brown-marbled and tiger groupers (Ismi et al. 2013).

The emergence of diseases is one of the main problems in the aquaculture. Emerging disease of epizootics frequently causes substantial, often explosive, losses among populations of fish, resulting in significant economic losses in commercial aquaculture and threats to valuable stocks of wild aquatic animals (Walker et al. 2010). Koesharyani et al. (2001) have compiled the viral, bacterial, parasitic, and noninfectious diseases in grouper. The hirudinea infection on grouper is one problem for parasitic diseases. Hirudinea has four orders, namely Acanthobdellia, Gnathobdellia, Pharyngobdellida, and Rhynchobdellida. The Rhynchobdellida Order has three families, i.e., Glossiphonidae, Ozobranchidae, and Piscicolidae. The Piscicolidae family is characterized by having a symmetrical, flattened cylinder body, an anterior suction and a posterior suction. Their habitats are freshwater and seawater, swimming by extending their body (Sawyer 1986). Species of leeches the family Pisciolidae are often parasitic seawater fish such as *Pterobdella amara*, *Aestabdella leiostomi*, *Piscicola* spp., and *Zeylanicobdela arugamensis* (Chandra 1991).

Marine leeches are an essential threat to the aquaculture industry (Ravi and Yahaya 2017). Heavily infested fish with leeches often have chronic anemia (Noga 2000). Grouper having infected leeches on its skin will rub the body on objects around it causing injuries and a large ulcer on the skin or in the mouth. Those conditions can cause secondary infection (Noga 2000; Johny and Roza 2006). Fishes mortality usually occurs within a 3-day period following infestation due to secondary infections with pathogenic bacteria such as *Vibrio alginolyticus* (Ravi and Yahaya 2017). Leeches infection also often transmits microbes and hemoparasites during feeding (Noga 2000). Marine leeches *Z. arugamensis* have been reported to have the ability to transmit the hemogregarine and trypanosomes

simultaneously between fish (Hayes et al. 2006).

Grouper culture using floating net cages in Pegametan Buleleng waters has been started in 2003. The number of sea cages in these waters is increasing due to the potential and reasonable price of grouper fish and high export demand. An outbreak of leeches was reported in grouper farm on August 2016. In this study, we identified the leech based on morphological and molecular approaches and determined the prevalence and intensity of leeches on hybrid groupers.

MATERIALS AND METHODS

Leeches sampling

Seven farms were selected to represent grouper culture in Pegametan Bay, Buleleng waters in September-October 2016. The position of farms were: Farm A at 8°07'10.7"S 114°36'47.1"E, Farm B at 8°07'03.0"S 114°36'42.2"E, Farm C. at 8°07'17.6"S 114°37'04.9"E, Farm D. at 8°07'47.3"S 114°36'06.9"E, Farm E. at 8°07'27.9"S 114°35'58.5"E, Farm F. at 8°07'40.5"S 114°36'09.3"E and Farm G at 8°07'40.9"S 114°35'44.6"E. All fish populations on the selected farm were sampled for the study. We defined population as fishes in a cage which have the same species, the same age and the same source of hatchery when stocked into the cage.

Leech observation

Thirty-six fishes were randomly sampled from each population to meet detection with a minimum prevalence rate of 10% with a 95% confidence level. For one population, fishes were sampled from three cages with twelve fishes in each cage. Fishes were collected from cages using scope net, then kept in a bucket. The species, length, and weight of fishes were recorded. The presence of leeches was observed with unaided eyes from all surface body of fish. The number of parasites was counted, and infected organs were recorded. The prevalence was calculated as the proportion of infected fishes among all the fishes in population. The intensity was calculated as the number of leeches found in the infected fish. For morphological identification, the leeches were collected alive and kept in containers with seawater for further identification. For molecular identification, ten parasites were fixed in 5 ml tubes containing 70% ethanol for further analysis.

Morphological identification

Morphological identification was performed using five fresh samples and five acetocarmine stained samples. Parasites were stained basically from Roberts et al. (2012) with 1% acetocarmine, and then destained using 1% HCl in 70% ethanol. The observations were conducted under a microscope and documented. Identification of species based on morphology and anatomy followed the guidelines of Sawyer et al. (1982) and Chandra (1983).

Molecular identification

The genomic DNA was isolated based on TNES method (Murwantoko et al. 2008). Approximately 50 mg of leech was ground in up 400 µl TNES on the microtube and added with three µl of Proteinase K (Roche) and incubated for 2.5 hours at 37 °C. After incubation, the mixture was centrifuged at 13500 x g for 5 minutes with Sorval Legend Micro 17 Microcentrifuge (Thermo scientific). The supernatant was collected and extracted with 300 µl of PCIAA solution (Phenol Chloroform Isoamyl Alcohol). After centrifugation at 13500 x g for 1 min, the aqueous phase was collected and added with 30 µl 5 M NaCl (1/10 volume of supernatant), and 600 µl cold absolute ethanol (2x volume of supernatant) then incubated for 24 hours in the refrigerator. The mixture was centrifuged at 13500 x g for 5 minutes, the supernatant was discarded, and the pellet was washed with 500 µl of ethanol 70%. After drying, the pellet was resuspended in 100 µl of TE containing 0.5 µl of RNAse.

The molecular identification was conducted based on mitochondrial cytochrome oxidase subunit I (COI) gene. LCO 1490 (GGT CAA ATA ATA AAG ATA TTG G) as the forward primer and HCO 2198 (TAA ACT TCA GGG TGA CCA AAA AAT CA) as the reverse primer (Lobo et al. 2013) were used. Amplification was performed in T100TM Thermalcycler (Biorad) with initial denaturation program at 95 ºC for 3 minutes, followed by 30 cycles of denaturation at 95 ºC for 30 seconds, annealing at 55 ºC for 30 seconds, extension at 72 ºC for 1 minute and the final extension at 72 ºC for 5 minutes. PCR product was electrophoresed in 1% agarose (Nacalai) in TAE solution with Fluorosave DNA stain (1st Base) using Mupid_2Plus electrophoresis tank (Advance). After electrophoresis, the gel was observed under UVP Transilluminator (Pasificimage Electronic). The PCR products were then sequenced through the sequencing service company. Aligned sequences were also subjected to nucleotide BLAST (Basic Local Alignment Search Tool) search to know the identity. Cluster tree was constructed under unweighted pair group method with arithmetic mean (UPGMA) using MEGA 7 software (Komar et al. 2016).

RESULTS AND DISCUSSION

Grouper culture

The culture of grouper using floating net cages in Pegametan bay has been started since 2003. In 2016 there were 24 farms with approximately 4000 cages as recorded by Association of Coastal Fish Farmer of Buleleng. The number of cages in each farm varied between 40 to 500 cages. The size of each cage ranged from 2 m x 2 m, 3 m x 3 m and 3 m x 6 m. A cage of 3 m x 3 m size was stocked with 500-600 fishes of 11-15 cm length, and cage of 3 m x 6 m was stocked with 700-800 fishes of 11-15 cm length.

The most commonly cultured grouper commodities were cantik hybrid grouper (*Epinephelus* sp) and cantang hybrid grouper (*Epinephelus* sp). Tiger grouper (*Epinephelus fuscoguttatus*), mouse grouper (*Cromileptes altivelis*), orange spotted grouper (*Plectropomus leopardus*), malabar grouper (*Epinephelus malabaricus*), and brown-marbled grouper (*Epinephelus microdon*) were

cultured in limited number. Based on information from the farms, the leech started to infect groupers with low intensity on few cages in April 2016. In August 2016, when there was a high tide, the leech infection spread to many floating net cages in the waters. Therefore, the sampling conducted around September to October was in condition with relatively high leech infection.

Fish samples

The grouper samples were taken from seven different farmers with total sample of 14 populations. The samples were composed by 9 populations of cantik grouper and 5 populations of cantang grouper. Based on the size, samples can be categorized on small, medium and big with 7, 5 and 2 populations, respectively (Table 1).

Location of leech infection

Leeches were easily observed and founded in mouth, eyes, operculum, skin, dorsal fin, anal fin, pectoral fins and tail (Figure 1). This parasite attaches to its host using its sucker and takes its host blood causing the leeches to become diverse in color as black and brown.

Table 1. Grouper samples from Pegametan cages

Farm	Pop.	Species	Length (cm)	Weight (g)	Category
A	A1	Cantik	15.9 +1.3	62.6 + 14.1	Small
	A2	Cantang	20.2 + 1.7	235.7 + 27.4	Medium
B	B1	Cantik	16.6 + 1.5	72.2 + 17.2	Small
C	C1	Cantik	17.1 + 0.7	75.1 + 7.4	Small
	C2	Cantik	29.9 + 1.4	434.5 + 78.1	Big
D	D1	Cantang	21.2 + 1.9	268.0 + 38.4	Medium
	D2	Cantik	14.1 + 0.9	46.2 + 9.7	Small
E	E1	Cantang	14.4 + 9.6	50.9 + 10.3	Small
	E2	Cantik	21.0 + 14.7	304.5 + 19.5	Medium
F	F1	Cantik	32.2 + 3.0	461.6 + 103.8	Big
	F2	Cantik	13.9 + 1.2	43.6 + 9.7	Small
G	G1	Cantang	14.5 + 1.1	51.1 + 10.5	Small
	G2	Cantik	21.3 + 1.1	252.7 + 20.4	Medium
	G3	Cantang	20.4 + 1.5	258.7 + 33.5	Medium

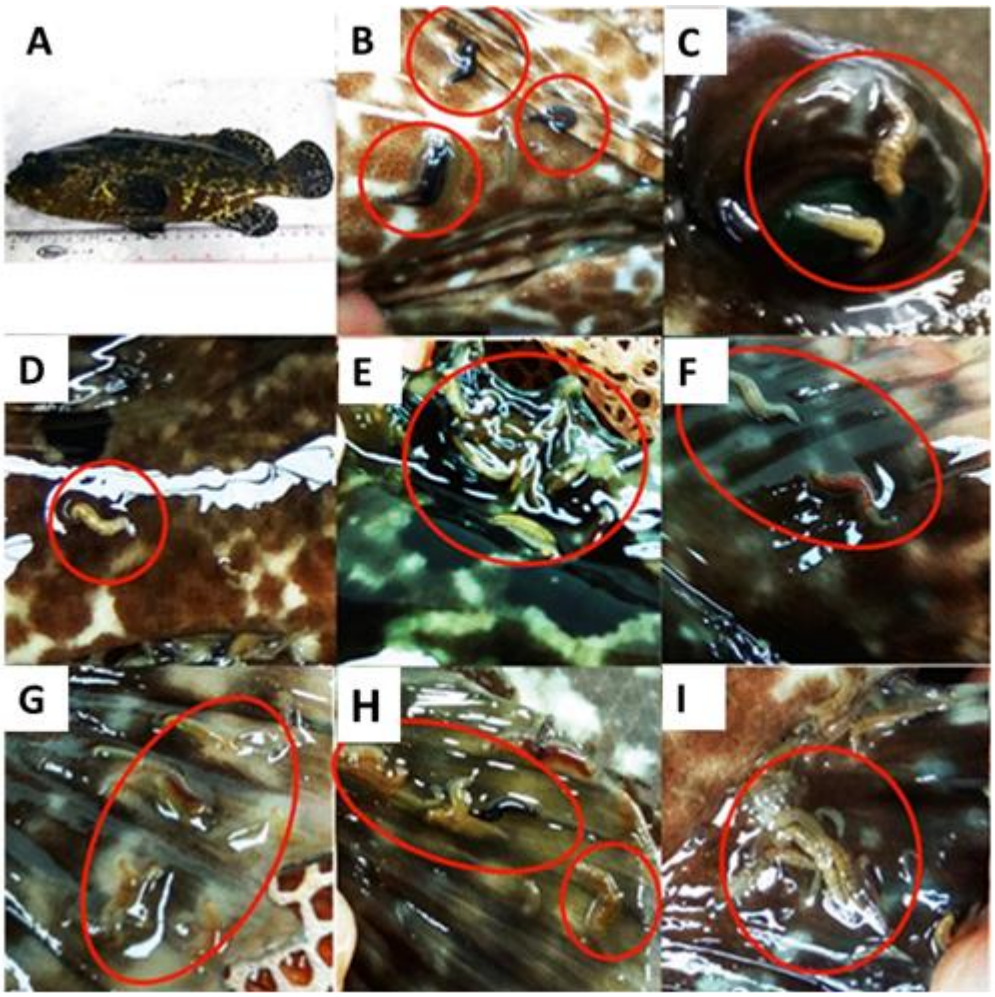


Figure 1. Infection by leech on fish body part (A: Infected grouper, B: Infection on operculum, C: Infection on eyes, D: Infection on the body surface, E & F: Infection on dorsal fin, G: Infection on caudal fin, H: Infection on pectoral fins and I: Infection on anal fin)

Prevalence and intensity

Leeches were found from all observed farms with different prevalence and intensity (Figure 2A). The average prevalence among farms was 62% with the highest prevalence was 86% (Farm C), and the lowest prevalence was 11% (Farm F). The average intensity among farms was 7.1 leeches fish^{-1}with the highest intensity was 11.2 leeches fish^{-1} (Farm F), and the lowest intensity was 0.9 leeches fish^{-1} (Farm D).

The prevalence and intensity levels of leech infection on each grouper sample population were varied (Figure 2B). Prevalence in the populations was also different even on the same farm. The highest prevalence was in population C2 and F1 grouper (100%), and the lowest was in population D2 (0%). The high prevalence variation among the population in farms occurred in Farm G (population G2 of 72.2% and population G1 of 8.3%) Fam F (Population F1 of 100%, population F2 of 19.4%). The intensity of leech infection in the population was also different even on the same farm. The highest inter-population variation in farms occurred in Farm F with population F1 of 21.2 leeches fish^{-1}and population F2 of 0.3 leeches fish^{-1}

The prevalence and intensity of leech infection on cantik grouper were, respectively, 69% and 9.3 leeches fish^{-1}, which were higher than those of cantang grouper with only 42% in prevalence and 2.6 leeches fish^{-1}in intensity (Figure 2C). This result suggests that cantik grouper is more susceptible to leech than cantang grouper. The highest prevalence of 100% was found in large grouper, and then 62% in medium grouper group and the lowest was 46% in small grouper. The highest intensity also showed similar pattern with the highest intensity was found in large grouper and the smallest was found in small grouper (Figure 2.D).

Morphological identification

The leeches can be observed on the fish body using unaided observation. The parasite has cylindrical shape, soft, elastic, and smooth body surface with light brown or black (Figure 3.A, 3.B). This parasite attaches to fish using its sucker and sucks its host blood. Adult of this species has a length of about 8-18 mm and a maximum width of the urosome of 0.5-2.0 mm. Anterior sucker has a diameter of 0.3-0.5 mm, and posterior sucker has a larger diameter that of 1.0-1.8 mm

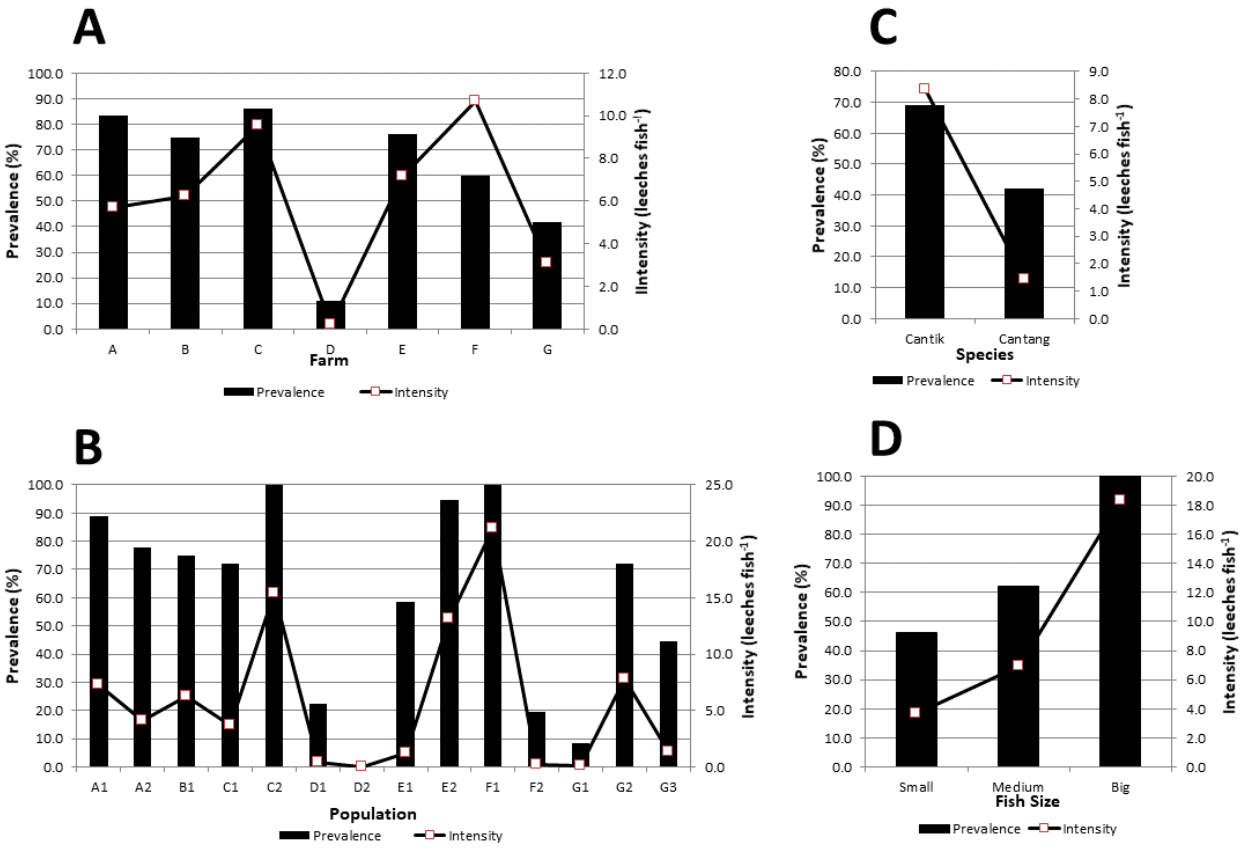
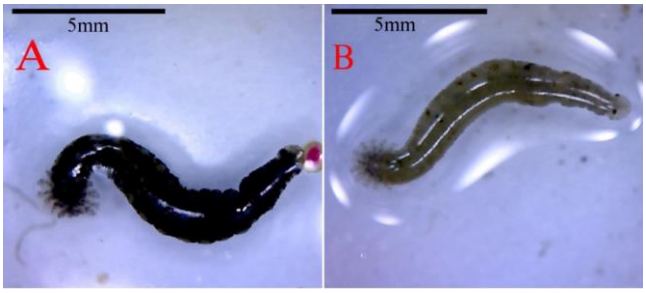


Figure 2. Prevalence and intensity of leech on the farm (A), population (B) species (C) and grouper size (D)



Fig**ure 3.** Leech found in grouper (A; Leech with black color; B: Leech with brown color)

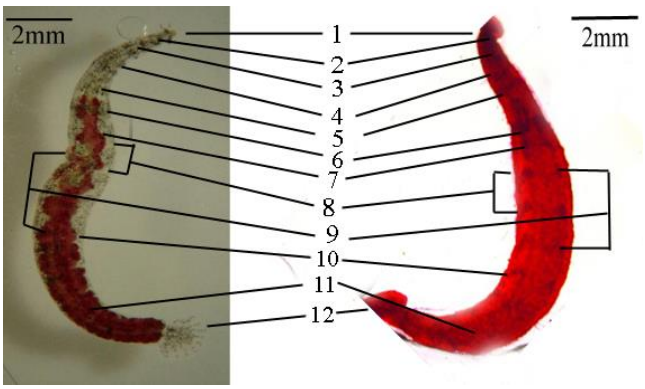


Figure 4. Morphology of leech (1 = Anterior sucker; 2 = Subesophageal ganglion mass; 3 = Proboscis; 4 = Ductus ejaculator; 5 = Ovary; 6 = First testicular ganglion; 7 = First Testis; 8 = Crop Abdomen; 9 = Pulsatile vesicle; 10 = 5th Testis; 11 = Posterior crop caecum; 12 = Posterior sucker)

Table 2. Leech determination according to Chandra (1983)

No	Description
1b	Has eyes and pulsating vesicles
4a	Eye pair
5a	Has no lateral branchiae
6b	Has pulsating vesicles
∴	*Zeylanicobdella arugamensis*

Table 3. Leech determination according to Sawyer et al. (1982)

No	Description
1b	Species that live in seawater or brackish
8b	Has no gill radius
9a	Has 10-12 pairs of pulsating vesicles along the lateral border of the abdomen
10b	Small size of about 1-2 cm
11b	Posterior sucker has a different sleeve with an anterior sucker and large size
12a	The lower body is smooth, about 12 segments in the body
∴	*Zeylanicobdella arugamensis*

This species has a pair of eyespots on the anterior sucker, 12 segments in the body, five pairs of testes and a pair of ovaries. The other part of the body of this species consists of subesophageal ganglion mass, proboscis, ovary, crop posterior caecum, pulsatile vesicles, testis (1-5), crop abdomen, testicular ganglion (1-5), and the ductus ejaculator (Figure 2). Posterior sucker (Figure 4(12)) different sleeve and large size than anterior sucker (Figure 4(1). Based on determination key of Chandra (1983) and Sawyer et al. (1982), this species is *Zeylanicobdella arugamensis* (Table 2 & Table 3)

Molecular identification

The genomic DNA was used as the template to amplify COI gene. The COI gene from leech was successfully amplified as indicated by the presence of a single band of DNA after agarose electrophoresis. This DNA fragment contained 725 nucleotides sequences that have been deposited in Genbank with accession number MH299847. The BLAST analysis showed that the sequence has a 100% identity with *Zeylanicobdella arugamensis* (KY4741721.1), while its homology with *Aestabdella abditovesiculata* (DQ414300.1), *Pterobdella amara* (DQ414334.1), *Myzobdella lugubris* (KY440059.1) was, respectively, 90%, 89%, and 86%**.** This Indonesian *Z. arugamensis* is closely related with *Z arugamensis* from Malaysia (Figure 5).

Discussion

The leeches can be seen visually attached to the fins, tail, body, operculum, mouth, and eyes of fishes. Some fishes showed hemorrhagic on their body surfaces, which is in line with the statements of Johnny and Roza (2006) that leech infection was found in the external part of the fish and caused hemorrhages leading to secondary bacterial infection. According to Ravi and Yahaya (2017), the most frequent effect of leech infection in fish are local bleeding and ulceration in fish tissues. This species is attached to the host using anterior and posterior suckers. They suck the blood of their host using their sucker, and in this study, the leech having fish blood was black (Figure 1). Leech that has sucked fish blood will escape from fish to find a place for spawning (Kua et al. 2010). After detached from the host, leeches were able to swim in the sea and able to survive without host for 5-7 days Cruz-Lacierda et al. (2000).

Leech parasites were found from all observed farms in Pegametan bay. The prevalence and intensity of leech that infected grouper in each farm were varied in values. The prevalence and intensity were also varied between population among farms and within farm. The highest prevalence was found in population C2 and F1 (100%), and the lowest prevalence was in population D2 (0%). The results indicated that infestation of leech was affected by location and or cultivation management. The prevalence and intensity of leech varied between species and size. Cantik grouper was more susceptible than cantang grouper. This shows that cantik grouper has a higher risk of disease infection than the cantang grouper. The bigger fish tended to have higher prevalence and intensity.

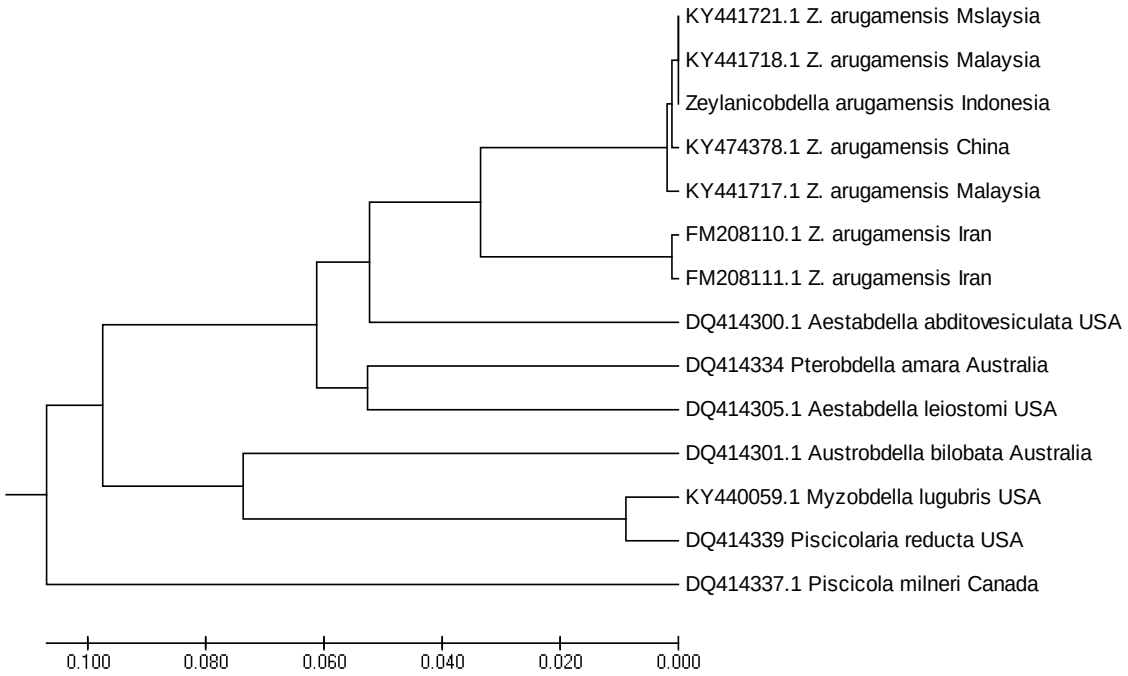


Figure 5. UPGMA tree using the mtDNA COI of Indonesia marine leech and added sequences from GenBank with indicated accession numbers

The average prevalence for these 14 populations was 59%, and average intensity was 6.9 leeches fish^{-1}. This prevalence and intensity were higher than that reported in muddy grouper in the Philippines with a prevalence of 30% and intensity of 2 leeches fish^{-1} (Cruz-Lacierda et al. 2000), and in red snapper with study of ectoparasite prevalence was 11.5% and intensity of 1.48 leeches fish^{-1} (Ravi and Yahaya 2017). This prevalence is lower than the that in white snapper in Malaysia (70%) (Kua et al. 2006).

Morphological and molecular identification based on COI sequence consistently showed that the leech belonged to *Zeylanicobdella arugamensis.* The COI sequences of *Z. arugamensis* on Genbank is limited, where up to April 2018, only ten entries were available. *Z. arugamensis* showed the genetic diversity, and at least 3 clusters are shown in Figure 5, Indonesian-Malaysian, Malaysian-China and Iran clusters. This genetic diversity seems to be correlated with the country location. *Z. arugamensis* had been reported to infect brackish-water fish Mozambique tilapia, *Oreochromis mossambicus* in Okinawa Japan (Nagasawa and Uyeno 2000), amphibious goby (*Scartelaos tenuis*) in southern Iran (Polgar et al. 2009). *Z. arugamensis* had been reported to infect cultured marine fish such as orange-spotted grouper (*Epinephelus coiodes*) in Philippine (Cruz-Lucierda et al. 2000), crimson snapper (*Lutjanus erythropterus*) in Malaysia (Ravi and Yahaya 2017), orange-spotted grouper, (*Epinephelus coiodes*) in Indonesia (Kleinertz and Palm 2015). In this study, we report the first time that *Z. arugamensis* can infect cantang and cantik hybrid groupers.

Several authors have documented study on leech infections on fish in Indonesia. Rosa and Johny (2006) have reported infection of the leech on *Epinephelus bleekeri* and *E. polyphekadion.* However, the species of leech was not reported. The intensity of *Piscicola* sp from tiger grouper (*E. fuscoguttatus*) and spotted coral grouper (*Plectropomus maculatus*) has been reported by Diana et al. (2004). The *Z. arugamensis* in Indonesia was reported by Kleinertz and Palm (2015) from orange-spotted grouper, *E. coiodes* with the species identification was only based on the morphology using microscope observation. Here we report the first time the identification of *Z. arugamensis* in Indonesia based on the COI nucleotide sequences and the sequences have been deposited in Genbank with accession number MH299847. Improvement of culture management should be addressed to control the leech. The lesions form this infection can cause secondary infection by bacteria, and this *Z. arugamensis* has been reported to be able to transmit the hemogregarine and trypanosomes simultaneously between fish (Hayes et al. 2006).

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Short Communication: The impact of Gamma radiation on *Tdc* and *Str* gene expressions in *Catharanthus roseus* regenerated plantlets

ZAHRA NOORMOHAMMADI[1,♥], MARYAM TABAN[1], FARAH FARAHANI[2]
[1]Department of Biology, Science and Research Branch, Islamic Azad University. Tehran, Iran. ♥email: marjannm@yahoo.com
[2]Department of Microbiology, Qom Branch, Islamic Azad University. Qom, Iran

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Ab*Str*act. *Noormohammadi Z, Taban M, Farahani F. 2018. Short Communication: The impact of Gamma radiation on* Tdc *and* Str *gene expressions in Catharanthus roseus regenerated plantlets. Biodiversitas 19: 1805-1810. Catharanthus roseus* L.G. Don, is the essential medicinal plant with considerable attention. This plant is a rich source of terpenoid indole alkaloids (TIAs). The main alkaloids in *C. roseus* are vinblastine, vincristine, and ajmalicine. The tryptophan decarboxylase (*Tdc*) and *Str*ictosidine synthase (*Str*) are key enzymes in TIA biosynthesis. In the present study, *Tdc* and *Str* gene expressions, as well as vinblastine production were evaluated in tissue culture regenerated plantlets in 4 groups; control, 60 Gy irradiation, 50 mg/L putrescine and 60 Gy irradiation + 50mg/L putrescine treatments. The results revealed significant increase in *Tdc* and *Str* gene expressions in 60 Gy irradiation + 50mg/L putrescine treated plantlets in comparison with control samples by using qPCR methods. HPLC analysis showed a higher amount of vinblastine in 60 Gy + 59 mg/L putrescine treated plantlets. Gamma radiation and putrescine as elicitor and polyamine, respectively, are able to improve vinblastine production in *C. roseus*.

Keywords: Gene expression, periwinkle, radiation, secondary metabolism, tissue culture

INTRODUCTION

The medicinal plant *Catharanthus roseus* L.G. Don, comprises more than 130 terpenoid indole alkaloids (TIAs) in different organs. They are used in treatments of hypertension and Hodgkin's disease, testicular cancer, ovarian cancer, breast cancer and head and neck cancer (Nejat and Vadamalai 2012; Almagro et al. 2015; Sears and Boger 2015). The main alkaloids in *C. roseus* are vinblastine, vincristine, and ajmalicine (Xing et al. 2011). They act as inhibitors during the metaphase of the cell cycle by binding to the microtubules (Keglevich et al. 2012). The tryptophan decarboxylase (*Tdc*) and *Str*ictosidine synthase (*Str*) are key enzymes in TIA biosynthesis. The *Tdc* converts tryptophan into tryptamine. The *Str*ictosidine synthase (*Str*) catalyzes the tryptamine with secologanin to become *Str*ictosidine, that leads to production of vindoline and catharanthine, which are precursors of vinblastine and vincristine (Ginis et al. 2012; Almagro et al. 2015).

The tissue and cell cultures provide a large amount of alkaloids with medicinal properties and active compounds for pharmaceutical indu*Str*ies (Sidhu 2011; Naz et al. 2015) Different tissue cultures including callus, cell, and shoot of Madagascar periwinkles are currently considered for vinblastine and vincristine productions (Moreno et al. 1995; Datta and Srivastava 1997; Miura et al. 1998; Ataei-Azimi et al. 2008). Using plant growth regulators like jasmonate, methyl-jasmonate, and salicylic acid, have also been used as elicitors to increase secondary metabolites (El-Sayed and Verpoorte 2007; Verma et al. 2012; Antonio et al. 2013).

Physical elicitors like gamma irradiation have been attentive as a new process to improve the secondary metabolites in plants (Fulzele et al. 2015). Irradiated *Atropa belladonna* L. seeds with various doses of gamma rays can produce altered plant phenotypes and can also increase the alkaloids percentage in the different organs of a plant, particularly in the leaves (Abdel-Hady et al. 2008). The only report showed an increase of vinblastine in roots and leaves of *C. roseus* by use of 0.5 and 5 Gy gamma irradiation (Sadowska et al. 1989).

Polyamines, such as putrescine, have roles in plant growth regulation, DNA replication, gene transcription, and translation, as well as membrane stabilization. The polyamines may vary in different species, tissue, and developmental stages. Abiotic *Str*esses can also induce it. Recently, exogenous polyamines are used to study relationship between endogenous polyamine and plant tolerance under abiotic *Str*ess (Zhang et al. 2009; Xu et al. 2011; Tabart et al. 2015).

The *Str* and *Tdc* gene expressions have been studied in different tissue culture treatments (Goddijn et al. 1992; Moghazee et al. 2014), as well as UV-B inducing *C. roseus* plantlets (Ramani et al. 2007; Aslam et al. 2010). The raising of vinblastine yield in cell culture of various tissues has also been reported through somatic embryogenesis by HPLC (Aslam et al. 2010).

The aim of this study was (i) to investigate the effect of gamma radiation (60Gy) and putrescine treatments on *Str* and *Tdc* gene expressions, and the measurement of

vinblastine in the leaves of *C. roseus*; (ii) the effect of putrescine on gamma radiation damages.

MATERIALS AND METHODS

Plant materials and treatment

F_1 seeds of *Catharanthus roseus* L.G. Don were provided by Pan American Company. The seeds divided to four groups for planting in MS media as following groups: (i) control (0 Gamma radiation + 0 mg putrescine), (ii) 60 Gy dose radiation + 0 mg putrescine, (iii) 0 gamma radiation + 50 mg/µL putrescine and (iv) 60 Gy radiation + 50 mg putrescine (El-Sharnouby et al. 2016). The irradiation was carried out by Gamma Cell, GC-220 in ^{60}Co gamma source with 9587 Ci activity and 2/28 Gy/s dose rate (Partonegaran-Saba company, Tehran, Iran).

Tissue culture and morphological characteristics

The *C. roseus* seeds sterilized under aseptic laminar flow hood with 75% Ethanol for 30 sec, then 5% Naclo for 3 min and finally washed 4-5 times with aseptic water. The seeds were cultured on Murashig and Skoog (MS) medium (Murashige and Skoog 1962) with 30 g/L sucrose and pH = 5.7 for 40 days. The explants were transformed to new MS medium and incubated under light treatments: illumination-5000 lx, photoperiod-16 h, temperature 25 ± 2°C for 45 days. The morphological characters, including surface area of leaves, stem length, root length, and fresh weight were measured for 4 groups by using digitizer software.

RNA extraction and qPCR assay

The fresh young leaves (3-5 leaves of each group) were used for RNA extraction. The RNA extraction protocol was based on CTAB method with modifications (Ghangal et al. 2009). The leaves (0.1 g) were milled in liquid nitrogen and transferred in 1 mL of pre-warmed CTAB extraction buffer [2% CTAB, 100mM Tris-HCl (pH 8.0), 2M NaCl, 25mM EDTA (pH 8.0)] containing 2% PVP and 25 µL 2-mercaptoethanol. After removing proteins with phenol/ chloroform/isoamyl alcohol (24: 1: 1), 0.2 volume of glacial acetic acid (1 M), 0.1 volume of sodium acetate (3M) and 2 volume of ethanol absolute (98%) were added to supernatant. They were incubated at-20 °C for 2h, then NaCl (5M) was added and centrifuged at 12000 rpm for 15 min at 4°C. The 70% ethanol solution was used for washing. After drying pellet, 50 µL of Diethyl Pyrocarbonate (DEPC) treated water was added. DNA contamination was removed by RNase-free DNase I treatment according to manufacturer protocol (Thermo Fisher Scientific, USA). The quantity and quality of RNAs were checked by UV spectrophotometer (260/280 nm) and 1% agarose gel electrophoresis.

The cDNA was synthesized from 1 µg of total RNA, using the RevertAid First *Str*and cDNA synthesis reagent (Thermo Fisher Scientific, USA) according to manufacturer instructions, using oligo (dT) and random hexamer as primers according to the manufacturers.

The *Tdc* and *Str* gene expressions, as well as *Elf1-α* gene as reference gene, were assayed in four group samples. At least 3 pooled samples of each treatment with two technical replicas were used for qPCR assay.

Real-time PCR (qPCR) was performed in 20 µL containing 1 µg cDNA, 1X Master Mix SYBR Green (TaKaRa, Japan), 0.5 µM of each primer. The following designed primer pairs were used: *Str* forward: 5́-GCCTTCACCTTCGATTCAAC-3́ and *Str* reverse: 5́-GATGCGTAGGCGAAGTCAGT-3́, *Tdc* forward: 5́-CGCCTGTATATGTCCCGAGT-3́ and *Tdc* reverse: 5́-GTTGCGATTTGCCAATTTTT-3́, *Elf1-α* forward: 5́-TGGGCCTACTGGTCTTACTAC-3́ and *Elf1-α* reverse: 5́-ACATACCCACGATTCAGATCCT-3́.

The qPCR thermal program was conducted in Rotor-Gene Q-pure Detection (Qiagen, USA) as follows: 95°C for 15 min, followed by 40 cycles of 95°C for 40 sec, 88°C for 30 sec and 72°C for 30 sec. Melting curves were generated at 65-95°C after 40 cycles to check specificity of primers.

Alkaloids extraction and HPLC assay

The young leaves of samples of each group studied were lyophilized for 24h. The dried sample (1 g) was added to 1 mL Methanol. They were mixed by vortex and sonicated for 30min in an ultrasonic bath (DL-60D). Then samples were centrifuged at 12,000 rpm for 10 min. The supernatant was filtered with 0.45 µm needle type PTEE membrane filter (Pan et al. 2016). The standard curve of 2 mg of vinblastine sulfate of over 97% purity (Sigma-Aldrich (St. Louis, MO), was depicted after preparation of it in the same way similar to sample preparation. The Agilent Technologies 1200 series in National Center for Genetic and Biological Resources, Karaj, Iran was used for HPLC assay. The column was an Agilent Eclipse XDB-C18 column. The mobile phase consisted of a mixture of 5 mM Na_2HPO_4 and Methanol at a flow rate of 1.5 mL per min. The injection volume was 30µL.

Statistical analysis

The morphological characteristics and vinblastine amounts were analyzed by ANOVA (Analysis of variance), followed by LSD test to study significant difference among the samples. The level of *Str* and *Tdc* gene expressions were measured by a relative quantification based on the relative expression of target genes versus a reference gene (*Elf1*-α).

RESULTS AND DISCUSSION

Morphological characteristics analysis

Morphological comparison of regenerated plantlets of four groups (control, 60 Gy dose radiation + 0 mg putrescine, 0 gamma radiation + 50 mg/µL putrescine and 60 Gy radiation + 50 mg putrescine 60 Gy irradiations) were studied. Morphological characters showed that regenerated plants treated by putrescine showed the highest means in stem and root lengths and putrescine + 60 Gy irradiation plantlets showed the highest amounts in fresh weigh, surface area of leaves in comparison with other treated plantlets while plantlets of control group revealed

the lowest mean values studied. The analysis of variance ANOVA test (Table 1) showed the significant differences in surface area of leaves and stem length between 4 groups studied (P<0.01). In detail, LSD test showed significant differences between all 4 groups in surface area of leaves except between putrescine and putrescine + 60 Gy irradiation plantlets. Length of stem was significant difference between control and other treated regenerated plants including 60 Gy irradiated plantlets, putrescine and 60 Gy irradiated plantlets, putrescine and putrescine + 60 Gy irradiated plantlets.

Str and *Tdc* gene expressions and vinblastine production

Relative comparison of *Tdc* and *Str* gene expressions plantlets of 4 groups were analyzed by qPCR method. Our finding showed up-regulating of *Str* gene transcripts in 60 Gy irradiation, putrescine, 60 Gy + putrescine in comparison to control samples significantly (p< 0.05, Figure 1). Meanwhile, the expression of *Str* gene was the highest in 60 Gy + putrescine treated plantlets. However, no significant differences were detected between treated groups in *Str* gene expression.

The *Tdc* gene expression was only up-regulated significantly (p<0.05, Figure 1) in 60 Gy + putrescine treated plantlets against control samples. The 60 Gy + putrescine treated plantlets were also up-regulated against 60 Gy irradiated as well as putrescine treated plantlets significantly. The *Tdc* gene expression level in three groups including control, 60 Gy irradiation and putrescine treated plantlets showed no significant differences (P>0.05).

The amount of vinblastine in leaves was measured by HPLC with about 24 min retention time based on standard curve (Figure 2, Table 2). The highest mean value of vinblastine was detected in control samples (233.86 µg/g plant) while the lowest mean belonged to 60 Gy irritated plantlets (11.99 µg/g plant). Apart from control samples, leaves of 60 Gy + putrescine treated plantlets (155.88 µg/g plant) showed higher vinblastine production than other treated samples. ANOVA and LSD tests showed significant differences between all 4 groups studied (data not shown).

Discussion

In the present study, we have used MS medium lack of any phytohormones and found proper plant growth in control and 3 treated groups studied. The main reason was to eliminate any hormone effects on morphological traits and gene expression. Morphological study on *C. roseus* plantlets showed higher value in length of stem and roots in putrescine treated plantlets (Noormohammadi et al. personal communication). This supported impact of exogenous putrescine on plant growth. Several reports have shown that exogenous polyamines can restore the metabolism of endogenous polyamines (Zhang et al. 2009; Xu et al. 2011). On the other hands, 60 Gy + putrescine plantlets showed higher mean value of fresh weight and surface area of leaves than 60 Gy gamma irradiation *per se*. It may prove the impact of putrescine on plantlets to relieve effect of gamma radiation. However, control samples showed the lowest mean value of all morphological traits. These results show improvement of morphological characters by putrescine even in plantlets which exposed to gamma radiation.

Table 1. ANOVA test based on morphological traits between 4 treated plantlets

Morphological traits		Sum of squares	df	Mean square	F	P value
Fresh Weight	Between Groups	.002	3	.001	1.924	.166
	Within Groups	.005	16	.000		
	Total	.007	19			
Surface area of Leave	Between Groups	194.891	3	64.964	22.915	.000
	Within Groups	45.359	16	2.835		
	Total	240.250	19			
Stem length	Between Groups	34.973	3	11.658	3.773	.032
	Within Groups	49.429	16	3.089		
	Total	84.402	19			
Root length	Between Groups	61.067	3	20.356	2.214	.126
	Within Groups	147.097	16	9.194		
	Total	208.164	19			

Table 2. The amounts of vinblastine in 4 groups with retention time, peak area, concentration (ppm) based on HPLC analysis

Sample	Retention time(min)	Peak area	Con. (ppm)	Mean µg / g plants
Control (10000 ppm)	24.925	46.07479	2.33864	233.864
Putrescine (10000 ppm)	24.918	10.9613	0.33101	33.101
60 Gy (10000 ppm)	24.916	7.2697	0.11994	11.994
60 Gy + putrescine (10000 ppm)	24.774	32.43533	1.5588	155.88

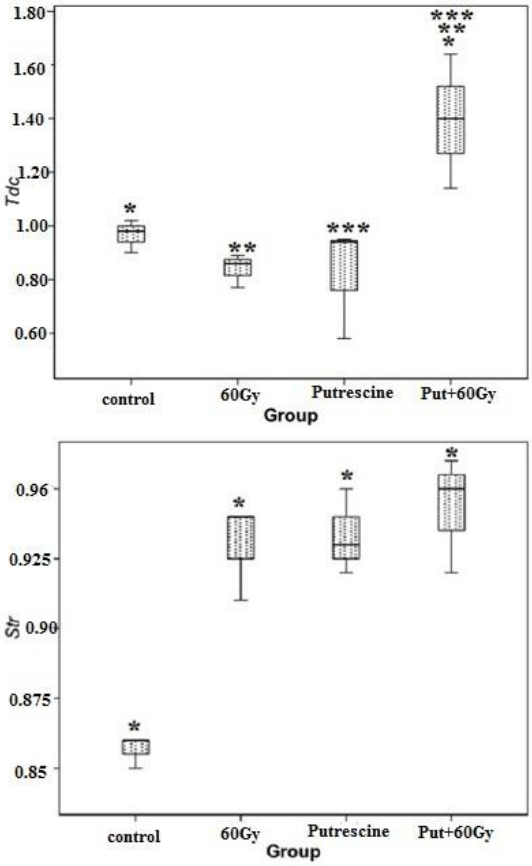

Figure 1. Bar plots of *Tdc* and *Str* gene expressions in 4 groups studied. X axis is 3 treated groups as well as control studied. Y axis is *Tdc* and *Str* gene expressions/reference gene ratio. * indicates significant differences between groups (P<0.05).

El-Sharnouby et al. (2016) also emphasized a negative effect of 30 Gy gamma irradiation on seed germination, number of shoots, shoot length of *C. roseus* plantlets while leaf area, fresh weight of leaves were increased. However, morphological characters are multigenic and multi-factorial traits which are affected by genotypes and environmental conditions. The impact of gamma irradiation on plant growth may be related to the compounds involved in multiplication of genomes and cell division (Elangovan and Pavadai 2014).

Previous studies have shown that physical elicitors like UV-B induced *Tdc* and *Str* gene expressions in *C. roseus* (Ouwerkerk et al. 1999 a,b). Ramani and Chelliah (2007) worked on cell suspension culture of *C. roseus* and exposure low dose of UV-B irradiation to evaluate the amount of catharanthine and transcription of genes encoding *Tdc* and *Str*. Their results demon*Str*ated that UV-B signaling leading to stimulation of *Tdc* and *Str* genes and the accumulation of catharanthine in *C. roseus* cell suspension cultures. The UV-B may induce ROS production and accumulation of catharanthine. The ROS generation could be induced by different elicitors like yeast and fungal elicitors in different plants (Dietrich et al. 1990; Felix et al. 1998; Tebayashi et al. 2001).

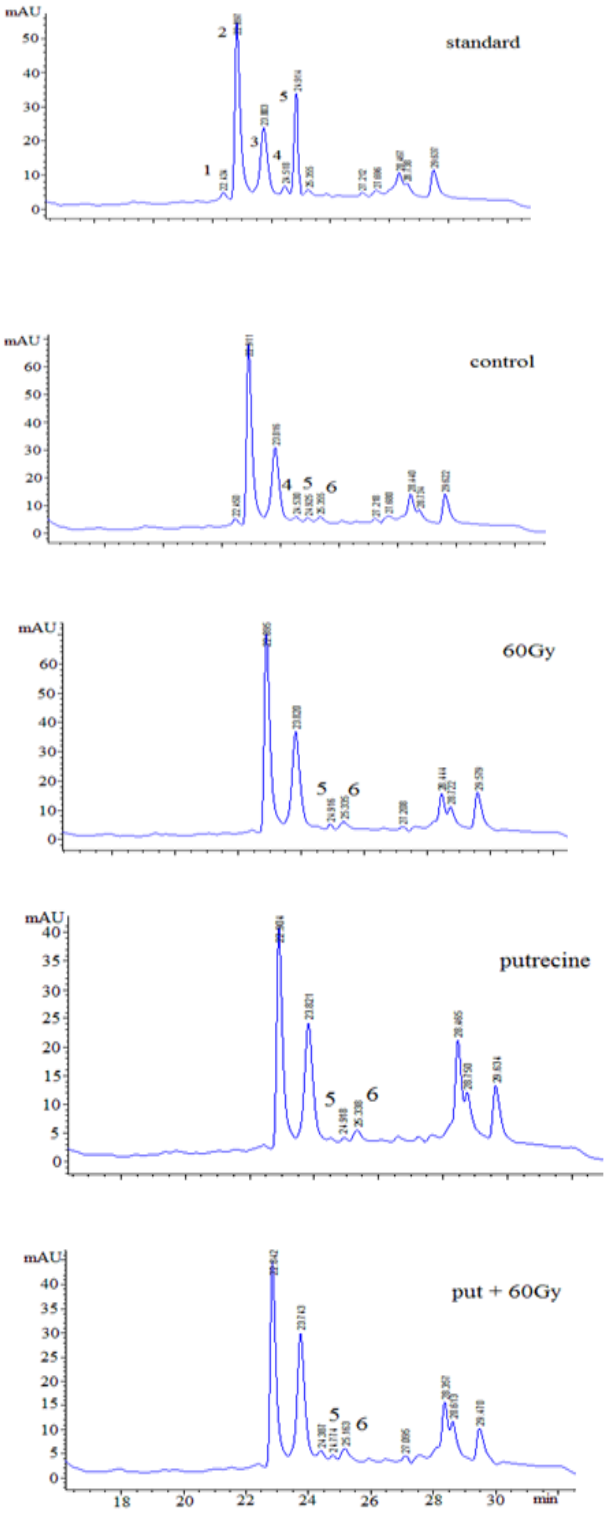

Figure 2. HPLC chromatographs of vinblastine of 4 groups studied and its standard. X axis is time (min) and Y axis is mAU/mA.

In our findings, gamma irradiation increased *Tdc and Str* gene transcripts in comparison to control samples. Meanwhile, gamma radiation with putrescine treatment showed highest gene expressions studied. It seems gamma radiation could act as elicitor and it would be boosted by

putrescine as a polyamine. Both may share elements in signal transduction for initiating TIA pathway.

The production of vinblastine in 3 treated groups also showed increase in 60 Gy irradiation + putrescine support gene expression results. Aslam et al. (2010) reported that somatic embryo-derived plantlets contained more vinblastine that leaves developed *ex vitro*. The amount of vinblastine in present study in gamma radiation and putrescine treated plantlets are more than those have been reported by Aslam et al. (2010). However, the differentiation and development of tissues are main factors which regulate the biosynthesis of alkaloids like vinblastine. Our findings suggest that polyamine and physical elicitor could have impact on terpenoid indole alkaloid pathway.

In this present investigation, we observed increase of mean values of morphological traits, *Tdc* and *Str* transcripts in treated plantlets in comparison to control plantlets. The vinblastine production in leaves showed that gamma radiation and putrescine as a polyamine could be change produce of this valuable alkaloid. The controversy data in this study between amount of *Str* and *Tdc* gene transcripts and vinblastine in different groups studied, may come from complicated biochemical pathway of vinblastine in *C. roseus* plants. However, the further studies are necessary to survey the biochemical pathways which gamma radiation may influence on them.

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Morphological, anatomical and isozyme variability among taro (*Colocasia esculenta*) accessions from southeastern part of Central Java, Indonesia

ARI PITOYO, ANGELIA ARUM PRAMETA, MARSUSI, SURATMAN♥, SURANTO
Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57126, Central Java, Indonesia. Tel./Fax. +62-271-663375, ♥email: suratman@staff.uns.ac.id

Manuscript received: 23 August 2018. Revision accepted: 9 September 2018.

Abstract. *Pitoyo A, Prameta AA, Marsusi, Suratman, Suranto. 2018. Morphological, anatomical and isozyme variability among taro (*Colocasia esculenta*) accessions from southeastern part of Central Java, Indonesia. Biodiversitas 19: 1811-1819.* The objective of this study was to evaluate morphological, anatomical and isozyme variability among taro accessions from southeastern part of Central Java (Indonesia). A total of 20 taro accessions were collected from a wide range of sites during field surveys. Morphological characters measurements were taken on vegetative structures such as roots, stems, leaves, and corms. Anatomical characters were observed from both paradermal and transverse sections of leaf. Identification of biochemical markers was done by using peroxidase and esterase isozyme system. A UPGMA dendrogram among accessions was constructed based on the genetic similarity matrix by applying a cluster analysis using a computer programme, NTSYS Version 2.00. The analysis of variance for morphological and anatomical characters revealed that there was significant difference for majority of the tested traits indicating that there was a variability among the taro accessions. Polymorphism was observed using isozymes of esterase (12 banding pattern) and peroxidase (8 banding pattern). Based on the dendrogram at a level of 62 % similarity, taro accessions were segregated into two major clusters. In Cluster I, the closest relationship was shown between SKH and SKA accessions that had 96 % coefficient of similarity. The ten accessions from Klaten, Sragen, and Karanganyar were then clustered separately as Cluster II with coefficient of similarity 73.52 %.

Keywords: anatomy, southeastern part of Central Java, isozyme, morphology, taro, *Colocasia esculenta*

INTRODUCTION

Taro (*Colocasia esculenta* (L.) Schott), belonging to the family Araceae, is an important root crop especially in the humid tropics and sub-tropics. It is one of the few crops that can adapt well to different agro-climatic conditions (Kreike et al. 2004; Asha Devi, 2012). Taro is an ancient crop of uncertain geographical and genetic origins in Southeast Asia (Matthews 2014). Presently, taro is naturalized as a tuber crop and leafy vegetable. However, it attains the importance of staple food in many African, Oceanic and Asian cultures (Purseglove 1972; Safo Kantaka 2004).

The edible part of taro such as corms, leaves, and petioles provide a good source of carbohydrate, protein, dietary fibre, minerals (calcium, phosphor, magnesium and iron) and vitamins (thiamin, riboflavin, niacin, ascorbic acid) (Wilson and Siemonsma 1996; Safo Kantaka 2004; Huang et al. 2007; Rao et al. 2010; Amagloh and Nyarko, 2012; Darkwa and Darkwa, 2013). The corm is usually sliced and fried into taro chips and is used in the preparation of soups, beverages, and puddings. The starch is used in baby foods and as cereal substitute. Taro leaves and leaf stalks are used as a leafy vegetable and potherb for soups and sauces. Taro leaves and corms are also credited for having medicinal values. In Mauritius, the boiled young leaves are eaten to treat arterial hypertension and liver affections, whereas juice is applied externally to treat eczema. In India, China and New Guinea, the corms are used to treat stomach-ache, diarrhea, and as a poultice on sores and skin diseases (Wilson and Siemonsma 1996; Safo Kantaka 2004).

Taro is an excellent multipurpose food crop for subsistence agriculture and home gardens, giving food security. Genetic improvement of taro has the potential to overcome production constraints, particularly resistance to pests and diseases. The success of genetic improvement of a crop, however, depends on the availability of genetic resources and their diversity (Okpul et al. 2004).

The assessment of genetic diversity is required in the crop breeding program. In order to assess genetic variability of plants, a variety of morphological, anatomical, biochemical and molecular markers are used. The traditional technique used to assess genetic variation among and within species, populations or accessions is based on differences in morphological traits (Acquaah 2012). Morphological character is still routinely used for preliminary evaluation because it is fast, simple, inexpensive and can be used as a general approach for assessing genetic diversity of plants (Beyene et al. 2005; Jingura and Kamusoko 2015). Anatomical characters are also valuable in taxonomy and identification of groups of plant (Mavi et al. 2011; Faria et al. 2012; Rahayu et al. 2012; Kumar et al. 2014; Arshed and Agoo 2017).

Isozymes are known as the classical biochemical marker and can be used to determine genetic variation and

relationship among cultivars, varieties, natural populations and accessions in germplasm collections (Fernandez de Souza and Primo 2001; Padmanaban et al 2013; Rizk and Soliman 2014). They are not influenced by environmental factors, making identification possible in early stages of development. Isozymes also can be studied easily, without requiring prior knowledge of the genome, using a small quantity of material with an efficient and inexpensive technique (Torres 1990; Johnson et al. 2010; Kovacevic et al 2010; Kumar et al. 2013).

Information on genetic diversity and relationship among and between individuals, accessions, populations, varieties, and species of plant are then important in guiding the improvement of plants, thus facilitating breeding material selection (Qi et al. 2008; Dharmar and De Britto 2011; Tang et al. 2014).

The objective of this study was to evaluate morphological, anatomical and isozyme variability among taro accessions from the southeastern part of Central Java (Indonesia). The combination of morphological, anatomical and isozyme markers to evaluate genetic variation in taro accessions from Java is the first study that is reported from this region.

MATERIALS AND METHODS

Plant Materials

A total of 20 taro accessions in the form of living plant collection were collected from a wide range of sites during field surveys (Table 1, Figure 1). The collected plants were then transplanted to the polybag and kept in screen house in the Department of Biology, Universitas Sebelas Maret for 12 weeks. The plants were grown under controlled environmental conditions with temperature regime of 28°/20 °C day/night, a relative air humidity of 80%, at a photon flux density of about 8.200 lux and at 126 m asl altitude. The young leaves of each accession were then used for isozymes extraction.

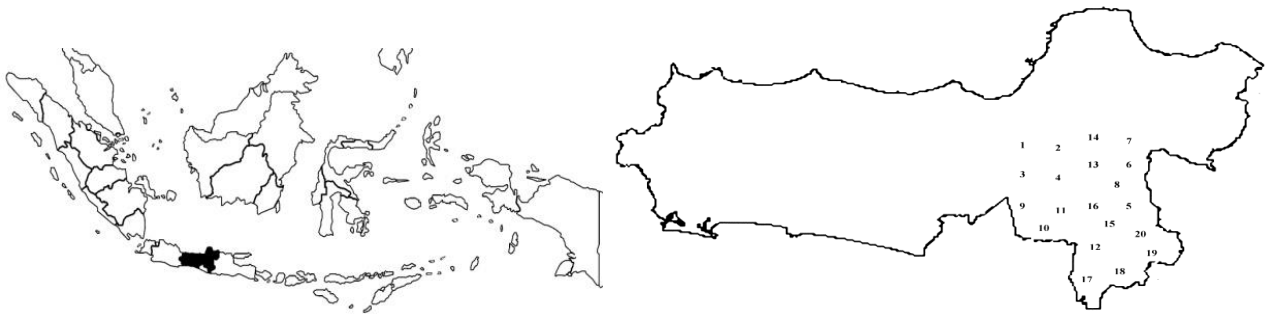

Figure 1. Map of the collection areas for taro (C. *esculenta*) accessions studied in southeastern part of Central Java. The number (1 to 20) indicates location of each collected accession

Table 1. The geographic variation of taro (*C. esculenta*) accessions originated from southeastern part of Central Java with climatic data for each collection site

No. code	Accessions	Collection site	Altitude (m asl.)	Air temperature (°C)	Light intensity (lux)	Air humidity (%)	Soil humidity (%)
1.	BYL 1	Boyolali	548	27	17.200	84	20
2.	BYL 2	Boyolali	583	26	13.500	82	10
3.	BYL 3	Boyolali	664	33	33.500	65	20
4.	BYL 4	Boyolali	800	28	5.400	74	20
5.	KRY 1	Karanganyar	275	30	7.600	50	50
6.	KRY 2	Karanganyar	449	28	9.600	74	10
7.	KRY 3	Karanganyar	641	29	5.200	66	70
8.	KRY 4	Karanganyar	845	28	7.800	60	10
9.	KTN 1	Klaten	491	26	18.900	91	11
10.	KTN 2	Klaten	492	26	12.900	84	15
11.	KTN 3	Klaten	726	25	1.700	80	10
12.	KTN 4	Klaten	802	25	1.700	96	15
13.	SRG 1	Sragen	197	27	2.400	91	80
14.	SRG 2	Sragen	304	23	3.200	100	75
15.	SKH	Sukoharjo	126	33	24.200	49	80
16.	SKA	Surakarta	119	30	3.900	80	50
17.	WNG 1	Wonogiri	233	27	54.900	69	10
18.	WNG 2	Wonogiri	381	28	5.400	84	80
19.	WNG 3	Wonogiri	600	25	7.400	81	75
20.	WNG 4	Wonogiri	677	27	6.900	68	82

Morphological characters analysis

Morphological characters measurements were taken on vegetative structures such as roots, stems, leaves, and corms of the taro plant. Measurements included plant height, leaf length, leaf width, sheath length, sheath width, petiole length, petiole width, root length, root width, and corm length: width ratio.All measurements were averaged and averages for each plant were used in subsequent analyses.

Anatomical characters analysis

The anatomical characters were observed from both paradermal and transverse sections of the leaf. The preparation of microscope slide sections was carried out as described by Munir et al. (2011), Arzani et al. (2013) and Kumar et al. (2014). Light microscopic (Model: Olympus, magnification of 10 x for ocular and a 40 x for objective) observations were used to observe the specimens from both studies. The observed anatomical characters were stomatal index, stomatal density, stomatal length, stomatal width, abaxial epidermis thickness, adaxial epidermis thickness, mesophyll thickness, palisade thickness, and palisade ratio. All measurements were averaged and averages for each plant were used in analyses of variance.

Isozyme analysis

Gel and buffer preparation

Acrylamide gel electrophoresis and buffer solutions (extraction buffer, tank buffer, running buffer) were prepared and carried out as described by Suranto (2001), Setyawan et al. (2014) and Suratman et al. (2016).

Isozyme Extraction

A total of 0.15 g young leaves of taro were ground in mortar using 600 µL of extracting solution and then transferred to a 1.5 mL microtube. Samples were centrifuged at 12000 rpm for 5 minutes, and supernatant was transferred to new microtube.

Electrophoresis

About 200 µL of supernatant was taken and 5µL of bromophenol blue (tracking dye) was added to each sample. About 10-24 µL of prepared samples (10-15 µL for peroxidase and 15-24 µL for esterase) was taken and loaded into each well of acrylamide gel. Loaded samples were electrophoresed at a constant current of 5 mA for peroxidase and 7 mA for esterase at room temperature for about 60 minutes. Electrophoresis was stopped when the bromophenol blue marker dye had traveled about 56 mm from the well toward the anode (Suranto 2001; Padmanaban et al. 2013, Setyawan et al. 2014).

Staining Procedures

After electrophoresis, the gels were stained for the appropriate enzyme systems (esterase and peroxidase) as described by Suranto (2001), Setyawan et al. (2014) and Suratman et al. (2016) with some modifications. Gels were immersed in the staining solutions until bands appeared. After appearance of the bands, the gel was transferred to a fixative solution that contained 100 mL of 50% methanol, 20 mL of 10% acetic acid and 40 mL of distilled water. The gel was stored at 4^0C in refrigerator (Tiwari and Bakshi 2015).

Data analysis

Analysis of variance was performed for observed quantitative morphological and anatomical characters data in order to test the significance of variability among accessions. The data from zymograms were entered as a matrix of presence/absence of bands for each enzyme system. A similarity dendrogram among accessions was constructed on the basis of genetic similarity matrix based on morphological, anatomical and isozyme markers by applying an Unweighted Pair Group Method with Arithmetic Averages (UPGMA) cluster analysis using a computer programme, Numerical Taxonomy and Multivariate Analysis System (NTSYS) Version 2.00 (Rohlf 1998).

RESULTS AND DISCUSSION

Morphological Analysis

The analysis of variance for morphological characters revealed that there was significant difference ($p < 0.05$) among taro accessions for majority of the tested quantitative morphological traits except for variation of sheath width, petiole width, root width and corm length: width ratio, indicating the existence of substantial amount of variability for the characters among the accessions. Plant height, leaf length, leaf width, sheath length, petiole length and root length showed wide variation while sheath width, petiole width, root width and corm length: width ratio showed a narrower range of phenotypic variation (Table 2).

Plant height exhibited wide range of variation among accessions and ranged from 25 cm (WNG 1) to 79.5 cm (KTN 1), the average being 48.75 cm. The leaf length varied significantly among accessions and displayed a range from 9.8 cm (SKH) to 50 cm (BYL 1), the average being 24.62 cm. Leaf width differed significantly among tested accessions and ranged from 8 cm (SKA) to 40.3 cm (BYL 1), the average being 20.96 cm. Sheath length also exhibited wide differences among accessions and ranged from 10 cm (SKA) to 44 cm (KTN 2), the average being 28.02 cm. Petiole length showed wide differences among accessions and ranged from 30.2 cm (WNG 1) to 84 cm (KTN 2), the average being 57.99 cm. Root length displayed a range from 9.5 cm (SKA) to 46 cm (BYL 3), the average being 23.35 cm. Sheath width values exhibited narrow differences among accessions and ranged from 0.28 cm (SKA) to 2.48 cm (KTN 1), the average being 1.56 cm. Petiole width values showed narrow variation and ranged from 0.31 cm (SKA) to 2.58 cm (BYL 1), the average being 1.34 cm. Root width displayed narrow differences among accessions and varied from 0.06 cm (WNG 3, WNG 4) to 0.41 cm (BYL 2, BYL 3, SRG 1, SKH), the average being 0.22 cm. Corm length: width ratio also displayed narrow differences among accessions and varied from 1 to 4.

Table 2. Morphological character variation among taro accessions from southeastern part of Central Java (Indonesia)

Accessions	PlH	LfL	LfW	ShL	ShW	PtL	PtW	RtL	RtW	CoR
BYL 1	60.5b	50.0c	40.3c	30.8b	1.76b	63.4ab	2.58c	44.5b	0.35b	4ab
BYL 2	47.6ab	31.0b	30.0b	35.0b	1.31b	56.3ab	2.32c	41.4b	0.41b	2a
BYL 3	43.3a	12.0a	11.3a	18.9a	1.52b	45.3a	1.21ab	46.0b	0.41b	2a
BYL 4	43.3a	38.3b	28.4b	13.5a	2.08bc	59.5ab	1.24ab	28.9ab	0.29ab	1a
KRY 1	60.3b	19.5a	16.5a	31.0b	1.80b	69.5b	1.6b	20.0a	0.09a	1a
KRY 2	45.0ab	24.7ab	23.1ab	35.0b	1.80b	63.5ab	1.43b	26.5ab	0.09a	1a
KRY 3	59.0b	21.6a	21.0ab	21.9ab	1.90b	69.3b	1.43b	21.0a	0.15a	2a
KRY 4	52.0ab	32.5b	25.0ab	30.0b	1.60b	65.4b	1.75b	20.0a	0.10a	1a
KTN 1	79.5b	30.5b	24.5ab	32.0b	2.48c	82.3b	1.15ab	18.0a	0.16a	4ab
KTN 2	63.0b	32.9b	29.8b	44.0b	2.23c	84.0b	1.12ab	20.2a	0.16a	4ab
KTN 3	65.0b	36.0b	30.3b	38.6b	2.08bc	70.2b	1.17ab	18.0a	0.15a	2a
KTN 4	65.0b	29.5b	23.6ab	40.0b	1.44b	65.7b	1.24ab	15.0a	0.25ab	2a
SRG 1	62.0b	20.0a	15.6a	36.7b	1.60b	68.0b	1.27ab	27.0ab	0.41b	2a
SRG 2	70.0b	18.4a	15.0a	38.1b	2.17bc	73.6b	0.98a	28.9ab	0.29ab	1a
SKH	31.5a	9.8a	8.3a	23.8ab	0.44a	38.0a	1.14ab	18.9a	0.41b	2a
SKA	26.0a	10.0a	8.0a	10.0a	0.28a	30.5a	0.31a	9.5a	0.29ab	1a
WNG 1	25.0a	22.0ab	20.0ab	19.7a	0.74ab	30.2a	1.14ab	20.0a	0.10a	1a
WNG 2	37.5a	10.1a	9.3a	20.8a	1.60b	45.4a	1.36ab	15.2a	0.10a	2a
WNG 3	30.5a	31.0b	29.4b	19.7a	0.74ab	34.3a	1.33ab	18.0a	0.06a	1a
WNG 4	37.0a	12.5a	9.8a	20.8a	1.60b	45.3a	1.11ab	10.0a	0.06a	1a
Average	48.75	24.62	20.96	28.02	1.56	57.99	1.34	23.35	0.22	2.50

Note : * PlH = plant height (cm); LfL = leaf length (cm); LfW = leaf width (cm); ShL = sheath length (cm); ShW = sheath width (cm); PtL = petiole length (cm); PtW = petiole width (cm); RtL = root length (cm); RtW = root width (cm); CoR = corm length: width ratio.
** Values followed by the different lower-case letter in the same column are significantly different (Duncan multiple range test, $p < 0.05$)

Table 3. Anatomical character variation among taro accessions from southeastern part of Central Java (Indonesia)

Accessions	StI	StD	StL	StW	AbT	AdT	MeT	PaT	PaR
BYL 1	15.72ab	7.44a	32.48b	21.22ab	33.32b	36.38b	438.46c	115.46b	0.50a
BYL 2	14.93ab	11.00a	29.06ab	22.63ab	25.84a	24.40a	335.89b	79.07a	0.44a
BYL 3	16.28ab	11.67a	27.97a	20.91a	29.26ab	22.66a	323.09b	63.60a	0.57a
BYL 4	19.41b	12.44ab	25.21a	19.62a	24.77a	28.16ab	338.50b	96.72ab	0.50a
KRY 1	11.91a	8.67a	27.97a	19.41a	23.92a	30.76ab	335.86b	87.13ab	0.44a
KRY 2	14.23a	10.00a	25.63a	19.20a	25.20a	27.46a	397.43c	100.43b	0.50a
KRY 3	17.52ab	15.00ab	24.79a	19.83a	24.57a	28.12ab	412.83c	85.40ab	0.44a
KRY 4	14.26a	10.00a	25.21a	19.41a	26.91a	20.48a	189.76a	51.19a	0.50a
KTN 1	21.18b	18.44b	23.20a	18.99a	23.27a	24.40a	335.86b	92.90ab	0.44a
KTN 2	16.49ab	11.00a	28.40ab	20.90a	29.04ab	25.71a	284.60ab	82.37a	0.40a
KTN 3	15.76ab	8.67a	26.70a	26.28b	29.70ab	23.77a	233.36a	69.94a	0.50a
KTN 4	15.76ab	11.67a	27.76a	19.41a	25.82a	20.70a	269.20ab	82.13a	0.50a
SRG 1	17.11ab	8.89a	28.83ab	20.91a	27.74ab	25.27a	292.30ab	84.30ab	0.44a
SRG 2	9.54a	10.33a	25.00a	19.62a	29.90ab	31.59b	271.86ab	69.92a	0.44a
SKH	10.42a	19.11b	25.63a	19.62a	22.62a	32.91b	271.76ab	67.54a	0.40a
SKA	15.78ab	13.67ab	25.42a	18.36a	26.49a	32.70b	266.73ab	73.19a	0.44a
WNG 1	15.37ab	11.00a	27.96a	22.84ab	28.19ab	24.20a	289.73ab	99.32ab	0.50a
WNG 2	14.42ab	9.56a	27.32a	20.69a	25.61a	22.01a	172.70a	79.08a	0.57a
WNG 3	17.67ab	7.67a	33.54b	23.51ab	33.12b	20.24a	205.13a	61.63a	0.57a
WNG 4	20.45b	7.44a	25.00a	19.62a	27.32ab	21.58a	220.56a	74.93a	0.44a
Average	18.09	11.18	27.15	20.65	27.13	26.18	294.28	80.81	0.48

Note : * StI=stomatal index; StD = stomatal density (pore/mm^2); StL= stomatal length (µm); StW = stomatal width (µm); AbT = abaxial epidermis thickness (µm); AdT = adaxial epidermis thickness (µm); MeT = mesophyll thickness (µm); PaT = palisade thickness (µm); PaR = palisade ratio.
** Values followed by the different lower-case letter in the same column are significantly different (Duncan multiple range test, $p < 0.05$)

Anatomical analysis

The analysis of variance for anatomical characters revealed that there was significant variation ($p < 0.05$) for all the tested characters among taro accessions, except for the difference of palisade ratio. However, accessions variation for palisade ratio was non-significant (Table 3).

Comparing the stomatal index, stomatal densities, stomatal length and stomatal width, there was significant

variability among all tested accessions. The lowest stomatal index value was distributed in SRG 2 accession (9.54) whereas the highest one was in the KTN 1 accession (21.18), the average being 18.09. BYL 1 accession displayed the lowest value of stomatal density (7.44 pores/mm^2) whereas the highest one could be found in SKH accession (19.11 pores/mm^2), the average being 11.18 pores/mm^2. Stomatal length also exhibited differences among accessions and ranged from 23.20 µm (KTN 1) to 33.54 µm (WNG 3), the average being 27.15 µm. Stomatal width differed significantly among tested accessions and was highest in the accession SKA (18.36 µm) and lowest in the accession KTN 3 (26.28 µm), the average being 20.65 µm.

Abaxial epidermis thickness exhibited variation and ranged from 22.62 µm (SKH) to 33.32 µm (BYL 1), the average being 27.13 µm. The adaxial epidermis thickness varied significantly among accessions and displayed a range from 20.24 µm (WNG 3) to 36.38 µm (BYL 1), the average being 26.18 µm. Mesophyll thickness differed significantly among tested accessions and was highest in the accession BYL 1 (438.46 µm) and lowest in the accession WNG 2 (172.70 µm), with the average being 244.48 µm. Palisade thickness also exhibited wide differences among accessions and ranged from 51.19 µm (KRY 4) to 115.46 µm (BYL 1), the average being 80.81 µm. Palisade ratio displayed narrow differences among accessions and varied from 0.40 to 0.57, the average being 0.48.

Isozyme Analysis

Polymorphism was observed in taro accessions from Central Java using isozymes of esterase and peroxidase. Esterase and peroxidase have been widely utilized to assess the genetic similarity and to reveal the variation of organisms at the various taxonomic levels. The two enzymatic systems showed a total of 20 banding pattern, distributed in the whole set of samples as esterase with 12 banding pattern and peroxidase with 8 banding pattern.

A total of 12 pattern zymograms (banding pattern A-L) of esterase at different Rf values varying from 0.08 to 0.95 were observed (Figure 2). Banding pattern A was distributed in two accessions (KTN 1, KTN 2) and consisted of two bands which were located at Rf 0.1 and Rf 0.35. Banding pattern B was distributed in three accessions (KTN 3, KTN 4, KRY 1) and consisted of two bands which were located at Rf 0.1 and Rf 0.3. Banding pattern C was distributed in two accessions (SRG 1, SRG 2) and consisted of two bands which were located at Rf 0.1 and Rf 0.25. Banding pattern D was distributed in three accessions (KRY 2, KRY 3, KRY 4) and consisted of two bands which were located at Rf 0.1 and Rf 0.27 from anodal zone. Banding pattern E was only distributed in WNG 4 accession and consisted of three bands which were located at Rf 0.08; Rf 0.21 and Rf 0.95. Banding pattern F was distributed only in WNG 3 accession and consisted of five bands which were located at Rf 0.08; Rf 0.21; Rf 0.29; Rf 0.45 and Rf 0.95. Banding pattern G was distributed in two accessions (WNG 2, BYL 1) and consisted of five bands which were located at Rf 0.08; Rf 0.21; Rf 0.29; Rf 0.30 and Rf 0.95. Banding pattern H was distributed only in WNG 1 accession and consisted of four bands which were located at Rf 0.08; Rf 0.21; Rf 0.29 and Rf 0.30. Banding pattern I was distributed only in BYL 2 accession and consisted of four bands which were located at Rf 0.08; Rf 0.21; Rf 0.29 and Rf 0.95.Banding pattern J was only distributed in BYL 3 accession and consisted of four bands which were located at Rf 0.08; Rf 0.21; Rf 0.29; and Rf 0.45. Banding pattern K was distributed only in BYL 4 accession and consisted of six bands which were located at Rf 0.08; Rf 0.21; Rf 0.29; Rf 0.35; Rf 0.45 and Rf 0.95. Banding pattern L was distributed in two accessions (SKH, SKA) and consisted of three bands which were located at Rf 0.08; Rf 0.21; and Rf 0.25.

Peroxidase analysis showed eight patterns zymogram (banding pattern A-H) distributed in different Rf value varying from 0.08 to 0.73. Eight isozymic banding pattern of peroxidase also was distributed separately in tested taro accessions (Figure 3). Banding pattern A was distributed only in KTN 1 accession and consisted of two bands located at Rf 0.08 and Rf 0.3. Banding pattern B was distributed in two accessions (KTN 2, KTN 4) and consisted of two bands located at Rf 0.3 and Rf 0.4 from anodal zone. Banding pattern C was distributed in seven accessions (KTN 3, SRG 1, SRG 2, KRY 1, KRY 2, KRY 3, KRY 4) and only consisted of one band located in Rf 0.3. Banding pattern D was distributed in three accessions (WNG 2, WNG 3, WNG 4) and consisted of two bands located at Rf 0.18 and Rf 0.73. Banding pattern E was distributed only in WNG 1 accession and consisted of four bands located at Rf 0.18; Rf 0.4; Rf 0.6 and Rf 0.73. Banding pattern F was distributed in two accessions (BYL 1, BYL 2) and consisted of five bands located at Rf 0.18; Rf 0.4; Rf 0.56; Rf 0.6; Rf 0.73. Banding pattern G was distributed in two accessions (BYL 3, BYL 4) and consisted of three bands located at Rf 0.18; Rf 0.4 and Rf 0.73. Banding pattern H was distributed in two accessions (SKH, SKA) and consisted of two bands located at Rf 0.18 and Rf 0.4.

Similarity among taro accessions

Based on the dendrogram at a level of 62 % similarity, it showed distinct separation of 20 taro accessions from Central Java into two major clusters (Figure 4). Cluster I comprised most of tested accessions which originated from all Boyolali accessions (BYL 1, BYL 2, BYL 3 and BYL 4), all Wonogiri accessions (WNG 1, WNG 2, WNG 3 and WNG 4), Sukoharjo (SKH accession) and Surakarta (SKA accession).

The closest relationship showed between SKH and SKA accessions that had 96 % similarity coefficient. The ten accessions that remained from all Klaten accessions (KTN 1, KTN 2, KTN 3, KTN 4), all Karanganyar accessions (KRY 1, KRY 2, KRY 3, KRY 4), and all Sragen accessions (SRG 1, SRG 2) were then clustered separately apart from the other as Cluster II with similarity coefficient of 73.52 % and considered to be genetically unique.

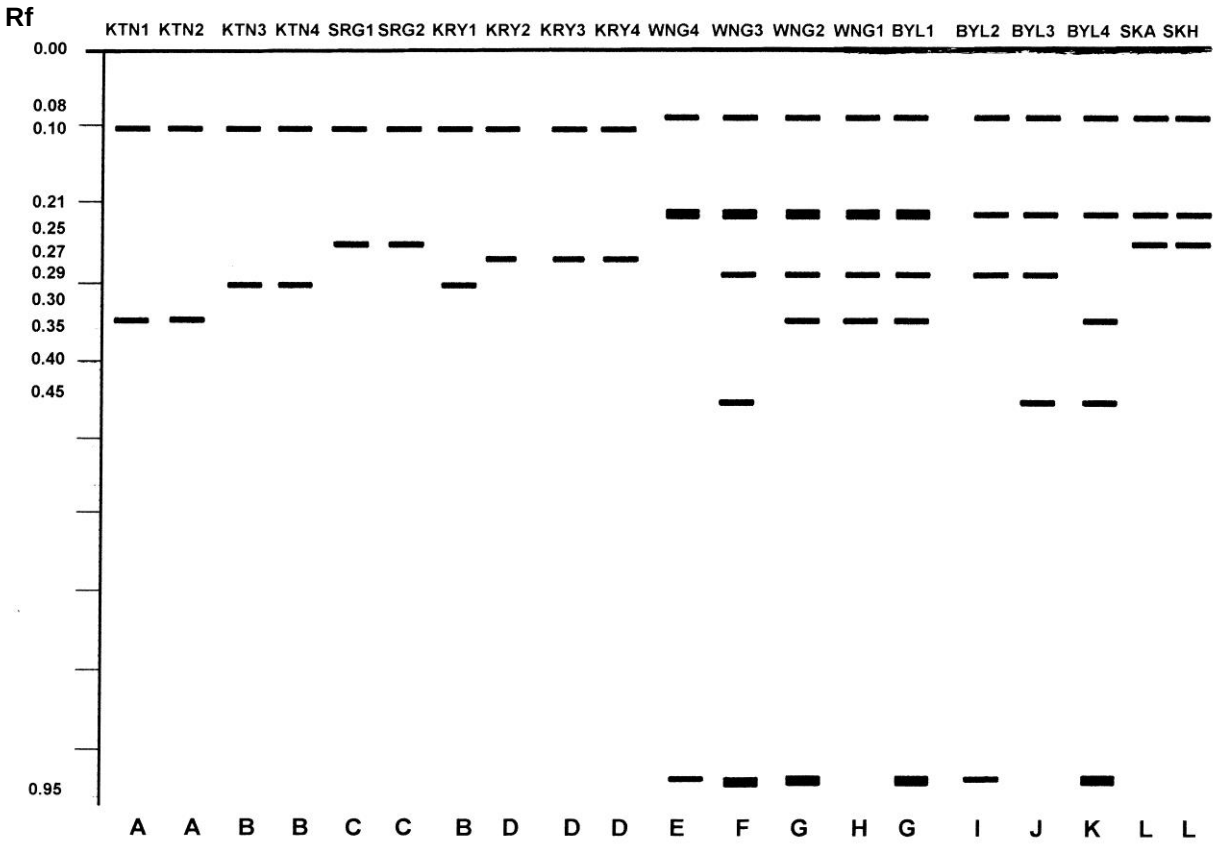

Figure 2. Esterase isozymic banding pattern of 20 taro accessions from southeastern part of Central Java (Indonesia)

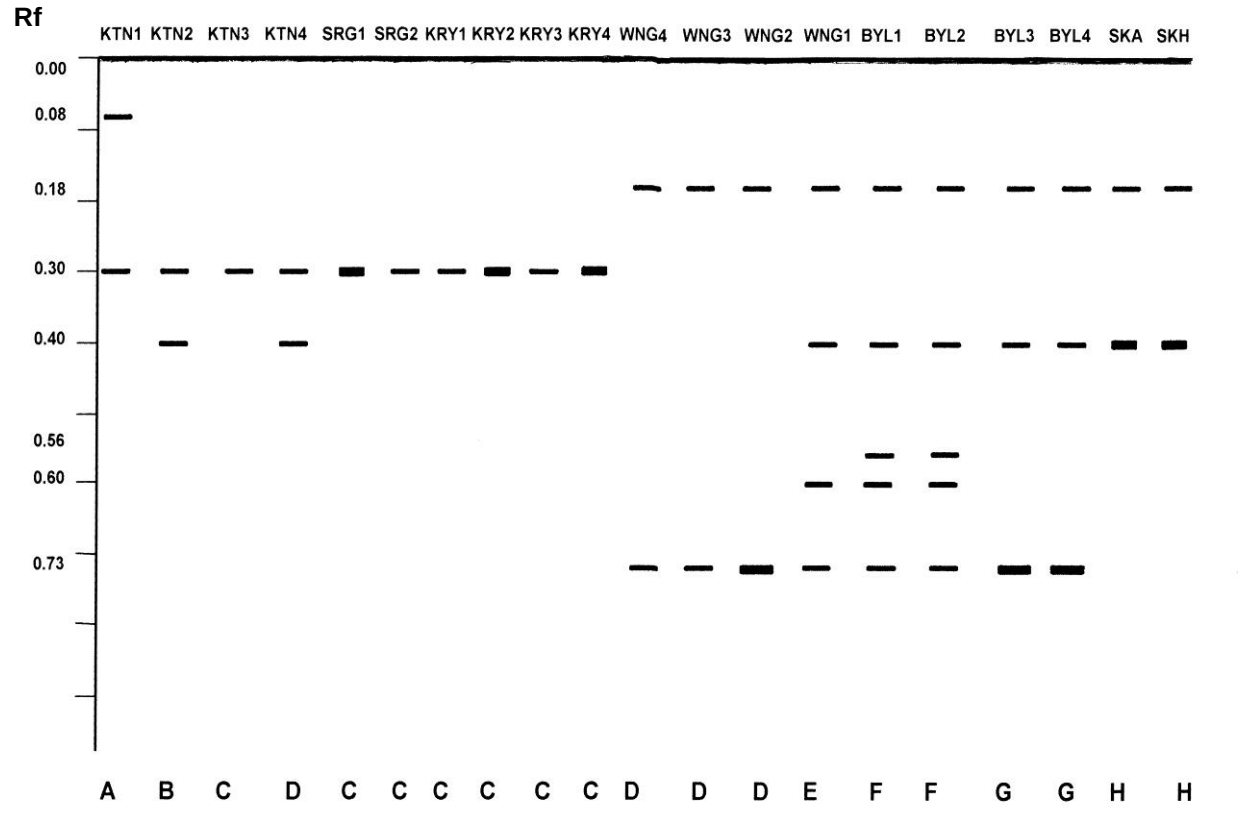

Figure 3. Peroxidase isozymic banding pattern of 20 taro accessions from southeastern part of Central Java (Indonesia)

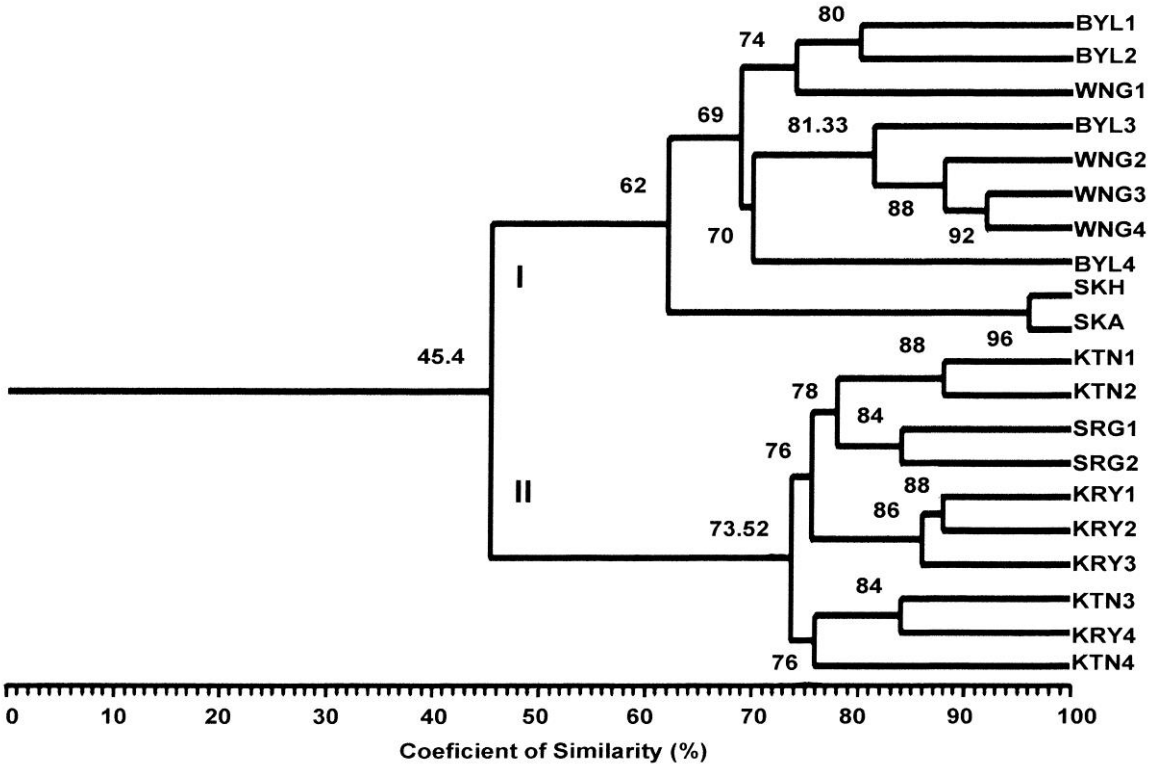

Figure 4. Relationship dendrogram among 20 taro accessions from southeastern part of Central Java (Indonesia) using morphological, anatomical and isozyme markers

Discussion

Analysis of variance for morphological characters used in the study indicates high genetic diversity among taro accessions. Thus, such methods have been successfully used to measure phenotypic diversity in germplasm collection. This indicates the existence of large diversity in taro, especially for quantitative characters. Indeed, these taro accessions collected from a wide range of sites during field surveys, when cultivated under the same microclimate, conserved the characteristics acquired previously. This indication then provided vital information on the morphological diversity (Mbouobda et al. 2007).

In general, this study revealed that the taro accessions showed variability in the majority of tested morphological characters. Morphological characters such as plant height, leaf length, leaf width, sheath length, petiole length, and root length also had high values. This implies that these characters could be employed for distinguishing variability in the accessions. Therefore, this indication showed that there is enough scope for selection of desirable characters, where variability exists (Beyene et al. 2013; Suratman et al. 2016).

The existence of significant variation among the accessions for the majority of the studied morphological characters is a sign of the presence of high degree of genetic variation implying great potential of the accessions in future breeding programs through selection (Nkansah et al. 2013; Roy et al. 2013; Sabaghnia et al. 2014).

Genetic variability as reflected from morphological characters is the raw material of crop breeding on which selection acts up to evolve into a superior genotype. Thus, the higher amount of variations expressed for a character in the breeding material, then the scope for its improvement through selection is greater (Osawaru et al. 2013).

Analysis of variance for anatomical characteristics revealed that there was significant variability for all the characters, except for variation of palisade ratio. The stomatal index, stomatal densities, stomatal length, and stomatal width varied significantly among all tested accessions. Stomata characteristics such as frequency and dimensions can be affected by type of species and environmental factors. Thus, the higher stomatal density or stomatal index can also be used as an indicator for higher transpiration rate, highest metabolism and absorption of mineral and water (Dong and Zhang 2000; Munir et al. 2011). Although stomatal features can be affected by complex environmental factors, stomatal differentiation and development are certainly determined by genetic factors (He et al. 1998; Hetherington and Woodward 2003). It has been reported that some stomatal features can be used as a selection marker for breeding program (Yang et al. 2004).

The leaf tissue layer thickness such as abaxial epidermis thickness, adaxial epidermis thickness, mesophyll thickness, and palisade thickness also showed variability among all examined accessions. The difference in leaf tissue layer thickness might be attributed to the

responses toward environmental factors (Brouillette et al. 2006; Donovan et al. 2007; Noman et al. 2014). Thus, these leaf anatomical characteristics can be used as a selection marker for genetic improvement of plants, especially to improve their adaptability to adopt varied environmental conditions.

The majority of the tested anatomical characters showed highly significant variation among all tested accessions although some anatomical characters might be influenced by environmental factors. This information indicated that there is enough scope for selection of accessions on the basis of these characteristics for genetic improvement (Kumar et al. 2014, Suratman et al. 2016).

Isozyme profiling of two enzyme systems viz. esterases and peroxidases enzyme were exploited to find out the variability among taro accessions. It was found that esterase and peroxidase isozymes are effective in differentiation among these accessions. Esterase showed most isozymic banding pattern variations (with 12 isozymic banding pattern) compared to peroxidase (with 8 isozymic banding pattern). Then, esterase exhibited significant variation among accessions in terms of number of bands and their thickness. Therefore, esterase is a useful diagnostic tool in this study for assessment of genetic variation in view of the extensive polymorphism for this enzyme (Tiwari and Bakshi 2015). Esterase is considered to be one of the most suitable enzyme systems for differentiation of group of plants (Rakshit et al. 2011; Sumathi and Balamurugan 2014).

Polymorphism is essential in use of isozymes as genetic marker. A large number of polymorphic zones reflect the validity of the isozyme data to study the genetic diversity (Kumar et al. 2013). Sher et al. (2010) stated that isozymes are still useful markers for genetic polymorphism identification due to its simplicity and validity for describing genetic structure of groups of plants.

The results from this present study concluded that the specific banding pattern observed in esterases and peroxidases can be used for accessions differentiation. Then, difference in the isozymes profile can reveal genetic diversity among accessions. However, an adequate level of genetic diversity is very essential for effective selection in a breeding programme.

The UPGMAdendrogram in this study showed that each group was comprised of geographically related accessions. However, the grouping did not always indicate the geographical origins similarity, but possibly showed the genetic similarity (Tikader and Kamble 2008). The genetic variability in taro accessions may be partly explained as a result of abiotic and biotic factors. Geographical, climatic or reproductive variables explain the partitioning of the diversity observed which may aid in improving the strategies for maximizing the efficiency of germplasm collection and preservation for breeding of taro accessions (Suratman et al. 2013).

One of the main applications of these clusters is the estimation of the genetic similarity among accessions and identification of parents for performing appropriate crosses, and reaching maximum heterosis in hybridization programs. Selection of better accessions can be made for species improvement based on its genetic similarity percentage. Two genetically similar accessions or more and possessing suitable characters for breeding activities can be chosen for this purpose (Prabha et al. 2010; Lombardi et al. 2014; Suratman et al. 2015).

Thus, genetic characterization based on morphological, anatomical and isozyme markers obtained in this study could be valuable for understanding of genetic variability among the examined taro accessions. It will also provide an important input into determining resourceful management strategies and help breeders in the taro improvement programme (Setyawan et al. 2014).

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The diversity of *Nepenthes* at the post-mining area in Sintang District, West Kalimantan, Indonesia

HENDRA SETIAWAN[1,♥], HILDA AQUA KUSUMA WARDHANI[1], KAMALUDIN[2], RIA ROSDIANA HUTAGAOL[2,♥♥], RACHMI AFRIANI[1]

[1]Department of Biology Education, Faculty of Teaching Training and Education, Universitas Kapuas. Jl. Y.C. Oevang Oeray No. 92, Baning Kota, Sintang, 78612, West Kalimantan, Indonesia, Tel: +62-565-22256, Fax: +62-565-202-0034, ♥email: hendra_setiawan09@yahoo.com
[2]Department of Forestry, Faculty of Agriculture, Universitas Kapuas. Jl. Y.C. Oevang Oeray No. 92, Baning Kota, Sintang, 78612, West Kalimantan, Indonesia, Tel: +62-565-22256, Fax: +62-565-202-0034, ♥♥email: riarose.h@gmail.com

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Abstract. *Setiawan H, Wardhani HAK, Kamaludin, Hutagaol RR, Afriani R. 2018. The diversity of Nepenthes at the post-mining area in Sintang District, West Kalimantan, Indonesia. Biodiversitas 19: 1820-1827. Nepenthe is* a tropical pitcher plant found in Indonesian archipelago and lives in nutrient leak habitat. One of *Nepenthes* nutrient leak habitat commonly found in Kalimantan Island is the post-mining area. This research aimed to know the habitat condition, species composition, species richness and diversity index of *Nepenthes* in Post-mining area in Sintang District, West Kalimantan Province. Thirty plots, each measuring 5 x 5-meter square, were established to survey the *Nepenthes* diversity in five locations of the post-mining area in Sintang District. There were 910 individuals of *Nepenthes* found spreading in two sub-Districts and five areas. Habitat condition in the post-mining area tended to be high in light intensity and temperature but low in humidity. Five *Nepenthes* species were found, namely *N. ampullaria*, *N. bicalcarata*, *N. gracilis*, *N. mirabilis*, and *N. rafflesiana*. Four natural hybrids were also found including *N. xhookeriana*, *N. xkuchingensis*, *N. xneglecta*, and *N. xtrichocarpa*. The species richness in post-mining area were 1.25 in Danau BTN (Bank Tabungan Negara/State Savings Bank) 0.95 in Dusun Kerangas, 0.58 in Sungai Ana Village, 0.21 in Jerora 1 and 0.20 in Danau Baning. The diversity index in Danau BTN, Dusun Kerangas, Sungai Ana, Jerora 1 and Danau Banning area were of low levels, respectively, 1.66, 1.3, 1.0, 0.64, and 0.68. Cluster analysis of species richness, diversity index, and abiotic factors showed that Danau Baning-Sungai Ana-Jerora 1 was grouped in the same cluster while Danau BTN and Dusun Kerangas were in a separate cluster.

Keywords: Diversity, *Nepenthes*, post-mining, Sintang District

INTRODUCTION

Nepenthes (Family of Nepenthaceae) is the largest genus of pitcher plants (Moran and Clarke 2010). The genus contained 114 species over the world (IUCN) and distributed from Eastward of Madagascar to New Caledonia at Pacific Ocean (Jebb and Cheek 1997). Indonesian Archipelago is a central diversity of *Nepenthes* with 32 endemic species in Borneo Island, 24 endemic species in Sumatera Island and some species found in Java, Sulawesi, and Papua Islands (Jebb and Cheek 1997; Clarke 2001; Clarke 2006;).

Nepenthes are tropical carnivorous plants that evolved it leaf extensions into jug shaped structures which contain a pool of digestive enzymes to attract, trap and digest animals for its nutritional benefits (Clarke 2006). *Nepenthes* pitchers are passive, gravity-driven traps that show distinct functional zonation on their inner surfaces (Moran and Clarke 2010). Beside prey capturing, an extra nutrition also can be extracted from other scenario such as litterfall with “carpets” of pitchers at ground level and animal faces (*Kerivoula hardwickii*’s faces of *N. hemsleyana* and *Tupaia Montana*'s faces of *N. lowii*) (Clarke 2006; Moran and Clarke 2010; Gaume et al. 2016). This condition (nutrition sequestering by the pitcher) is vital because *Nepenthes* commonly found in lack nutrient habitat (Latiff et al. 2014).

The post-mining area is one of nutrient-deficient habitat where the *Nepenthes* is easily found. This area was formed by abandoned illegal gold mining or other mining activity in open space, usually in *kerangas* forest or watershed. This habitat is similar to *Kerangas* forest which generally has siliceous and acidic soil, higher temperature and lower humidity, all of which are preferred by *Nepenthes* (Latiff et al. 2014). In Sintang District, West Kalimantan Province, numbers of post-gold mining areas are increasing every year because of the local government policy to create a better ecosystem and fulfill the vision of "the Sustainable Sintang District." This research aimed to determine the species composition, species richness and diversity index of *Nepenthes* in Post-mining area in Sintang District.

MATERIALS AND METHODS

This research was conducted during February-July 2018 in five locations of post-mining areas in Sintang District, West Kalimantan Province. The post-mining areas are located in two sub-districts, i.e., Sintang Sub-district and Sepauk Sub-district. There were four post-mining areas in Sintang Sub-district namely post-mining Sungai Ana, post-mining Danau BTN (Bank Tabungan Negara/State Savings

Bank), post-mining Jerora 1, and post-mining Danau Baning. There were no longer mining activities in these four locations, and the areas are now being used for other activities such as tourism (swimming pool at the ex-mining hole), settlement area, and swallow-nest farm. Another location is in Sepauk Sub-district at Kerangas Village, where mining activity (gold and sand mining) is still ongoing but the sampling plots were placed on around the mining area. In each location, a plot of 5 m x 5 m size was set up to observe the *Nepenthes*. The location was determined by using a purposive sampling technique to ensure the presence of *Nepenthes* (Lestariningsih and Setyaningsih 2017). The research site was surveyed comprehensively in localities with *Nepenthes* presence, but the plots were set up randomly and discontinuously (Latiff et al. 2014; Lestariningsih and Setyaningsih 2017). Well-known *Nepenthes* were identified directly in the fields while unknown species were collected as herbarium specimen for further identification. Data collection in plots included several variables such as habitat description (another biodiversity in the location of *Nepenthes* presence) and microclimate (humidity, light intensity, and air temperature) which were later analyzed by PAST ver. 2.14's software (Hammer et al. 2001). Morphological data were analyzed descriptively, species richness was analyzed with Margalef's index (Aslam 2009; Endrawati et al. 2017), and diversity index was calculated using Shannon-Wiener's diversity index (Aslam 2009; Olopade and Rufai 2014; Komara et al. 2016).

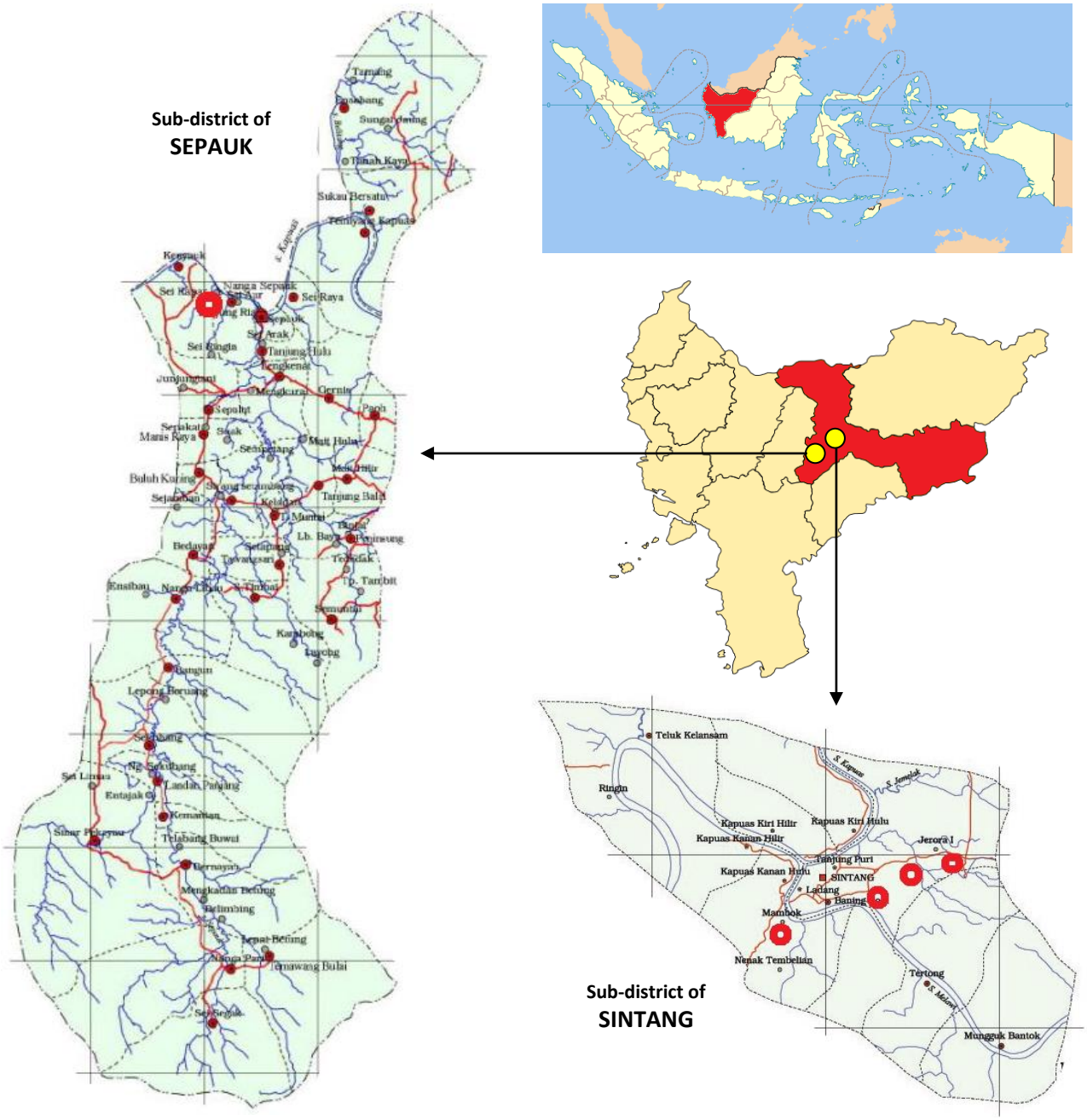

Figure 1. Study area in two sub-districts (Sintang and Sepauk Sub-district) of Sintang District, West Kalimantan Province, Indonesia. Red dots mean study sites

Margalef's index of species richness = (S-1)/ ln N

Where:
S : total species number
N : total of individual number in the sample
ln : natural logarithm

Shannon-Wiener's diversity index : H : - ∑ Pi ln Pi

Where:
Pi : S / N
S : total species number
N : total individual number in the sample
ln : natural logarithm

RESULTS AND DISCUSSION

Habitat conditions

All *Nepenthes* habitat referred to in this research is the post-mining areas in Sintang District. Some of this habitat was active illegal mining areas, and others were shifted to other activities like residential area and tourism. Before the illegal mining activity took place, all of this habitats were *Kerangas* forest. The soil in *Kerangas* forest was generally siliceous and acidic, the canopy was much lower then Dipterocarpaceae forest and tended to be more uniform (Clarke 2006).

In this research, habitat condition of the post-mining area was represented by air temperature, humidity, and light intensity (Table 1). All the variation of microclimate is dependent on the canopy and vegetation over the area. In an open area with less vegetation, air temperature and light intensity tended to be higher while the humidity was low (Table 1). This condition affected the presence of *Nepenthes*. In the area with high air temperature and light intensity like Sungai Ana, Danau Baning and Jerora 1, the number of species found tended to be less than that in closed canopy area with lower air temperature and light intensity like Danau BTN and Dusun Kerangas (Table 1). Clarke (2006) found that the climate of undisturbed *Kerangas* (the area before mining activity) is superficially similar to that in mixed Dipterocarpaceae forests. This type of climate supports the living of more *Nepenthes* as compared to the open canopy area.

N. gracilis and *N. mirabilis* were prone to be on the habitat with open canopy area while *N. ampullaria* and *N. bicalcarata* preferred the area with close canopy and high in humidity (Table 1). *N. rafflesiana* were found in both open and closed canopy areas. Most of *Nepenthes* hybrid was found in close canopy area while only one other species was found in open canopy area (*N. xneglecta*). *N. xneglecta* is a hybrid of *N. gracilis* and *N. mirabilis*. Although both *Nepenthes* commonly live in the same habitat, the hybrid was rarely found. The high abundance of the parents may likely be the reason for the existence of this hybrid (Clarke 2006).

Nepenthes compositions

There were five *Nepenthes* species and four natural hybrids that were found in five locations within 30 plots. *Nepenthes* found in the post-mining area included *N. ampullaria* Jack, *N. bicalcarata* Hook.f, *N. gracilis* Korth, *N. mirabilis* (Lour.) Druce, and *N. rafflesiana* Jack. var. typical. Moreover, four natural hybrids of *Nepenthes* were also found, i.e., *N. ampullaria* x *N. rafflesiana* (*N. xhookeriana*), *N. ampullaria* x *N. mirabilis* (*N. xkuchingensis*), *N. gracilis* x *N. mirabilis* (*N. xneglecta*), *N. ampullaria* x *N. gracilis* (*N. xtrichocarpa*). A total of 910 *Nepenthes* individuals were found in this research; the highest number was *N. gracilis* (401 ind) followed by *N. mirabilis* (245 ind) in the second place (Figure 2).

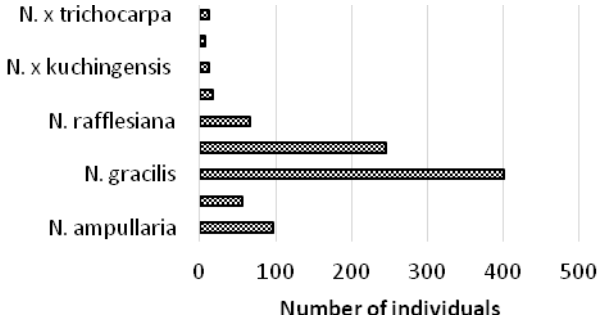

Figure 2. *Nepenthes* composition in the Post-mining area

Table 1. Humidity, air temperature, light intensity and number of individuals in each survey location

Location	Hm (%)	Temp (°C)	Lig (lux)	Number of individuals									Total of ind
				N.amp	N.bic	N.gra	N.mir	N.raf	N.xho	N.xkh	N.xne	N.xtr	
Sungai Ana (open canopy)	52.17	36.43	25970	0	0	58	68	43	0	0	6	0	175
Jerora 1 (open canopy)	52.33	34.07	43915	0	0	82	43	0	0	0	0	0	125
Danau Baning (open canopy)	53.00	33.83	31556	0	0	57	75	0	0	0	0	0	132
Danau BTN (closed canopy)	65.67	32.68	14823	34	49	126	13	20	17	9	0	12	280
Dsn Kerangas (closed canopy)	73.83	29.7	8241	62	6	78	46	3	0	3	0	0	198
Total				96	55	401	245	66	17	12	6	12	910

Note: Hm: humidity, Temp: air temperature, Lig: light intensity, N.amp: *N. ampullaria*, N.bic: *N. bicalcarata*, N.gra: *N. gracilis*, N.mir: *N. mirabilis*, N.raf: *N. rafflesiana*, N.xho: *N. xhookeriana*, N.xkh: *N. xkhucingensis*, N.xne: *N. xneglecta*, N.xtr: *N. xtrichocarpa*, Total of ind: total of individuals

Figure 3. Nepenthes species in the Post-mining area at Sintang District, West Kalimantan, Indonesia. A. *NEPENTHES ampullaria*, B. *N. bicalcarata*, C. *N. gracilis*, D. *N. xhookeriana*, E. *N. xkuchingensis*, F. *N. mirabilis*, G. *N. xneglecta*, H. *N. rafflesiana*, I. *N. Xtrichocarpa*. Bar = 2.5 cm

Description of *Nepenthes*

Each *Nepenthes* has a specific morphologic character that makes it distinct from one to another. This particular characteristic was used as a simple field identification of *Nepenthes*. The most variations appeared in the pitcher morphology and its colors (Clarke 2006). Some descriptions of *Nepenthes* found in post-mining habitat were different from those of *Nepenthes* in other habitats. The specific characters are presented below.

Nepenthes ampullaria

Nepenthes ampullaria has a unique lid of the pitchers which are cuneate and reflexed. These *Nepenthes* pitchers vary in colors such as light green, dark green, red, dark brown, bright green with red peristome, red with green peristome, green with red and brown blotches, red with brown blotches and some other combinations. In the post-mining area, *N. ampullaria* tended to be smaller than that in undisturbed habitat. The smaller size of *N. ampullaria* is an adaptative mechanism resulted from the lack of nutrient and to prevent the evaporation in an open area (Clarke 2006; Bauer et al. 2011).

Nepenthes bicalcarata

Nepenthes bicalcarata has a specific character that is distinct from other *Nepenthes* species, i.e., a pair of large thorns under the pitcher lid. This pair of large thorns is used to attract the nectar preys to enter the pitcher. The pitcher of *N. bicalcarata* is commonly found in two different forms; an upper pitcher and a lower pitcher in one individual to capture different types of preys. *N. bicalcarata* plants are the largest of genus *Nepenthes*, although the pitchers are not the largest one (Clarke 2006). *N. bicalcarata* has the largest stem and leaf in the *Nepenthes* genera. The stem of this plant can reach five m long in the post-mining area in Sintang District while other *Nepenthes* species can only achieve less than one meter in length.

Nepenthes gracilis

Nepenthes gracilis was commonly found in post-mining areas in Sintang District. Its pitcher is relatively smaller than that of other lowlands *Nepenthes* (less than 15 cm height). Its stem is triangular and climbs other vegetation to get better light. In the post-mining area, *N. gracilis* was found as a pioneer plant after the mining activity was closed. In this research, *N. gracilis* was always seen with *N. mirabilis* (Table 1) because both *Nepenthes* live in the same habitat (Clarke 2006; Setiawan et al. 2015).

Nepenthes mirabilis

Nepenthes mirabilis was the most adaptive *Nepenthes* in the post-mining area along with *N. gracilis*. It was found in different colors from light green to dark brown. Sometimes, it was found in full red from stem, leaves, and pitcher, especially in the profoundly disturbed habitat like post-mining area (Figure 3). This *Nepenthes* has most familiar flowers and fruits over seasons (Handayani 2017). In the post-mining area, the arthropods and wind factors facilitate the spread of the seed to entire habitat at the post-mining area.

Nepenthes rafflesiana

Nepenthes rafflesiana is lowland *Nepenthes* species in which lower and upper pitcher has many differences. The lower pitcher was more bulbous and had a pair of wing running from top to bottom. The upper pitcher looked more attractive with horn-shape and had a big lid to cover the peristome. Pitcher mouth of this *Nepenthes* was oblique and elongated into the neck at the back. Pitcher of *N. rafflesiana* was varying in colors from green to brown and blotches cover the pitcher's body (Listiawati and Siregar 2008). In the post-mining area, the color of *N. rafflesiana* pitcher was generally light green to dark green with red/brown blotches. This color variation is less than that in undisturbed habitat as an adaptation from habitat stress in the heavily disturbed post-mining area (Clarke 2006).

Nepenthes xhookeriana

Nepenthes xhookeriana is a natural hybrid from *N. ampullaria* and *N. rafflesiana*. The characteristic of *N. xhookeriana* is the combination of both of its parents. The pitcher was round and smaller than that of *N. rafflesiana*. The peristome of the pitcher was extended but less than that of *N. rafflesiana*. The lid of the pitcher was similar to that of *N. ampullaria* but broader and taller than one. The upper pitcher was ovoid and smaller than lower pitcher. The appearance of upper pitcher in *N. xhookeriana* was a contrast of *N. ampullaria* because *N. ampullaria* rarely produced it. The upper pitcher of *N. xhookeriana* was predicted from *N. rafflesiana* parentals (Clarke 2006).

Nepenthes xkuchingensis

Nepenthes xkuchingensis is a natural hybrid from *N. ampullaria* and *N. mirabilis*. According to Clarke (2006), this hybrid species was commonly found in Borneo but somehow appeared among both parental populations. In the post-mining area at Sintang District, it was only found in one location; in Danau BTN pal IV at Kapuas Kanan Hulu Village. The lower pitcher is more like that of *N. mirabilis* with well-developed wings and expanded peristome. The leaf size ranged between both parents and was smoother than that of *N. ampullaria*.

Nepenthes xneglecta

Nepenthes xneglecta is known as a natural hybrid from *N. gracilis* and *N. mirabilis*. According to Clarke (2006), even though both plants were commonly found in the same habitat but natural hybrids were rarely found. *N. xneglecta* is more like *N. gracilis* in shape, but in size, it is more like *N. mirabilis*. The leaf of its hybrid has the same characteristic as that of *N. mirabilis*. The stem of *N. xneglecta* is more cylindrical than *N. gracilis*.

Nepenthes xtrichocarpa

Nepenthes xtrichocarpa is a natural hybrid of *N. ampullaria* and *N. gracilis*. It was found in sandy-siliceous habitat in Danau BTN pal IV at Kapuas Kanan Hulu Village. The pitcher is relatively the same size as *N.*

gracilis but slightly wider in all aspects. The peristome is more like that of *N. ampullaria*. The lower pitcher is usually found in clumps cover the ground layer. The upper pitcher appears as a prove that *N. gracilis* parental is more dominant in this hybrid species. The upper pitcher is more like a "cup" with the lower part is smaller than the upper part.

Species richness

The post-mining area with the highest species richness (1.25) was Danau BTN in Kapuas Kanan Hulu Village followed by Dusun Kerangas and Sungai Ana Village with species richness of, respectively, 0.95 and 0.58. The lowest species richness was found in Jerora 1 (Jerora 1 Village) and Danau Baning (Banning Kota Village), respectively, 0.21 and 0.20 (Figure 4).

All of this *Nepenthes* species were found in post-mining areas affected by different community activities. Danau BTN is a post-mining area already covered by secondary vegetation like *Resam* fern (*Gleichenia* spp.), *Geronggang* (*Cratoxylum arborescens*), and *Jonger* (*Ploiarium alternifolium*). The dead root, stem and leaf of *Resam* fern are good growth media for lowland *Nepenthes*. Geronggang and *Jonger* are canopy trees in *Kerangas* habitat. The vegetation is an essential aspect of *Nepenthes* growth as it affects microclimate, soil organic matter, and biodiversity in that specific area/habitat (Clarke 2001; Clarke 2006).

The diversity index was used to determine the diversity of *Nepenthes* in each plot. The Shannon-Wiener Diversity Indices in five locations of the post-mining area showed some differences. The highest diversity index was found in Danau BTN (1.66) followed by Dusun Kerangas (1.30), Sungai Ana (1.00), Danau Baning (0.68), and Jerora 1 (0.64) (Figure 5). All of these diversity indices were categorized as less diverse (Gandhi and Sundarapandian, 2014). The diversity of *Nepenthes* in the post-mining area was impacted by habitat condition. In the location with high vegetation canopy cover like Danau BTN, the *Nepenthes* tended to be more diverse than that in open areas like Jerora 1 and Danau Baning since the soil nutrients in vegetated area (Danau BTN) was higher (than the disturbed area), which provided enough nutrient for plants growth (Singh et al. 2015).

Cluster analysis

Cluster analysis was used to identify the similarity between each location based on species richness, Shanon Wienner index, and abiotic factors. Cluster analysis divided the sites into three clusters, in which, Danau Baning, Sungai Ana and Jerora 1 were in one group, and Dusun Kerangas and Cluster Danau BTN were in a separate group (Figure 6).

Danau Baning, Sungai Ana and Jerora 1 are post-mining areas characterized by open canopy area, high temperature, and light intensity and low humidity. This cluster was also characterized with *N. mirabilis* as the dominant species. *N. mirabilis* was known as the most adaptive species in the genus and can live in a harsh habitat with high light intensity and temperature (Clarke 2006).

The cluster containing Danau BTN was characterized by the existence of *N. bicalcarata*, *N. xkuchingensis*, *N. xhookeriana*, *N. xtrichocarpa*. This area was a vegetated area with specific *Kerangas* secondary vegetation like *Gleichenia* spp and *Cratoxylum arborescens*. This vegetation makes a good condition (microclimate) for the growth of these *Nepenthes*.

Dusun Kerangas was a post-mining area, some of which were covered by secondary *Kerangas* vegetation. Different from Danau BTN, there were less *Gleichenia* spp. in Dusun Kerangas, but the area was covered by *Cratoxylum* spp. and other three species. The humidity was relatively higher, and the light intensity was the lower than other areas (Clarke 2006).

Based on biplot analysis, the group of Danau Baning-Sungai Ana-Jerora 1 was characterized by very dominant *N. mirabilis* in this area (Figure 7). Most of *N. mirabilis* was found in this group area. Dusun Kerangas was placed in a separate group characterized by high humidity and *N. ampullaria*. *N. ampullaria* was known as a species which tends to prefer a close canopy where it scrambles among low bushes (Clarke 2006). The group comprising Danau BTN was a group with many characters such as *N. bicalcarata*, *N. xhookeriana*, *N. xtrichocarpa*, and *N. kuchingensis*. Danau BTN was a vegetated habitat that supports the growth of *Nepenthes*. The existence of many *Nepenthes* in the same habitat provided [a] higher opportunity to find a hybrid species (Clarke 2006).

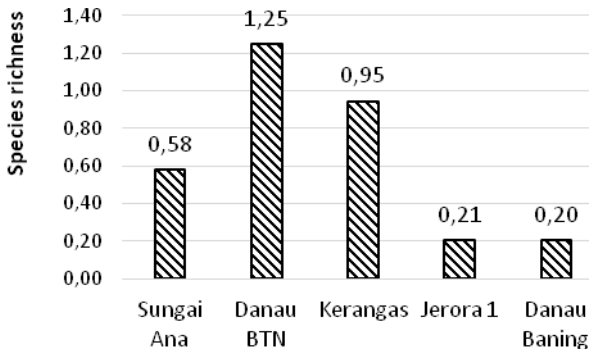

Figure 4. Species richness of *Nepenthes* in the post-mining area

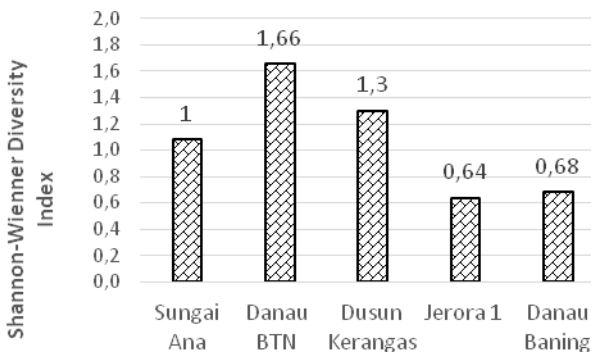

Figure 5. Shannon Wienner diversity index in the post-mining area

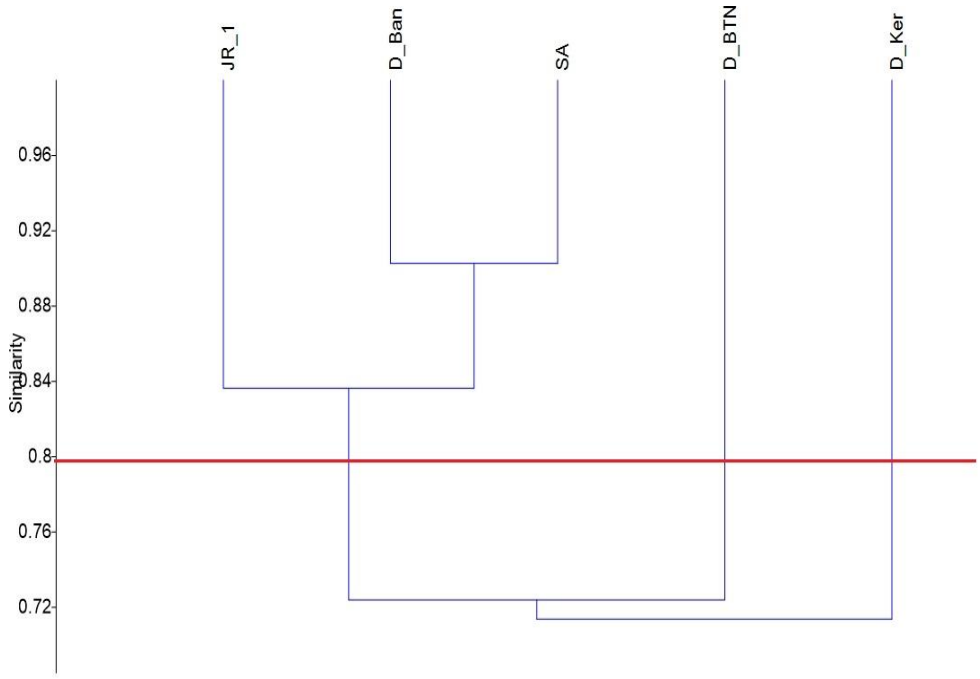

Figure 6. Cluster analysis of Species richness, diversity index, and abiotic factors. Note : JR_1= Jerora 1; D_Ban = Danau Baning; SA = Sungai Ana; D_BTN = Danau BTN; D_Ker = Dusun Kerangas

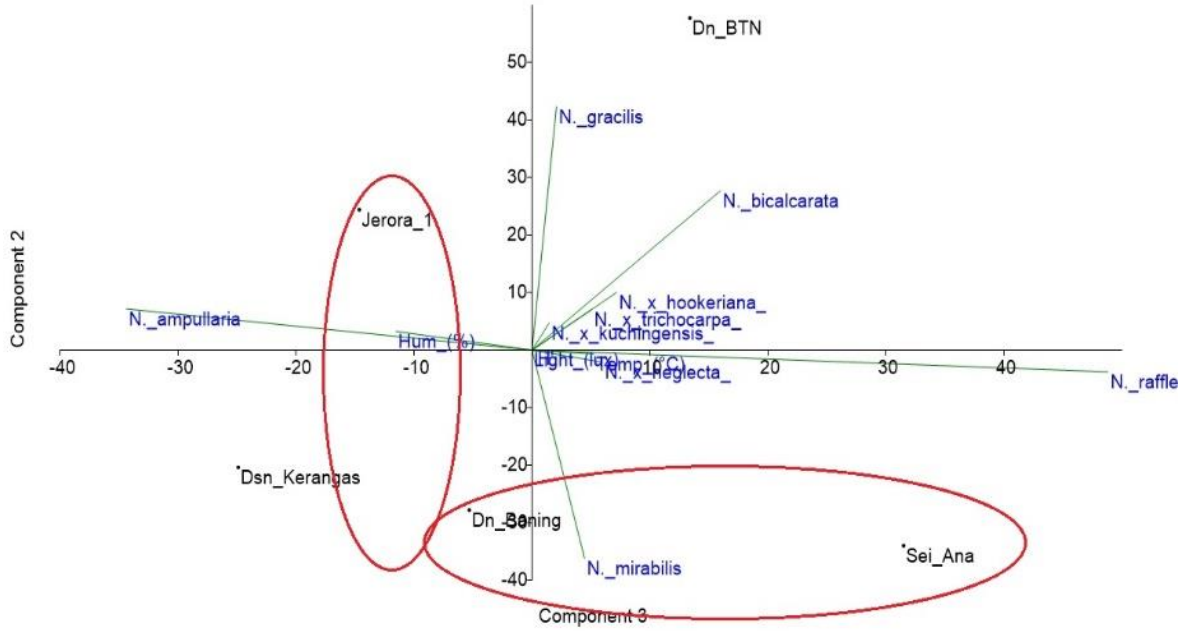

Figure 7. Biplot analysis of Species richness, diversity index, and abiotic factors

In conclusion, *Nepenthes* found in the post-mining area at Sintang Regency consisted of five *Nepenthes* species and four natural hybrids namely *N. ampullaria* Jack., *N. bicalcarata* hook.f., *N. gracilis* Korth., *N. mirabilis* Lour (Druce), *N. rafflesiana* Jack., *N. xhookeriana*, *N. xkuchingensis*, *N. xneglecta*, and *N. xtrichocarpa*. The condition of the post-mining area promoted the *Nepenthes* growth both in open canopy and closed canopy areas. There were 910 individuals of *Nepenthes* found with *N. gracilis* and *N. mirabilis* the top two in individual number (401 and 245 number of individuals, respectively). Species richness showed the number of species found in the

specific location. The highest species richness was found in Danau BTN (1.25), followed by that in Dusun Kerangas (0.95), Sungai Ana Village (0.58), Jerora 1 (0.21) and Danau BTN (0.20). The diversity index in Danau BTN, Dusun Kerangas, Sungai Ana, Jerora 1 and Danau Baning were on a low level, respectively, 1.66, 1.3, 1.0, 0.64, and 0.68. Based on cluster analysis of species richness, H' and abiotic factors, Danau Baning-Sungai Ana-Jerora 1 was grouped in the same cluster while Danau BTN and Dusun Kerangas were in a separate cluster. This means that Danau Baning-Sungai Ana-Jerora 1 had proximity habitat characterized by highly dominant *N. mirabilis* in this areas. Whereas, Danau BTN was characterized by dominant *Nepenthes* hybrid and Dusun Kerangas was characterized by existence of *N. ampullaria.*

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Isolation, molecular characterization and extracellular enzymatic activity of culturable halophilic bacteria from hypersaline natural habitats

SUZAN AHMED BIN-SALMAN[1], REDA HASSAN AMASHA[1,♥], SAMYAH D. JASTANIAH[1], MAGEDA M. ALY[1], KHALIL ALTAIF[2]

[1]Department of Biology, Faculty of Science, King Abdulaziz University. Al Ehtifalat St, Jeddah 21589, Saudi Arabia. ♥email: ramashah@kau.edu.sa
[2]Faculty of Pharmacy, Middle East University. Queen Alia Airport Street, Amman, Jordan

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Abstract. *Bin-Salman SA, Amasha RH, Jastaniah SD, Aly MM, Altaif K. 2018. Isolation, molecular characterization and extracellular enzymatic activity of culturable halophilic bacteria from hypersaline natural habitats. Biodiversitas 19: 1828-1834.* Saline habitats, like the Dead Sea, are unusual extreme environments, due to their extreme salinity. Most saline habitats originate from the evaporation of seawater, and have a nearly neutral to slightly alkaline pH (such as the Red Sea (pH8.3) and Arabian Gulf, pH8.3). Ten halophilic bacterial strains (two Gram-negative) belonging to the family of Halomonadaceae and (eight Gram-positive), belonging to the family of Bacillaceae, were isolated from the Red Sea, Arabian Gulf, and Dead Sea by subjecting the isolates to a high salinity medium, followed by identification using 16S rRNA gene sequencing. Four of isolates were designated on the basis of their tolerance to high salinity; SBR_1 exhibited 97% homology to *Halomonas aquamarina*, SBR_2 showed 97% homology to *Sediminibacillus* sp., (Red Sea), SBA_9 exhibited 94% homology to *Halobacillus* sp., (Arabian Gulf) and SBD_{17} gave 98% homology to *Halobacillus dabanensis* (Dead Sea). The isolates were also characterized by their physiological parameters, SBR_1 showed optimum growth at 30°C, pH8.5 and1.5M NaCl, SBR_2 at 30°C, pH6.0 and 1M NaCl. Optimum conditions for SBA_9 were 35°C, pH6.5 and 1M NaCl and for SBD_{17}, 37°C, pH7.0 and 1M NaCl.

Keywords: 16S rRNA gene sequence, Arabian Gulf, Dead Sea, extremophiles, halophiles, Red Sea

INTRODUCTION

Microorganisms living in extreme environments are referred to as extremophiles. So-called psychrophiles and thermophiles grow best at low and high temperature, respectively, alkaliphiles and acidophiles are adapted to alkaline and acidic conditions, barophiles grow best at high pressure, radioresistant organisms can live in high radiation environments while halophiles are salt-tolerant organisms (Rampelotto 2013). Extremophiles possess a number of strategies which allow them to live in such harsh environments, such as their ability to produce hydrolytic extremozymes which become increasingly attractive for modern biotechnology, industry, and medicine (Karray et al. 2018). For example, polymer-degrading enzymes and DNA polymerases are produced by thermophiles; these are stable and active at high temperatures. Proteases and lipases, found in psychrophiles are active at lower temperatures, while enzymes produced by acidophiles and alkaliphiles can be useful in the production of detergents (Babu et al. 2015). Halophiles or halophilic microorganisms grow in hyper-saline concentrations and include representatives of the eukarya, bacteria, and archaea (Mohammadipanah et al. 2015). The pink-red color of hypersaline environments worldwide, is due to halophilic microorganisms, and the most generally observed halophiles are either belong to the archaea or to some bacterial genera, such as *Haloquadratum*, *Halobacterium*, *Halomonas*, and *Salinibacter*, as well as the green alga, *Dunaliella salina* (Ma et al. 2010; Oren 2011; Waditee-Sirisattha et al. 2016). Halophiles can be divided into three main groups, due to their salt requirements; extreme halophiles prefer to grow at 5 M of NaCl, moderate halophiles at 3 M of NaCl and slight halophiles at 1 M of NaCl (Ventosa et al. 2015).

The difficulty of studying microorganisms in natural environments via culturing and other traditional methods have hindered our full realization of microbial diversity (Alnaimat et al. 2017). One milliliter of seawater may contain 10^6 of microorganisms, which have not yet been identified, thereby making necessary the use of modern molecular methods for determining the vast variety and structure of microbial populations. These techniques and methods can be used on both culturable and non-culturable microorganisms isolated from a variety of environments including seawater and soil (Fakruddin and Mannan 2013). A number of molecular techniques have been devised for characterizing and identifying the phylogenetic and functional diversity of microorganisms, the most commonly used being the analysis of 16S rRNA genes for prokaryotes, which are selectively amplified by the Polymerase Chain Reaction (PCR) from the whole genomic DNA extracted from environmental sample, with or without, the need to culture microorganisms (Rastogi and Sani 2011; Li and Zhao 2013). The aim of this study was to isolate bacteria from the Red Sea, the Arabian Gulf

(also known as the Persian Gulf) in Saudi Arabia and the Dead Sea in Jordan, and then identify any halophilic bacteria isolated, using 16S rRNA gene sequencing. In addition, the study involved physiological characterization of halophilic bacterial isolates.

MATERIALS AND METHODS

Site description and sample collection

Samples were collected in May 2016 and September 2016. Three samples of water and three samples of sediment were aseptically collected from six different sites at the southern part of Red Sea (Site1, N: 21°29'14.8", E: 39°07'58.0"; Site2, N: 21°29'05.8", E: 39°08'004"; Site3, N: 21°28'50.2", E: 039°07'52.2"; Site4, N: 22.144268, E: 38.974901; Site5, N: 22.174521,E: 38.965919; Site6, N: 21° 29'23.5", E: 039°07'58.0"), at various depths (17m, 21m, 12m, 14m and 11m) with maximum distance estimated at nearly (1.8km), located in Jeddah city, Saudi Arabia. One sample of water and the other of sediment were collected from coast of Arabian Gulf, located in Khobar city (N: 28°24'01.2", E: 49°18'28.6"), Saudi Arabia. Three samples of water, three samples of sediment and three samples of saline mud were also collected from two different sites at the northern part of Dead Sea (N: 31°42'27.0", E: 35°34'52.7"), at two depths of (1.5m-3.0m), located in Balqa province, Jordan Recorded temperatures at the sampling sites varied between 34°C, 38°C and 30°C, respectively.

Isolation, purification, and preservation of halophilic bacteria

For isolation of halophilic bacteria,1.0g of both of sediment and mud were suspended in 9ml sterile dH_2O, followed by 9ml of sterile distilled water was poured aseptically into test tubes. For the preparation of the serial dilution series (up to 10^4), Culture media (saline nutrient, Zobell marine, casein, and seawater) were inoculated with 0.1ml of the diluted solutions of each sample and was spread on the surface of the medium (Nieto et al. 1989; Lee et al. 2003; Satbhai et al. 2015). All plates were incubated at 37°C and growth was monitored during 24h, 48h, and 72h. The different types of colonies were picked off and transferred to fresh medium in order to obtain pure cultures.

The cultures were finally purified on the same media from which they were isolated and all isolates were preserved at 4ºC. Simultaneously, the isolates were grown in broth, and 1ml of cultures were transferred with 1ml of 50% glycerol for long preservation at (-20ºC).

Phenotypic characterization of the selected halophilic bacteria

Selected halophilic bacteria were morphologically characterized using standard techniques (Gram stain, colony shape, size, and color on S.N agar plates, motility, and endospore-forming, etc.) according to Bergey's Manual of Determinative Bacteriology (Holt et al. 1994).

Physiological and biochemical characteristics

On saline nutrient agar plates, four genera of halophilic bacteria SBR_1, SBR_2, SBA_9, and SBD_{17} were purified. Biochemical tests of them were performed using API 20E® kit, and other tests for identification of each bacterial isolates. These tests include indole production, Voges Proskauer, methyl red, oxidase and catalase test, and their capacity for fermentation mannitol salt. Physiological tests were also performed, such as growth-temperature range, optimal pH, NaCl tolerance and growth of organic source. These tests were carried out as recommended by (Ventosa et al. 1982; Delgado-García et al. 2013).

Hydrolytic activities of the halophilic bacterial isolates

In order to detect the enzymatic production for halophilic bacteria, isolates were tested on agar plates. Amylase production was performed on starch media according to (Amoozegar et al. 2003), protease was applied to skim milk media according to (Amoozegar et al. 2008), lipase was carried out on Luria-Bertani media with Tween-80 according to (Martin et al. 2003), nuclease was determined by DNase media according to (Onishi et al. 1983), L-asparaginase was on modified M9 medium according to (Shirazian et al. 2016), phosphatase was on National Botanical Research Institute's-phosphate media according to (Nautiyal 1999), for chitinase was used to colloidal chitin according to (Shaikh and Deshpande 1993).

Antibiotic resistance profile

Isolates were exposed to antibiotic resistance screening by the disc diffusion method on Mueller-Hinton agar medium, supplemented with 10% NaCl for halophilic bacteria. The following antibiotics (Mast Group Ltd, Merseyside, U.K.) were used: Cephalothin (30 µg), Cotrimoxazole (25 µg), Imipenem (10 µg), Erythromycin (15 µg), Teicoplanin (30 µg), Ciprofloxacin (5 µg), Vancomycin (30 µg), Ampicillin (10 µg), Augmentin (30 µg), Cefoxitin (30 µg), Gentamicin (10 µg), Amikacin (30 µg), Cefepime (30 µg), Piperacillin (100 µg), Ticarcillin (75 µg), PenicillinG (10 units), Clindamycin (2 µg), Ceftazidime (30 µg), Aztreonam (30 µg) and Tobramycin (10 µg) (Andrews 2008).

Molecular and phylogenetic analysis of the isolated strains

DNA extraction of isolated samples was performed using (Key prep-Bacterial DNA Extraction Kit) (QIAamp® DNA Blood Mini Kit 50). The extracted DNA was used as a template for PCR to amplify 16S rRNA. The extraction method was performed according to the instructions of the manufacturers. 16S rRNA gene was amplified with the bacterial forward primer 27 F (5`-ATG GAG AGT TCG ATC CTG GC-3`) and reverse primer 1303 R (5`-TCC CTC ATT ACG AGC TTG TAC ACA-3`) (MACROGEN). Amplification of 16S rRNA was performed in a total volume of 25µl containing 2.0µL Genomic DNA, 12.5µL of 2x Green MasterMix (Go Taq®),1.0µL Forward Primer, 1.0µL Reverse Primer, and 8.5µl of sterile distilled water. The PCR reaction mixtures, after incubation at 95°C for 5 minutes as an initial

denaturation, were cycled 33 times through the following temperature profile: denaturation for 30sec at 95°C; annealing for 1 minute at 61°C; and elongation for 1 minutes at 70°C with final incubation for 7 minutes at 70°C, after which 10µL of PCR amplification analyzed by 1% agarose gel electrophoresis. In addition, 3µl of 100bp ladder loading was used to confirm the correct sized product. PureLink® Quick Gel Extraction Kit (Invitrogen™) was used to purify PCR products according to the manufacturer's protocol. The purified PCR products were sequenced using the commercial service of MACROGEN, Korea. The resulting 16S rRNA gene sequences were compared with those in GenBank using the blast program (NCBI) and phylogenetic trees were reconstructed.

Statistical analysis

Statistical analyses were performed using the statistical package of a scientific data (SigmaPlot software, version 14.0). The triplicate data were represented as means and error bars showing standard errors of the means. Graphs were constructed and statistical analysis performed with GraphPad Prism (version 6.01f or Windows; GraphPad Software, La Jolla, CA, USA).

RESULTS AND DISCUSSION

Fifty-eight halophilic bacterial strains were isolated under aerobic conditions of seventeen samples taken from diverse high-saline environments varying between water, sediment and mud, i.e. from The Red Sea and Arabian Gulf-Kingdom of Saudi Arabia, which are saline and alkaline environments (pH 8.39-pH 8.35) and the Dead Sea in Jordan, which is a hyper-saline and acidic environment (pH 6.03) making them harsh environments even for microorganisms. During early summer May, twenty-four bacterial isolates were obtained from the Red Sea samples. In the same time, nine bacterial isolates were obtained from the Arabian Gulf samples. At the end of the summer September, twenty-five bacterial isolates were obtained from Dead Sea samples. For the specific isolation of halophilic bacteria, a range of media containing a range of NaCl was used. Saline nutrient medium (1M NaCl) yielded ten strains of halophilic bacteria. In this study, it was decided to fully characterize only four of the ten strains, since these were most suited to grow at high concentrations of NaCl.

Phenotypic characterization of halophilic bacteria

Morphological characters of the isolates SBR_1, SBR_2, SBA_9, and SBD_{17} were described after growth on saline nutrient agar medium within 24h and represented in Table 1. The colonies of SBR_1were purple-colored, opaque, surface of smooth, raised with a regular-circular edge, the colonies of SBR_2 had pale pink colored, opaque, surface of smooth, flat, with a regular-circular edge, the colonies of SBA_9 had yellowish-orange colored, opaque, surface of smooth, raised with a regular-circular edge, and the colonies of SBD_{17} had yellowish-orange colored, opaque, surface of smooth, flat, with a regular-circular edge. Examination of the isolates under light microscope showed that the cells of SBR_1 from overnight culture were Gram-negative, motile, none spore-forming, they have capsule and monobacilli, while cells of SBR_2 were Gram-positive, motile, none spore-forming, they have capsule and diplobacilli or monobacilli, SBA_9 cells were Gram-positive, motile, spore-forming, they have capsule and diplobacillior monobacilli, whereas cells of SBD_{17} were Gram-positive, motile, spore-forming, they have capsule and diplobacillior monobacilli.

A range of biochemical and physiological tests was carried out to identify the genus of isolates SBR_1, SBR_2, SBA_9 and SBD_{17}, the results are shown in Table 1.

Table 1. Summary of the morphological and the biochemical characteristics of SBR_1, SBR_2, SBA_9, and SBD_{17}

Morphological and biochemical characteristics	SBR_1	SBR_2	SBA_9	SBD_{17}
Source of isolate	Sediment (Red Sea)	Sediment (Red Sea)	Water (Arabian Gulf)	Mud (Dead Sea)
Form	Regular-Circular	Regular-Circular	Regular-Circular	Regular-Circular
Margin	Entire	Entire	Entire	Entire
Elevation	Raised	Flat	Raised	Flat
Surface	Smooth	Smooth	Smooth	Smooth
Color	Purple	Creamy	Yellowish orange	Yellowish orange
Opacity	Opaque	Opaque	Opaque	Opaque
Gram's reaction	Negative	Positive	Positive	Positive
Cell shape	Bacilli	Bacilli	Coccobacilli	Bacilli
Spore-forming	Negative	Negative	Positive	Positive
Capsule	Positive	Positive	Positive	Positive
Motility	Motile	Motile	Motile	Motile
Pigment production	Negative	Negative	Negative	Negative
Catalase	Positive	Positive	Positive	Negative
Oxidase	Negative	Negative	Negative	Negative
Indole production (IND)	Positive	Positive	Positive	Positive
Citrate(CIT)	Negative	Positive	Positive	Positive
Methyl red (MR)	Negative	Positive	Positive	Positive
Voges-Proskauer (VP)	Negative	Negative	Negative	Negative
Fermentation of Mannitol salt	Negative	Positive	Positive	Positive

Negative patterns of SBR_1 results were obtained in the oxidase, Voges-Proskauer, citrate, and methyl red tests, while it was positive for catalase and indole production. Negative results of SBR_2 and SBA_9 were obtained in the oxidase and Voges-Proskauer tests but they were positive for catalase, indole production, citrate, and methyl red. Negative responses of SBD_{17} were for catalase, oxidase, and Voges-Proskauer tests while positives were for indole production, citrate and methyl red.

As illustrated in Figure 1, the physiological characteristics for SBR_1, SBR_2, SBA_9 and SBD_{17} were showed that they grow under anaerobic conditions, the growth temperatures were SBR_1 = (4-40°C), SBR_2 = (25-45°C), SBA_9 = (25-40°C) and SBD_{17} = (35-45°C) The other clear difference between the strains was their ability to grow at different pH levels, SBR_1= (pH 7.0-9.0), SBR_2= (pH 5.5-7.5), SBA_9= (pH 6.0-8.0) and SBD_{17}= (pH 6.5-8.0) as clearly shown in Figure 2. Whereas the different NaCl concentration showed that SBR_1, SBR_2, SBA_9, and SBD_{17} are moderate halophiles (i.e., they have an absolute requirement for NaCl in the growth medium) (Figure 3), no significant effects were observed when the isolated strains were grown in the presence of 1M of NaCl concentration. The organic source gave the highest growth of strains with yeast extract compared to peptone or casein.

Enzymatic production of halophilic bacteria

The halophilic bacterial isolates SBR_1, SBR_2, SBA_9, and SBD_{17} were investigated for their ability to hydrolyze extracellular enzyme substrates on solid medium supplemented with 1M NaCl (Table 2). The SBR_1 strain produced lipase, L-asparaginase, and chitinase enzymes, while the SBR_2 strain produced proteinase and nuclease. While the two strains SBA_9 and SBD_{17} produced amylase, proteinase, lipase, and nuclease.

Determination of the resistance to antibiotics

Halophilic isolate tested for antibiotic susceptibility was shown to be resistant (Table 3).

Molecular and phylogenetic analysis of the isolated strain

PCR amplification of the 16S rRNA gene produced fragments of approximately 1500 base pairs in size (Table 4). The resulting 16S rRNA gene sequences from halophilic isolates were compared and the closest match was detected using BLAST program (NCBI). The highest sequence similarities for the halophilic isolates were as follows: Strain SBR_1 showed 97% similarity with *Halomonas aquamarina*, accession number EU684464.1; Strain SBR_2 showed 97% similarity with *Sediminibacillus* sp., accession number KM199865.1; Strain SBA_9 showed 94% similarity with *Halobacillus* sp, accession number FJ477402.1 while strain SBD_{17} showed 98% similarity with *Halobacillus dabanensis*, accession number KT008293.1. As clearly shown in Figure 4, the neighbor-joining phylogenetic tree based on 16S rRNA gene sequences confirms that halophilic isolates fell into three genera; *Halomonas*, *Sediminibacillus*, and *Halobacillus*.

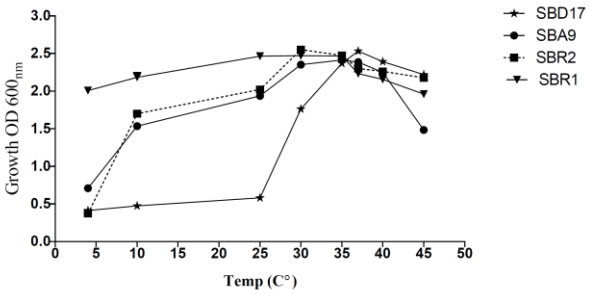

Figure 1. Effect of different temperatures on the growth of the isolated strains

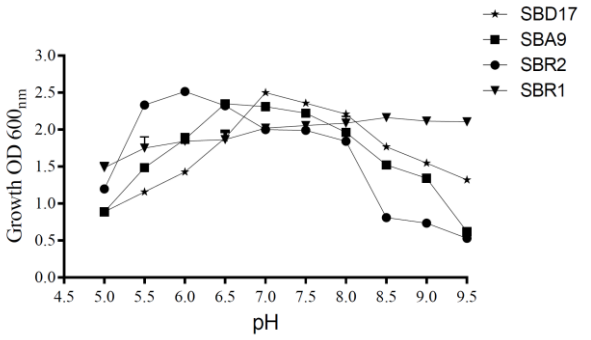

Figure 2. Effect of different pH values on the growth of the isolated strains

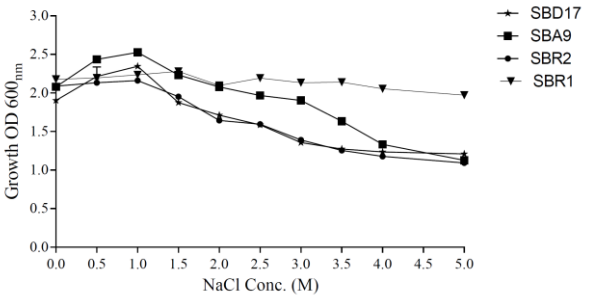

Figure 3. Effect of different NaCl Concentrations on the growth of the isolated strains

Table 2. Hydrolytic activities of the halophilic bacterial isolates

Enzyme	SBR1	SBR2	SBA9	SBD17
Hydrolysis of starch	-	-	+	+
Hydrolysis of skim milk	-	+	+	+
Hydrolysis of fat	+	-	+	+
Hydrolysis of DNA	-	+	+	+
Hydrolysis of L-asparagine	+	-	-	-
Hydrolysis of phosphate	-	-	-	-
Hydrolysis of chitin	+	-	-	-

Table 3. Antibiotics resistance pattern of the isolated strains

Isolated strains	Resistance pattern*
SBR_1	AP^{10}, AUG30, FOX30, GM^{10}, KF^{30}, TS^{25}, AK^{30}, CAZ^{30}, ATM^{30}, CIP^{5}, VA^{30}, PRL^{100}, IMI^{10}.
SBR_2	AP^{10}, AUG^{30}, FOX^{30}, GM^{10}, KF^{30}, TS^{25}, AK^{30}, CPM^{30}, TC^{75}, PG^{10}, E^{15}, CD^{2}, TEI^{30}, CAZ^{30}, ATM^{30}, CIP^{5}, TN^{10}, VA^{30}, PRL^{100}, IMI^{10}.
SBA_9	AP^{10}, AUG^{30}, FOX^{30}, GM^{10}, KF^{30}, TS^{25}, AK^{30}, CPM^{30}, TC^{75}, PG^{10}, E^{15}, CD^{2}, TEI^{30}, , CAZ^{30}, ATM^{30}, CIP^{5}, TN^{10}, VA^{30}, PRL^{100}, IMI^{10}.
SBD_{17}	AP^{10}, AUG^{30}, FOX^{30}, GM^{10}, KF^{30}, TS^{25}, AK^{30}, CPM^{30}, TC^{75}, PG^{10}, E^{15}, CD^{2}, TEI^{30}, CAZ^{30}, ATM^{30}, CIP^{5}, TN^{10}, VA^{30}, PRL^{100}, IMI^{10}

Note: * 10 µg Ampicillin (AP), 30 µg Augmentin (AUG), 30 µg Cefoxitin (FOX), 10 µg Gentamicin (GM), 30 µg Cephalothin (KF), 25 µg Cotrimoxazole (TS), 30 µg Amikacin (AK), 30 µg Cefepime (CPM), 75 µg Ticarcillin (TC), 10units PenicillinG (PG), 15 µg Erythromycin (E), 2 µg Clindamycin (CD), 30 µg Teicoplanin (TEI), 30 µg Ceftazidime (CAZ), 30 µg Aztreonam (ATM), 5 µg Ciprofloxacin (CIP), 10 µg Tobramycin (TN), 30 µg Vancomycin (VA), 100 µg Piperacillin (PRL), 10 µg Imipenem (IMI).

Table 4. Comparative analysis of 16S rRNA gene sequences of halophilic isolates from the Red Sea sediment, the Arabian Gulf water, the Dead Sea mud, using highly matched species available in BLASTN

Isolated strain	Sequence length	Highly matched bacteria accession no.	GenBank accession no.	Similarity (%)
SBR_1	1470	*Halomonas aquamarina*	EU684464.1	97%
SBR_2	1475	*Sediminibacillus* sp.	KM199865.1	97%
SBA_9	800	*Halobacillus* sp.	FJ477402.1	94%
SBD_{17}	1519	*Halobacillus dabanensis*	KT008293.1	98%

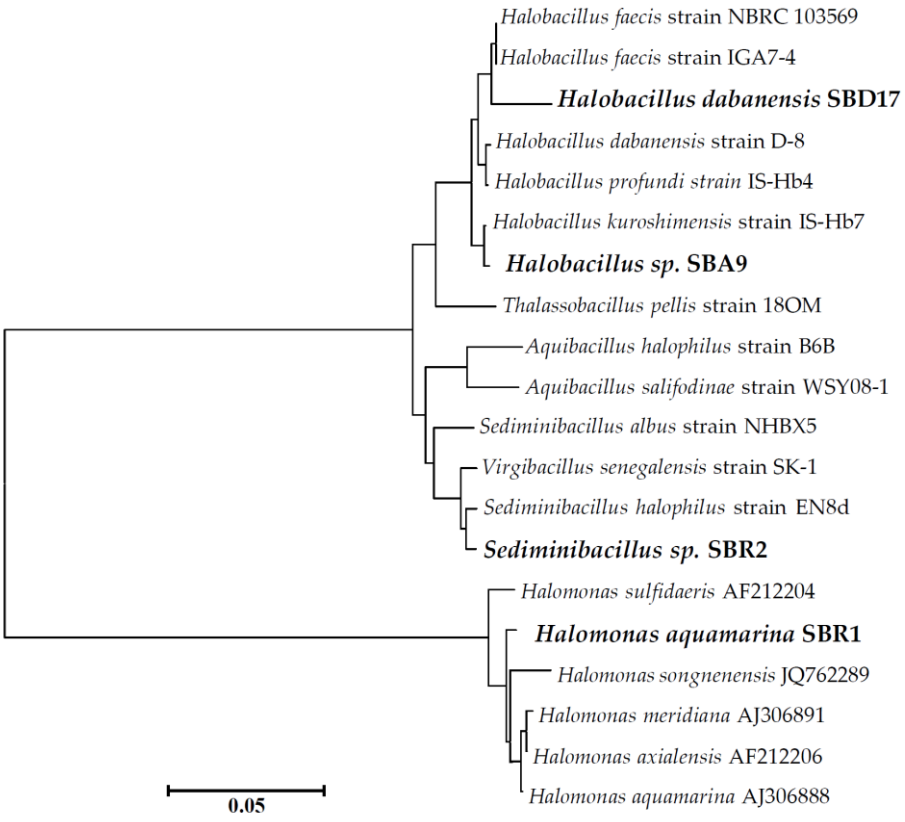

Figure 4. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences, showing the interrelationships of the isolated bacterial strains. The GenBank accession number of each strain is shown in parenthesis. The tree was generated using the neighbor-joining (NJ) method (Saitou and Nei 1987) contained in the MEGA6 software package (Tamura et al. 2013). Bootstrap values based on 500 replications are listed at nodes. Scale bar represents 0.05 substitutions per nucleotide position

Discussion

Traditional methods of bacterial cultivation have been widely used to investigate microbial diversity in a range of environments, including extreme habitats that might include potentially new bacterial species. It is well known that the microbial community represents a wide gene pool that can be explored for several beneficial applications, such as the production of biomolecules and bioremediation (Poli et al. 2017). Generally, halophilic microorganisms are organisms that grow optimally at high salt concentrations. For optimal growth, extreme halophiles require 2.5-5.2 M (15-30%) NaCl (Siglioccolo et al. 2011). This work aimed to investigate the microbial diversity discovered in the Red Sea region, the Arabian Gulf, Saudi Arabia and in Dead Sea region, Jordan. In the current study, focus was placed on identification and characterization of halophilic bacteria that they were isolated from sediment, mud and water samples. After initial characterization of the isolates showed them to be halophilic bacteria. Based on the reported results, the morphological and biochemical characteristics of halophilic bacterial isolates were displayed in Table 1. And the halophilic bacterial isolates at the different concentrations of NaCl was evaluated by growing in (SNB) at 37°C for determination of the growth range of salinities. Figure 3 showed the salinities range of isolates was at the moderate level (0.5-3.0 M NaCl). Beside the high salt tolerance capacity, that up to 3M, the growth capacity of halophilic bacterial isolates at different temperature, SBR_1(4-40°C), SBR_2 (25-45°C), SBA_9 (25-40°C) and SBD_{17} (30-45°C) and at pH values, SBR_1 (7.0-9.0), SBR_2 (5.5-7.5), SBA_9 (6.0-8.0) SBD_{17} (6.5-8.0) were demonstrated in the results (Figures 1 and 2), suggesting that the isolates obtained might be tolerant for growth not only with salt but with a wide range of temperature and pH values. the ability of halophilic isolates for producing some enzymes like amylase, protease, lipase, DNase, L-asparaginase and chitinase is shown in Table 2.

In addition, the results reveal that halophilic bacterial isolates tested for antibiotic susceptibility had multiple antibiotic resistances (Table 3). In order to identify the strains, molecular methods were used. In the current study, four moderately halophilic bacterial isolates were identified using 16S rRNA analysis, which is acknowledged as the method of choice for identifying novel isolates to the genus and particularly species level. The results were compared with those described in a range of identification schemes and the literature in general (Hotl et al. 1994; Amoozegar et al. 2003; Liu et al. 2005; Tamegai et al. 2006; Wang et al. 2009; Guzmán et al. 2010). The halotolerant bacteria, *Halobacillus salinus* isolated from a salt lake in Korea can, for example, grow at 23% NaCl (Echigo et al. 2005). In the last decade, a few studies have been demonstrated the ability of halophiles to grow in non-saline habitats. Usami et al. (2007) for example, have isolated two strains of halophilic bacteria from non-saline soil in Japan, designated $BM2^T$ and HN2. The cells of strain $BM2^T$ were found to be Gram-positive, rod-shaped, aerobic, and motile. Growth occurred at 5-25% (w/v) NaCl, with optimal growth occurring at 10-15% (w/v) NaCl at 20-50 °C and pH of 7-10. 16S rRNA gene sequencing, used to analyze the phylogeny of strain $BM2^T$showed 98% sequence similarity to *Alkalibacillus haloalkaliphilus* $DSM5271^T$ (Al'Abri 2011).

In an earlier study, conducted by Savage et al. (2008), the occurrence of novel halophilic archaeon, has been reported in the Zodletone Spring in south-western Oklahoma, USA, this being characterized by its low-salt, as well as rich-sulfide content. A novel halophilic archaeon of strain $BZ256^T$ was isolated, and the cells were found to occur as non-flagellated, non-motile, cocci, and forming *Sarcina*-like clusters. They grew at a 1.3-4.3 M salt concentration and with optimum growth at almost 3.5M NaCl and required at least 1 mM Mg^{+2}, a pH range of 5.0-8.5 and a temperature range of 25-45°C. The 16S rRNA gene sequence of strain $BZ256^T$ revealed that it was related to *Halogeometricum borinquense*. Strain $BZ256^T$ stands for a member of a novel genus and species within the family Halobacteriaceae, proposed as *Halosarcina pallida* gen. nov, sp.nov. (Savage et al. 2008). Phylogenetic analyses are derived from the comparison of the nucleotide sequences unknown with already identified ones, found in GenBank, that are accessible worldwide (Clarridge 2004). Phylogenetic analysis is represented using phylogenetic tree construction (Brinkman and Leipe 2001). In this study, the sequences data were used to produce a phylogenetic tree providing the basis for efficient phylogenetic investigation of each genus. Figure 4 is an example of phylogenetic analysis of *Halomonas aquamarina* strain, *Sediminibacillus* sp. strain, *Halobacillus* sp. strain, and *Halobacillus dabanensis* strain. Phylogenetic analysis of SBR_1, SBR_2, SBA_9, SBD_{17} by using the BLASTN algorithm at NCB1 indicated that the halophilic bacterial isolate SBR_1 belongs to the genus *Halomonas* and is closely similar to *Halomonas aquamarina* (i.e., the halophilic bacterium obtained here from the Red Sea sediment is *Halomonas aquamarina*). SBR_2, from the Red Sea sediment, belongs to the genus *Sediminibacillus* sp, while SBA_9 was shown to be belonging to the genus, hence, the halophilic bacterium obtained from Arabian Gulf water in the present study could be identified as *Halobacillus* sp. Finally, SBD_{17} isolated from Dead Sea mud, was shown to belong to the genus *Halobacillus* and closely similar to *Halobacillus dabanensis*.

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Short Communication: Floristic composition and relationships between plant species abundance and soil properties in common hazel (*Corylus avellana*) mountainous forest of northern Iran

GHADER POURRAHMATI[1,♥], ASADOLLAH MATAJI[1,♥♥], HASSAN POURBABAEI[2], ALI SALEHI[2]

[1]Department of Forest Ecology and Silviculture, Science and Research Branch, Islamic Azad University. Tehran, Iran.
♥email: gh_pourrahmati@yahoo.com, ♥♥ a_mataji2000@yahoo.com
[2]Department of Forestry, Faculty of Natural Resources, University of Guilan. Guilan, Iran.

Manuscript received: 12 July 2018. Revision accepted: 17 September 2018.

Abstract. *Pourrahmati G, Mataji A, Pourbabaei H, Salehi A. 2018. Short Communication: Floristic composition and relationships between plant species abundance and soil properties in common hazel (Corylus avellana) mountainous forest of northern Iran. Biodiversitas 19: 1835-1841.* Mountainous forests are valuable terrestrial ecosystems because of their useful services for the human being. Here, we explored the floristic composition and the relationships between plant species abundance distribution and soil physical and chemical properties in common hazel (*Corylus avellana* L.) in the mountainous forest of northern Iran. Within the forest stand, 30 quadrats (20 m × 20 m and 1 m × 1 m for woody and herbaceous species, respectively) were selectively sampled along an altitudinal range from 1300 m to 1800 m a.s.l. to assess plant species composition and abundance, and soil samples were taken to perform chemical and physical analyses. The results showed that a total of 43 herbaceous and 15 woody species belonging to 23 and 8 families were identified. The abundance of herbaceous species was significantly correlated with soil properties (pH and total N). Furthermore, the abundance of woody species had a non-significant correlation with soil properties.

Keywords: CCA, *Corylus avellana*, distribution, mountainous forests, soil physical and chemical properties, species abundance

INTRODUCTION

Mountainous areas have been introduced as the prominent ecosystems since the Earth Summit in Rio de Janeiro in 1992. The services of mountainous forests, including provisioning, regulating, and cultural, are very vital for the human being (Price et al. 2011; Smith et al. 2015). The natural geographical distribution of the common hazel covers a vast area in the world, including Albania, Armenia, Austria, Azerbaijan, Belarus, Belgium, Bosnia and Herzegovina, Bulgaria, Croatia, Czech Republic, Denmark, Estonia, Finland, France (Corsica, France (mainland)), Georgia, Germany, Greece (Greece (mainland)), Hungary, Iran, Italy (Italy (mainland), Sardegna, Sicilia), Latvia, Liechtenstein, Lithuania, Luxembourg, Macedonia, Moldova, Montenegro, Netherlands, Norway, Poland, Portugal, Romania, Russian Federation (Central European Russia, Chechnya, Dagestan, East European Russia, European Russia, Ingushetiya, Kabardino-Balkariya, Kaliningrad, Karachaevo-Cherkessiya, Krasnodar, North European Russia, Northwest European Russia, Severo-Osetiya, South European Russia, Stavropol), Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey (Turkey-in-Asia), Ukraine (Krym, Ukraine (main part)), United Kingdom (Great Britain, Northern Ireland) (Thompson et al. 1996). Naturally, common hazel grows in habitats with sandy soil and little nutrient elements in some mountainous areas in the north of Iran (Pourbabaei and Adel 2015).

The plant species are known as a primary component of ecosystem functioning, which are affected by the environmental factors (Montoya and Raffaelli 2010). According to the tolerance theory, each plant species is able to survive and reproduce successfully only within a definite range of environmental conditions (Good 1931). There is an interrelationship between plants and soil (Kirkpatrick et al. 2014; Srinivasan et al. 2015), so that, the vegetation growth correlates with some soil properties and vegetation also affects the soil properties (Johnson et al. 2014; Lee et al. 2014). Soil controls the hydrological, erosional, biological, and geochemical cycles in an ecosystem (Keesstra et al. 2012; Brevik et al. 2015; Smith et al. 2015) thereby affecting the plant species richness and composition (Rankin et al. 2007; Silva et al. 2013; Keymer and Lankau 2017). In mountainous areas, soil properties (i.e., physical and chemical features) along with topography can play an important role to develop plant communities (Miyamoto et al. 2003; Ravanbakhsh and Moshki 2016). According to the previous studies, the soil properties have been introduced as effective factors on variations in species abundance (Marcuzzo et al. 2013; Qian et al. 2014; Toure et al. 2015; An et al. 2015; Noumi 2015; Ghaderi et al. 2016). Vegetation is one of the most important factors affecting the stability of ecosystems (Ghaderi et al. 2016). Therefore, understanding the factors that govern patterns of plant species distribution and

abundance is a mandatory requirement to control the establishment and distribution of plant communities in the mountainous areas of Iran.

In the mountainous ecosystems of northern Iran, the main soil challenges which impose particular constraints to plant establishment include the rocky substrates, soil scarcity, low water content, and low levels of nutrients. Hazel forests are unique and rare ecosystems in northern Iran and highly valuable because of their major contribution to the improvement of livelihoods and welfare of rural communities, protective function against natural hazards and conservation of many endemic and rare plant species. The most studies related to plant and soil relationships focused on tropical rainforests (Peña-Claros et al. 2011); there is less data base about temperate mountainous forests. As far as we know, there is no studies have paid attention to floristic composition and plant and soil relationships in the common hazel forest of Talesh in northern Iran. This study aimed to identify the floristic composition and investigate the relationships between plant species abundance and soil properties to address the following main question: Is there any relationships between the herbaceous and woody species abundance and soil physical and chemical properties?

MATERIALS AND METHODS

Study area

We conducted our study in the common hazel forest which is located on the northern slopes of the Alborz Mountains overlooking the Caspian Sea. The forest stand is located on the mountainous regions of Talesh (latitude: 37˚53′16″ N, longitude: 48˚39′28″ E) along an altitudinal gradient from 1300 m to 1800 m asl. (Figure 1). In this region, mean annual precipitation, temperature, and relative humidity were about 1300 mm, 13.19˚C, and 55%, respectively, and also soil pH was moderately acidic to neutral, soil texture was sandy loam, soil colour was dark brown (7.5YR4/2), and maximum soil depth was 60 cm.

Figure 1. The geographical location of study area in the mountainous areas of Talesh, Guilan, northern Iran

Data collection

Sampling of understory vegetation

We took a total of 30 sample plots (20 m × 20 m) selectively in the forest stand along an altitudinal range from 1300 m to 1800 m a.s.l. (Pourbabaei et al. 2012; Zhang et al. 2016). The forest stand was divided into five zones based on 100 m interval in the altitudinal gradient. The woody species with diameter at breast height (≥ 10 cm) in the plots were measured and the species was identified. Furthermore, individual shrubs and saplings (< 10 cm) were enumerated within the plots (Ihlen et al. 2001; Poorbabaei and Poorrahmati 2009). In order to survey the herbaceous layer, the sampling plot area (1 m × 1 m) was obtained according to the method of species/area curve (Cencini et al. 2012). The coverage percent and type of each herbaceous species was estimated using Van der Maarel criterion (Acosta et al. 2007).

Soil sampling and analysis

After litter removal from the mineral soil, we collected three soil samples (12.8 cm diameter, 0-20 cm depth) at three locations near the plot center using a soil auger for assessment of the soil chemical and physical properties. Then samples corresponding to each sampling plot were mixed to create a single sample (Vockenhuber et al. 2011). The soil samples were air-dried and sieved to pass a 2 mm sieve to remove the rocks, gravel and debris. The soil samples were analyzed for organic carbon (OC), electrical conductivity (EC), saturation moisture percentage (SP), texture, pH, total N, potassium (K^+), phosphorus (P^+), bulk density (BD), particle density (PD). The soil analyses were conducted in the Soil Testing Laboratory of Natural Recourses Faculty at University of Guilan. The percentage of organic carbon (OC) was determined using the Walkley and Black method (Lo et al. 2011), total N using the Kjeldahl procedure (Flowers and Bremner 1991), phosphorus (P) test was based on extraction with ammonium lactate (AL) at acidic pH (Olsen and Sommers 1982), available K was analyzed using a flame atomic absorption spectrophotometer (Cox et al. 1993), electrical conductivity (EC) was determined using the sodium saturation ratio (Van Reeuwijk 1992), pH was determined in a 1:5 soil water ratio suspensions using a digital pH-meter (Model 691, Metrohm AG Herisau Switzerland) (Thomas 1996), bulk density, particle density, water content, and total porosity were determined by oven-drying method, and the soil texture was determined by the hydrometer method (Bouyoucos 1962).

Data analysis

The CCA method was performed using a PC-Ord software version 5 (McCune et al. 2002) to study the plant abundance and soil relationship (Ter Braak 1986). The significance of axes was tested using the Monte Carlo permutation test (Ter Braak and Šmilauer 2002). The significance of eigenvalues of first canonical axis was tested using the Monte Carlo permutation test. The inter-set correlations from the ordination analysis were evaluated to assess the importance of various soil properties, including organic carbon (OC), electrical conductivity (EC), saturation moisture percentage (SP), texture, pH, total N, potassium (K^+), phosphorus (P^+), bulk density (BD), and particle density (PD) (Gazer 2011).

RESULTS AND DISCUSSION

Species composition

The results indicated that a total of 43 herbaceous and 15 woody species belonging to 23 and 8 families were found in the forest stand. The most species-rich families were the Asteraceae, Fabaceae, and Rosaceae, with 4, 3, and 11 genera and 5, 7, and 11 species, respectively (Tables 1 and 2).

Plant and soil relationship

Herbaceous species abundance and soil relationship

To analyze the herbaceous species abundance distribution and soil relationship, the CCA method was performed. The cumulative percentage variances for the axes of CCA (and their eigenvalues) are: 13.6 (0.26), 23.0 (0.18), and 29.4 (0.12) for axes 1, 2, and 3, respectively (Table 3). The correlation calculated for the first three axes of CCA includes: 0.94, 0.87, and 0.83. The Monte Carlo permutation test showed that the relationship was significant (Table 4; $p = 0.03$). Furthermore the effects of pH and total N on species abundance distribution were significant (Figure 2; $P < 0.05$).

Woody species abundance and soil relationship

The CCA method was performed to analyze the woody species abundance distribution and soil relationship. The cumulative percentage variances for axes of CCA (and their eigenvalues) are: 16.7 (0.05), 25.1 (0.02), and 29.8 (0.01) for axes 1, 2, and 3, respectively (Table 5). The correlation calculated for the first three axes of CCA includes: 0.80, 0.64, and 0.59. The Monte Carlo permutation test showed that the relationship was non-significant (Figure 3; Table 6; $P = 0.49$).

Table 2. The recorded woody species in the study area

Family	Scientific name	Abbrev.	Life form
Aceraceae	*Acer campestre* L.	Acer cam	Ph
Caprifoliaceae	*Viburnum lantana* L.	Vibu lan	Ph
Corylaceae	*Corylus avellana* L.	Cory ave	Ph
Cornaceae	*Cornus mas* L.	Corn mas	Ph
Ebenaceae	*Diospyros lotus* L.	Dios lot	Ph
Fagaceae	*Quercus iberica* M.Bieb.	Quer ibe	Ph
Oleaceae	*Fraxinus excelsior* L.	Frax exc	Ph
Rosaceae	*Cerasus avium* (L.) Moench.	Cera avi	Ph
	Crataegus ambigua A.K.Becker.	Crat amb	Ph
	Malus orientalis Uglitzk.	Malu ori	Ph
	Mespilus germanica L.	Mesp ger	Ph
	Prunus divaricata Ledeb.	Prun div	Ph
	Rosa canina L.	Rosa can	Ph
	Sorbus torminalis (L.) Crantz	Sorb tor	Ph

Table 1. The recorded herbaceous species in the study area

Family	Scientific name	Abbreviations	Life form
Alliaceae	*Allium ursinum* L.	Alli urs	Geo
	Allium paradoxum (M.Bieb.) G.Don.	Alli par	Geo
Apiaceae	*Heracleum persicum* Desf.	Hera per	Hem
Aspleniaceae	*Phyllitis scolopendrium* (L.) Newman.	Phyl sco	Geo
	Asplenium adiantum lanceolatum Hoffm.	Aspl adi	Geo
Asteraceae	*Achillea millefolium* L.	Achi mil	Hem
	Centaurea hyrcanica Bornm.	Cent hyr	Hem
	Hieracium lactucella Wallr.	Hier lac	Hem
	Hieracium umbrosum Jord.	Hier umb	Hem
	Taraxacum officinale F. H. Wigg.	Tara off	Hem
Campanulaceae	*Campanula latifolia* L.	Camp lat	Hem
Caprifoliaceae	*Sambucus ebulus* L.	Samb ebu	Geo
Clusiaceae	*Hypericum androsaemum* L.	Hype and	Ch
Convolvulaceae	*Calystegia sepium* (L.) R.Br.	Calys sep	Geo
Cyperaceae	*Carex stenophylla* Wahlenb..	Care ste	Hem
Dennstaedtiaceae	*Pteridium aquilinum* (L.) Kuhn.	Pter aqu	Geo
Fabaceae	*Lathyrus latifolius* L.	Lath lat	Hem
	Lathyrus laxiflorus Kuntze.	Lath lax	Hem
	Trifolium medium L.	Trif med	Hem
	Trifolium pratense L.	Trif pra	Hem
	Trifolium repens L.	Trif rep	Cr
	Vicia sativa L.	Vici sat	Hem
	Vicia orobus DC.	Vici oro	Hem
Lamiaceae	*Mentha pulegium* L.	Ment pul	Hem
	Nepeta involucrata Bornm.	Nepe inv	Hem
Liliaceae	*Convallaria majalis* L.	Conv maj	Geo
Orchidaceae	*Epipactis atrorubens* (Hoffm.) Besser.	Epip atr	Geo
Poaceae	*Dactylis glomerata* L.	Dact glo	Hem
	Echinochloa crus-galli (L.) P. Beauv.	Echi cru	Th
	Eremopoa persica (Trin.) Roshev.	Erem per	Th
Polygonaceae	*Rumex crispus* L.	Rume cri	Hem
Primulaceae	*Primula veris* L.	Prim ver	Hem
	Primula vulgaris L.	Prim vul	Hem
Ranunculaceae	*Actaea spicata* L.	Acta spi	Hem
	Primula vulgaris Huds.	Prim vul	Hem
Rosaceae	*Agrimonia eupatoria* L.	Agri eup	Hem
	Fragaria vesca L.	Frag ves	Hem
	Geum heterocarpum Boiss.	Geum het	Hem
Rubiaceae	*Galium aparine* L.	Gali apa	Th
	Galium odoratum Scop.	Gali odo	Geo
	Galium rotundifolium L.	Gali rot	Geo
Scrophulariaceae	*Veronica hederifolia* L.	Vero hed	Th
Violaceae	*Viola alba* Besser.	Viol alb	Hem

Table 3. Monte Carlo test results, analyzing eigenvalue significance for herbaceous species

Axis	Eigenvalue	Mean	Minimum	Maximum	*P*
1	0.26	0.19	0.12	0.26	0.006
2	0.18	0.14	0.09	0.22	
3	0.12	0.11	0.07	0.17	

Table 4. Monte Carlo test results, analyzing herbaceous species abundance distribution-soil correlation

Axis	Correlation	Mean	Minimum	Maximum	*P*
1	0.94	0.88	0.72	0.97	0.03
2	0.87	0.84	0.70	0.95	
3	0.83	0.82	0.65	0.94	

Table 5. Monte Carlo test results, analyzing eigenvalue significance for woody species

Axis	Eigenvalue	Mean	Minimum	Maximum	*P*
1	0.05	0.04	0.02	0.07	0.3
2	0.02	0.03	0.01	0.05	
3	0.01	0.01	0.00	0.03	

Table 6. Monte Carlo test results, analyzing woody species abundance distribution-soil correlation

Axis	Correlation	Mean	Minimum	Maximum	*P*
1	0.80	0.80	0.65	0.98	0.49
2	0.64	0.73	0.55	0.93	
3	0.59	0.65	0.46	0.86	

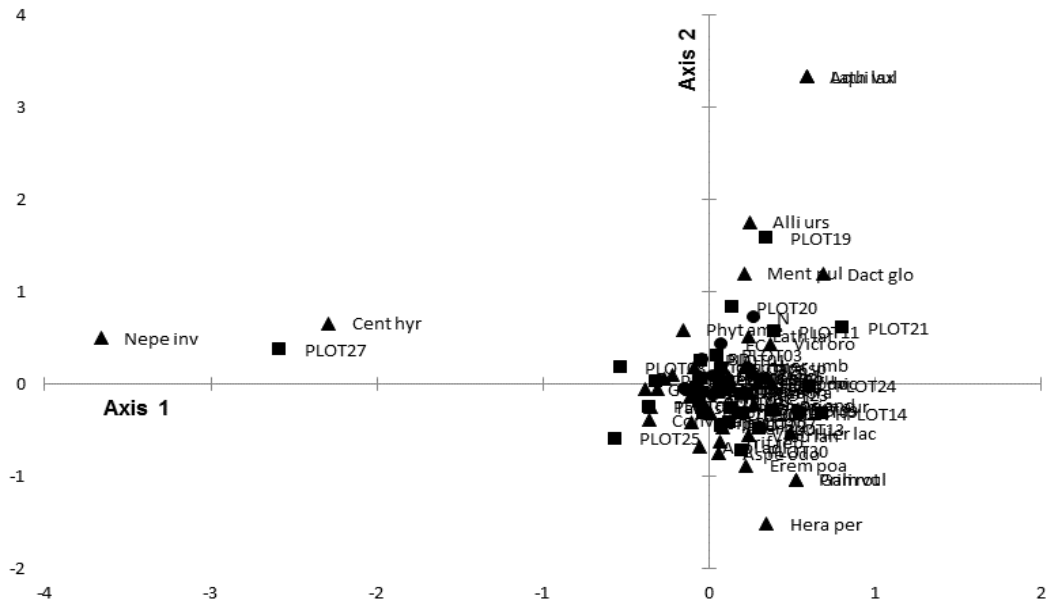

Figure 2. CCA-ordination diagram of herbaceous species abundance distribution related to soil physical and chemical properties. EC (electrical conductivity), OC (organic carbon), pH (acidity), SP (saturation moisture percentage), N (nitrogen), P (phosphorus), K (potassium), PD (particle density), S (sand), Si (silt), Cl (clay), and BD (bulk density). The triangles represent species, circles represent soil factors, and squares represent plots.

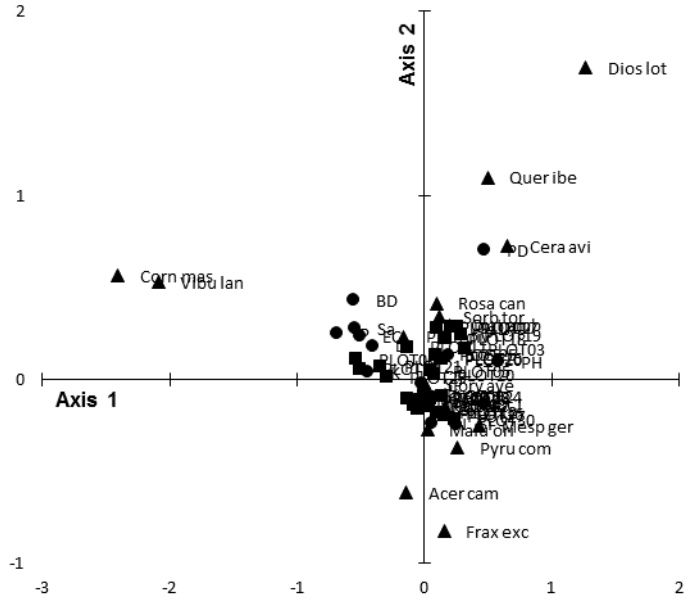

Figure 3. CCA-ordination diagram of woody species abundance distribution related to soil physical and chemical properties. EC (electrical conductivity), OC (organic carbon), pH (acidity), SP (saturation moisture percentage), N (nitrogen), P (phosphorus), K (potassium), PD (particle density), S (sand), Si (silt), Cl (clay), and BD (bulk density). The triangles represent species, the circles represent soil factors, and the squares represent plots

Discussion

The study of relationships between vegetation and environmental factors gives valuable knowledge of why certain plant species are found in some locations but not in others (Ravanbakhsh and Moshki 2016). This knowledge can help us in afforestation planning and biodiversity conservation in mountainous areas of northern Iran. A total of 43 herbaceous and 15 woody species belonging to 23 and 8 families were identified in the forest stand. The most species-rich families were the Asteraceae, Fabaceae, and Rosaceae, with 4, 3, and 11 genera and 5, 7, and 11 species, respectively.

The soil physical and chemical properties have a significant influence on plants growth and development

(Muenchow et al. 2013; Qian et al. 2014; An et al. 2015), and plants mutually play clear and predictable role in determining the soil nutrient (Fu et al. 2015; Novak et al. 2017). The soil properties cause heterogeneity over space and time and regulate the vegetation abundance (Silva and Batalha 2008; Brinkmann et al. 2009; Otýpková et al. 2011; Zhang et al. 2015). According to the previous researches, some soil variables have been identified as a determinant of species abundance distribution, for example, pH (Rodríguez-Loinaz et al. 2008; Hofmeister et al. 2009; Laganière et al. 2009; Royer-Tardif and Bradley 2011; Haberl et al. 2012; Pourbabaei and Adel 2015; Ullah et al. 2015), available K and total N (Qian et al. 2014; An et al. 2015), organic matter (Liu et al. 2012), rock content and bulk density (Wang et al. 2016), soil depth (Zhang et al. 2016), phosphorus content and electrical conductivity (Khan et al. 2016), concentration levels of K, Ca, P, CEC, and fertility index (Nadeau and Sullivan 2015), and CEC, OM, Fe, P, Mg, pH, Mn, Pb, Zn, Cu, sand, and clay proportion (Vincent and Meguro 2008).

The results showed the significant influence of some soil properties on the abundance of herbaceous species within the forest stand. The abundance of herbaceous species was closely correlated with the soil properties including pH and total N, while, we found no relationship between the woody species abundance and soil properties. This result may be due to many woody species are capable of growing across a wide range of soil or it seems that factors other than soil features are influential in the woody species abundance in the forest stand.

In conclusion, Our results provided information about plant diversity and the relationship between plant species abundance and soil physical and chemical properties in the common hazel forest of northern Iran. The distribution of vegetation largely depends on the capacity of plants to adopt resistance strategies in order to colonize the different soil conditions. The results indicated that a total of 43 herbaceous and 15 woody species belonging to 23 and 8 families were identified in the study area. The most species-rich families were the Asteraceae, Fabaceae, and Rosaceae, with 4, 3, and 11 genera and 5, 7, and 11 species, respectively. The herbaceous species abundance distribution was strongly correlated with soil properties, including the soil pH and total N. Furthermore, there was no correlation between the woody species abundance distribution and soil factors.

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Habitat utilization of the Sumatran rhinos (*Dicerorhinus sumatrensis harrissoni*) in Kutai Barat forest, East Kalimantan, Indonesia

MUKHLISI[1,♥], TRI SAYEKTI NINGSIH[1], ULFAH KARMILA SARI[1,] YUYUN KURNIAWAN[2], RIDWAN SETIAWAN[2], AHMAD MUSLIM[2]

[1]Research and Development Institute for Natural Resources Conservation Technology. Jl. Samboja-Sepaku No.42, Sungai Merdeka, Semboja, Kutai Kartanegara 75271, East Kalimantan. Tel.: +62-542-7217663, Fax.: +62-542-7217665, ♥email: muci_musci@yahoo.co.id
[2]WWF Indonesia**,** Graha Simatupang Tower 2 Unit C, 7th-11th Floor Jl. T.B. Simatupang, South Jakarta 12540, Jakarta, Indonesia

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Abstract. *Mukhlisi, Ningsih TS, Sari UK, Kurniawan Y, Setiawan R, Muslim A. 2018. Habitat utilization of the Sumatran rhinos (*Dicerorhinus sumatrensis harrissoni*) in Kutai Barat forest, East Kalimantan, Indonesia. Biodiversitas 19: 1842-1850.* Sumatran rhino population in Kutai Barat forest faces a high threat of extinction. Conservation efforts can be developed more effectively and efficiently by understanding the condition of their natural habitat. This study aimed to analyze various factors influencing habitat utilization of the Sumatran rhino in Kalimantan, specifically in Kutai Barat forest. We used past survey data of rhino presence carried out by WWF from 2014 to 2016. GPS coordinates were recorded for all signs of rhinos, such as camera trap images, footprints, bites mark on food plant, dung piles, urine, scratch, twisting, and lying signs. Rhino occurrence GPS coordinates were plotted on a map of the Sumatran rhino's habitat distribution in Kutai Barat using a grid of 2 x 2 km^2 size. Spatial analyses were run using ArcGIS 10.6. We used a habitat selection index formula to analyze habitat preference and biner logistic regression to develop Resources Selection Function (RSF). We found that the preferred habitat of the Sumatran rhino was in the secondary forest with medium and high vegetation densities. The most influential habitat variables on the presence of Sumatran rhinoceros in the Kutai Barat forest were the slope and distance from wallow. The Sumatran rhinoceros were more likely to be found in the sloping areas and the areas closer to the wallows.

Keywords: Conservation, habitat, Kutai Barat, secondary forest, Sumatran rhino

INTRODUCTION

The Sumatran rhino (*Dicerorhinus sumatrensis*) is the world's most threatened species of all rhinoceroses (Nardelli 2014; Mays et al. 2018). IUCN has listed the species as critically endangered (CR) (Havmoller et al. 2015). D. sumatrensis was, in the past, widely distributed throughout Southeast Asia and South Asia. The population now has just concentrated in Sumatra and Borneo Islands, which are a part of Indonesia (Miller et al. 2015). Illegal hunting and habitat loss are some main contributors causing the sharp decrease of rhino's population during the last 30 years from 800 individuals to less than 100 individuals (Nardelli 2014; Miller et al. 2015).

Numerous records reveal that the Sumatran rhinos mainly occur in coastal swamp forest, lowland, and mountain forest (van Strien 1985; Pusparini et al. 2015). According to Plair et al. (2011), the Sumatran rhino is a solitary species. However, this species could occasionally be found in a small group consisting of two or more individuals (van Strien 1985; Plair et al. 2011). The Sumatran rhinos appear to exhibit habitat preferences influenced by food abundance, topography, the source of minerals and water, and human presence (van Strien 1974; van Strien 1985; Pusparini et al. 2015; Kretzschmar et al. 2016). Therefore, within the utilized habitat, different environmental factors may interact to affect their presence. Putra (2014), for instance, found that the distance from roads and slope significantly affected habitat utilization of the Sumatran rhinos in Aceh. Similarly, Rusman (2016) recorded that the Sumatran rhino in Bukit Barisan National Park preferred to live close to the river, far from settlements, and relatively flat areas.

The Kalimantan forest harbors the smallest number of the Sumatran rhino population worldwide (Miller et al. 2015). A review of available literature indicates that only 7-15 individuals occur in the forest of Kutai Barat and Mahakam Ulu, East Kalimantan Province (WWF 2014). The patchy forest is a considerable factor for this phenomenon. In fact, the fragmented forest of Kutai Barat is only able to maintain 1-3 individuals of the Sumatran rhinos. Those rhinos inhabit a logging concession characterized by lowland secondary forest, with Dipterocarpaceae growing in abundance (Mukhlisi et al. 2017). Nevertheless, rhinos do not use entire habitat as their home range. Spatial evidence of the rhino presence in the Kutai Barat forest shows that this species is interested in a particular area within their home range (WWF 2014; field observation). This, in turn, has led to the suggestion that habitat utilization of the Sumatran rhinos in Kutai Barat is also affected by environmental gradients.

Study on ecology of the Sumatran rhinos in Kalimantan is limited. Previous studies have only focused on food ecology (Atmoko et al. 2016; Mukhlisi et al. 2017). WWF (2014) documented the distribution of the Sumatran rhino population in Kutai Barat and Mahakam Ulu forests. Regarding habitat utilization, studies have dealt with this issue in Sumatera (Putra 2014; Pusparini et al. 2015;

Rusman 2016). Accordingly, there is a need to understand habitat preferences and utilization of the Sumatran rhinos in Kalimantan, particularly in the Kutai Barat forest. Since the Sumatran rhinos show different responses to environmental conditions (Pusparini et al. 2015), research related to this field is urgent to enrich ecological information.

Research on habitat utilization of the Sumatran rhinos can help ecologists and managers determine strategies for ex-situ and in-situ conservation. Preferred habitat with an optimum condition is definitely capable of maintaining a sustainable population. Knowledge of habitat preferences can be applied to assist habitat and population management of the Sumatran rhinos in the wild such as Intensive Protection Zone (IPZ), which is an exclusive area for in-situ conservation with strict protection (Pusparini et al. 2015; Rusman 2016). In addition, information on habitat preferences is also beneficial to support the establishment of restricted breeding areas and corridors. This study, therefore, aimed to analyze various factors influencing habitat preferences of the Sumatran rhinos in Kalimantan, particularly in the Kutai Barat forest.

MATERIALS AND METHODS

Study area

The Kutai Barat forest was situated in Kutai Barat District, East Kalimantan, Indonesia. The forest was located in two active logging concession areas identified as one of the rhino's habitat patches in East Kalimantan (WWF 2014). In general, forest cover in the study area can be classified as lowland dipterocarp forest, with comprised of 177 tree species (Mukhlisi et al. 2017). The annual average temperature was around 26.5-27 °C, whereas the annual rainfall was 350 mm for the last 30 years (BMKG 2018).

Procedures

Information on the rhino presence was derived from the last survey carried out by WWF during 2014-2016. Evidence of habitat use was denoted by the presence of footprints, bite marks, dung piles, urine, scratching, twisting, and lying signs. Each presence sign was recorded in the form of GPS coordinates. In total, 294 coordinates were representing the rhino's signs (Ginanjar 2016, pers.com). The habitat components were divided into eight variables including distance from roads (X1), rivers (X2), wallows (X3), salt licks (X4), slope (X5), elevation (X6), Normalized Difference Vegetation Index/NDVI (X7), and soils (X8) (Santosa et al. 2013; Putra et al. 2014; Pusparini et al. 2015; Rusman 2016). To simplify the analysis process, each variable was classified (Table 1). We considered the distance from roads, rivers, wallows, salt licks, and elevation as interval data. Meanwhile, slope and NDVI were categorized as ordinal data.

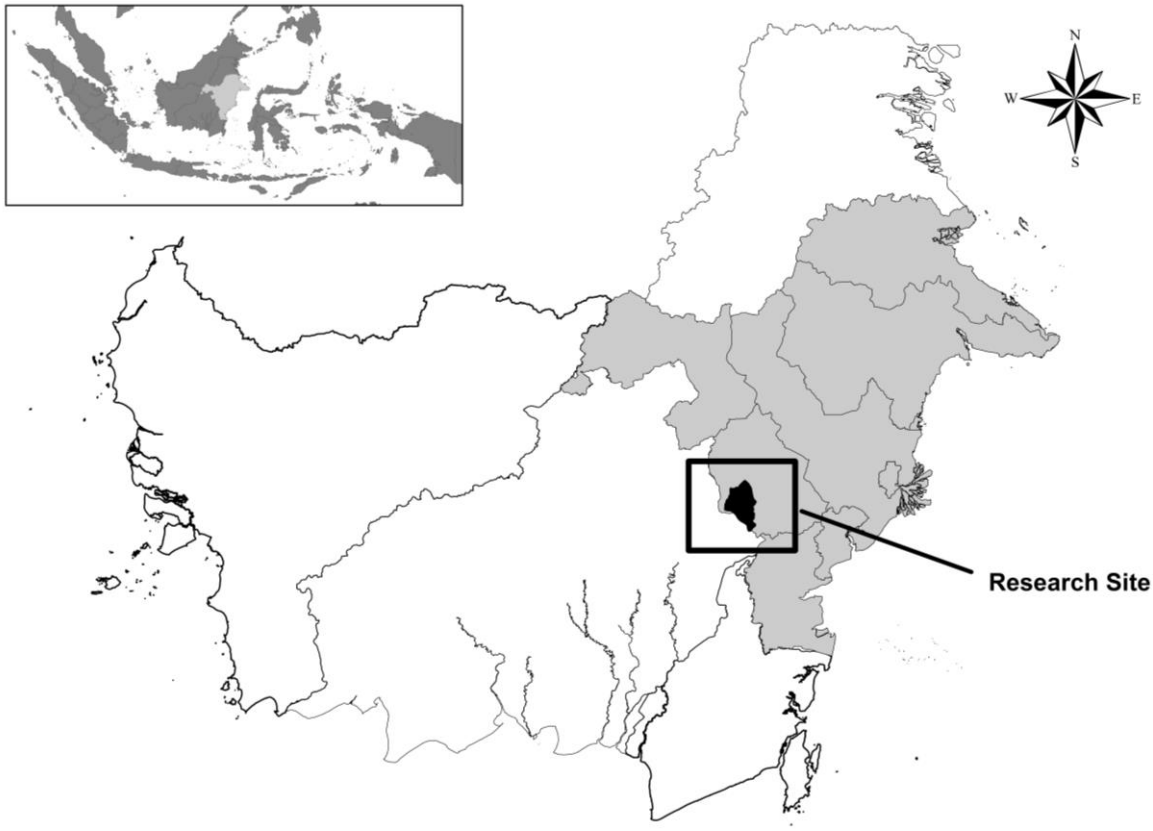

Figure 1. Study area of the Sumatran rhinos (*Dicerorhinus sumatrensis harrissoni*) in Kutai Barat forest, East Kalimantan, Indonesia

The observed signs were plotted onto the map of Sumatran rhino habitat distribution in Kutai Barat District. The result then was overlaid with each map of habitat variable using ArcGIS version 10.6. The problem emerged when we described habitat features of each sign on the combined map. To overcome this, we divided each habitat variable map into grid cell of 2 x 2 km^2 before overlay operation. We assigned each grid cell with 1 (Y = 1) if there was a sign for rhinos and 0 (Y = 0) for the opposite. A 2 x 2 km^2 grid cell was based on the estimate of the lowest Sumatran female rhino's home range (Putra 2014). Totally, the study site was covering an area of 44,272.03 Ha. There was 18,352.01 Ha with the rhino's presence signs and 25,920.02 Ha with no sign of rhinos.

Distance from roads, rivers, wallows, and salt licks were analyzed using Euclidean Distance tools in ArcGIS 10.6. Two variables-slope and elevation-were extracted from DEM, which was derived from Shuttle Radar Topographic Mission (SRTM) at a resolution of 30 m. NDVI was assessed from Landsat 8 using the following equation with acquisition 9th Sept 2016 Path 117 Row 061.

NDVI = (NIR-RED)/(NIR+RED)

NDVI shows the degree of vegetation density as Rusman (2016) also used the same variable for analyzing the availability of rhino food in Sumatra Island. Finally, to understand the real condition of each habitat variable, we did fieldwork in 2016. For particular variables such as wallow, we did detail measurement of water pH, length, width, and distance from rivers or the nearest water spring. We also carried out a 1500 mL water sample collection on salt licks and rivers for mineral content analysis.

Data analysis

We employed a Chi-Square test with a confidence level of 95% to know the relationship between habitat types and habitat use frequency. In this paper, we categorized habitat types into the shrub and secondary forest. Furthermore, to analyze habitat preferences of rhinos, we used the formula of Neu et al. (1974):

$$\text{Selection index } w = \frac{r}{a}$$

$$\text{Standardised index } B = \frac{w}{\sum w}$$

Where: w is habitat selection index, r is rhino occurrence frequency, and a is the proportion of habitat used. For the selection index, we would conclude that rhinos will prefer to employ a particular habitat if w > 1. With regards to standardized index (B), this index always has a value of 1. Therefore, it will show the comparison of habitat selection index within each tested habitat.

A multicollinearity test was used to detect correlation among independent variables of habitat. No multicollinearity will happen if p > 1 or variance inflation factor (VIF) is less than 10. Once there was no indication of multicollinearity, the Resources Selection Function can be identified based on logistic regression as the following formula (Manly et al. 2002):

$$\pi = \frac{\text{Exp } (\beta o + \beta 1X1 + \beta 2X2 + \cdots \beta pXp)}{1 + \text{Exp } (\beta o + \beta 1X1 + \beta 2X2 + \cdots \beta pXp)}$$

Where: π is the probability of the presence of rhinos, β0 to βp are coefficients estimated from the available data, and X1 to Xp are habitat variables. To this end, all statistical processes were run using PSPP, an open source statistical software.

RESULTS AND DISCUSSION

Distribution of habitat utilization

According to the map of observed signs of rhinos during 2014-2016, we argue that rhino habitat was stretching from the east to the west of the Kutai Barat forest (Figure 2 and 3). Habitat utilization pattern of rhinos resulted from animal preference and adaptation. Particularly adaptation, this term is interesting since the forest was experiencing severe human pressure. From Landsat imagery, it was clear that the forest is adjacent to coal mining, palm oil plantations, settlement, and agricultural areas (Figure 2). The condition leads to forest fragmentation resulting in patchy forest separated from rhino habitat in the northern part of Mahakam Ulu District. Besides, illegal hunting and rampant logging tended to increase considerably. The fact eventually caused rhinos to select safe and secure habitat with good quality. Distribution of habitat utilization by rhinos in the study site is presented in Table 1.

The road networks in the study site were the consequence of industrial timber operations in the past. Some of them were still active, whereas others were no longer used. Nevertheless, roads significantly affected the habitat preferences of the Sumatran rhinos. Table 1 indicates that rhinos preferred to utilize habitat with distance < 2,000 m from road networks. Santosa et al. (2013) found a strong correlation between Javan rhino and roads since their food plants were more abundant near road networks. Gaps in the forest created by roads allow pioneer species to regenerate, providing food preferred by rhinos such as *Macaranga* spp, *Homalanthus populneus*, *Ficus obscura*, and *Pternandra rostrata* (Atmoko et al. 2016; Mukhlisi et al. 2017). Although roads have a substantial effect on rhino behavior, it seems that human presence could also influence the rhino habitat use. Rhinos will avoid roads frequently used by human (Pusparini and Wibisono 2013; Putra 2014; Pusparini et al. 2015).

Three main rivers (Piraq, Naja, and Tenaik) flow around the Kutai Barat forest, containing numerous small streams. Those rivers are mostly perennial. Visual observation revealed that rhino signs were mostly found near the river (< 1,000 m). This finding supports the fact that behavior of rhinos in the wild is affected by water availability. Rivers play an important role in providing a source for drinking, wallowing, and bathing, which in turn will influence habitat preference of rhinos (van Strien 1974; Ng 2001; Putra 2014; Pusaprini et al. 2015; Rusman 2016). Rivers may facilitate a temporary water hole

creation which is often used by animals such as rhinos for maintaining body temperature, skin protection, and avoiding insect bite (van Strien 1974; Ng 2001). We also noticed that rivers are crucial for defecation. Feces and urine resulted from this process let rhinos communicate with each other. Chemical components released through excretion are beneficial for detecting age and sex of other individuals (Linklater et al. 2013).

The average length and width of wallows were approximately 1.70-4.80 m and 1.40-4.70 m, respectively. Measurements were recorded on 11 out of 123 wallows which were identified during 2014-2016 (Table 2). The average size of wallows seemed to be small as a consequence of the small body size of the Sumatran rhino. However, wallows made by the Sumatran rhino in the Kutai Barat forest were larger than those made by a captive bred rhino in Selangor, Malaysia (Ng 2001). Ng (2001) reported that the size of wallows was around 1.25-4.00 m in length and 1.20-3.70 m in width. The difference could be due to the use of wallows by other animals and rolling behavior expressed by rhinos. The Sumatran rhino needs at least 2-3 hours for wallowing before foraging (Ng 2001). According to this finding, it is plausible to conclude that the distance from wallows is vital for rhino presence. Van Strien (1985) reported that the Sumatran rhino would create a wallow in every 50 m of its movement.

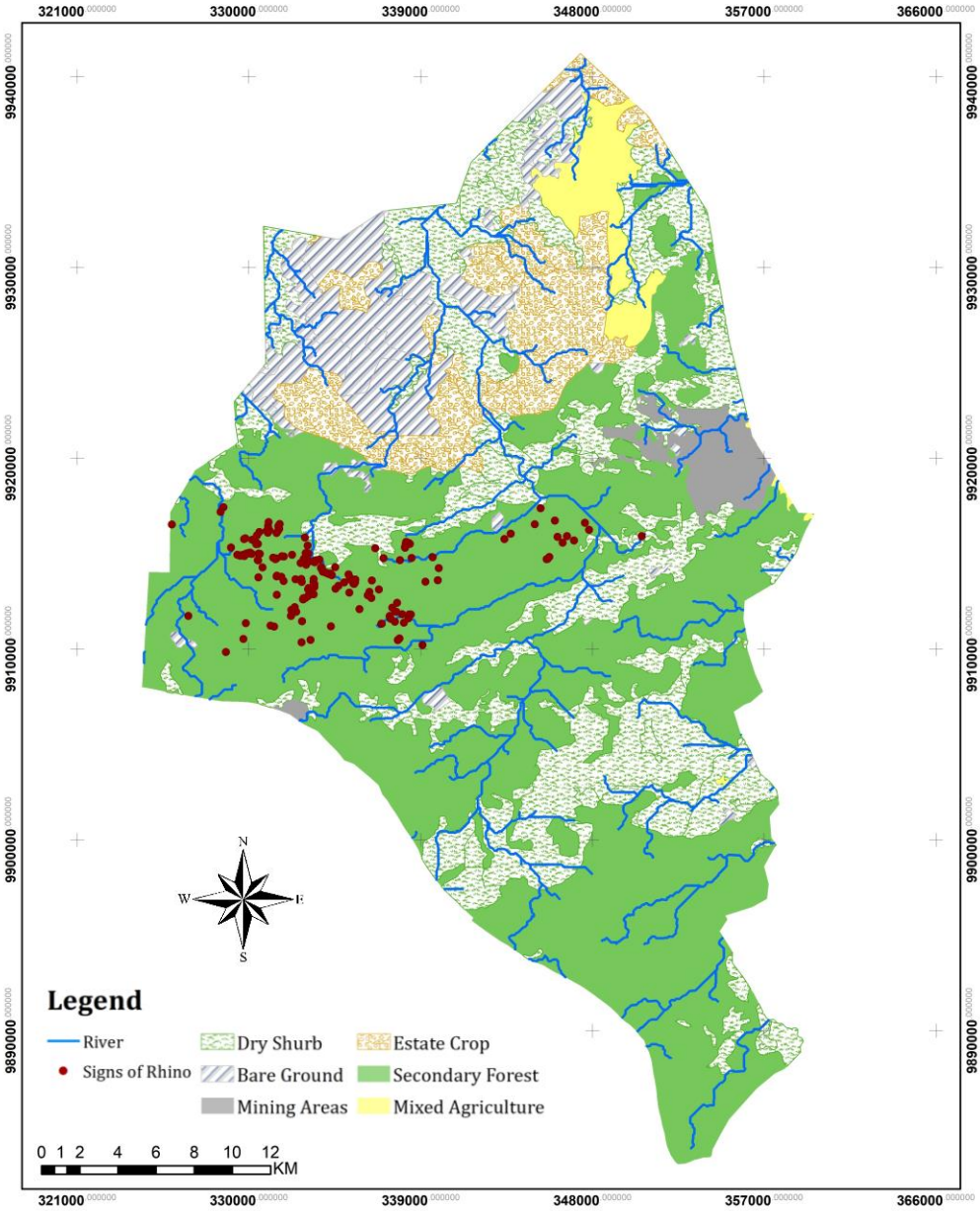

Figure 2. Distribution of rhino's signs in Kutai Barat forest, East Kalimantan, Indonesia

Figure 3. Signs of rhino presence in the Kutai Barat forest, East Kalimantan, Indonesia. A. Dung piles, B. Wallow, C. Scratch, D. Footprint, E. Bites mark on food plant (Photo by RS/WWF Indonesia)

Salt licks are beneficial for many animals like rhinos since they can provide a source of minerals. Van Strien (1974) and Matsubayashi et al. (2006) revealed that three main minerals found in a salt lick are magnesium (Mg), potassium (K), and phosphate (P). Those minerals can help rhinos maintain their digestive system. Minerals such as sodium (Na) and potassium (K) are also useful in balancing ions in the body. To know the mineral content of rhino habitat in the Kutai Barat forest, we tested several locations such as rivers, wallows, and salt licks (Table 3).

Table 2 shows that P, Na, and Mg concentration in rivers, wallows, and salt licks were higher than other mineral compounds. This current study also indicates that wallows had the highest level of salt mineral. On the other hand, the mineral content of salt licks particularly P, Na, and Mg were higher than those found in rivers. This phenomenon indicated that the source of salt mineral for rhinos was not only from salt licks but also from wallows. Apart from those given locations, food plants can provide salt mineral for rhinos. This reason might explain the fact that signs of rhinos were mostly found within the distance of > 2,000 m from salt licks. The visit rates to salt lick seemed to increase in the dry season or for unhealthy rhinos. van Strien (1985) stated that the frequency of visits

of rhinos to a mineral source was one visit/month, but females with their young tended to be more frequent. For adult rhinos, the visitation to salt mineral sources is essential for social life (van Strien 1985).

The slope is mostly flat in the study site. Moderate slope ranging between 8-25% is an ideal habitat for the Sumatran rhinos, supporting feeding and moving activity. An area with moderate slope will allow sunlight to penetrate deeply to the forest floor. The process will accelerate the regeneration process of forest floor vegetation. Furthermore, forest gaps are more likely to occur in an area with 15.01-25% in the gradient due to soil instability and fall trees. Similar to forest floor vegetation; the forest gap can stimulate pioneer tree species to regenerate and grow rapidly, affecting the abundance of rhino's food plants. Rhinos also avoid higher slopes because it is a part of their energy storage strategies. Consequently, it is common that rhinos will follow contour lines (van Strien 1985; Santosa et al. 2013). Previous studies found that the most encountered gradient of the Sumatran rhino in Aceh was less than 40%, whereas their relatives in Bukit Barisan Selatan National Park preferred to live in areas typified by a moderate slope (Rusman 2016).

The rhino habitat in the Kutai Barat forest was characterized by lowland tropical forest. However, in general, the Sumatran rhinos were able to tolerate a wide range of elevations up to 2,000 m above sea level (Putra 2014). Furthermore, WWF (2014) found that the Sumatran rhinos in Mahakam Ulu used habitat up to 700 m above sea level. The lack of food availability may constrain rhinos to survive in a habitat with an elevation of > 2,000 m above sea level (van Strien 1985). Interestingly, Pusparini et al. (2015) stated that habitat preference had no strong correlation with elevation. Rhinos in the Kutai Barat forest were absent within elevation > 200 m above sea level. A possible explanation for this discrepancy might be that habitat preferences related to altitude could be specific depending on each site condition, suggesting that temperature decline due to an increase in elevation is not an impediment factor influencing rhino adaptation. One of the issues that emerge from these findings is the food availability which tends to have a more significant impact on the rhino presence.

NDVI is based on the density of the green patch of the area indicated on satellite imagery with the value ranging from -1 to 1 (Rouse Jr. et al. 1974; Xue and Su 2017). According to a land cover map released by the Ministry of Environment and Forestry, the habitat of rhinos in the Kutai Barat forest is classified as secondary forest. Yet, we investigated some areas dominated by shrubs resulted from the past logging. There were at least 53 species of identified food plants in the study site (Atmoko et al. 2016). Furthermore, Mukhlisi et al. (2017) also reported that two vegetation transect ever created around rhino habitat had a relative abundance of 1.79-1. 82 ind. ha^{-1} with dominant species of *Koilodepas brevipes*, *Palaquium sericeum*, *Pternandra rostrata*, *Diospyros* sp., *Dillenia excelsa*, and *Baccaurea lanceolata*.

Table 1. Habitat use distribution by the Sumatran rhinos in the Kutai Barat forest

Variables		Classification	Rhino presence signs (%)
Distance from road	1	< 1,000 m	70.07
	2	1,001-2,000 m	29.93
	3	>2,000 m	0.00
Distance from river	1	< 1,000 m	75.85
	2	1,001-2,000 m	24.15
	3	>2,000 m	0.00
Distance from wallow	1	< 1,000 m	87.76
	2	1,001-2,000 m	10.20
	3	>2,000 m	2.04
Distance from the salt lick	1	< 1,000 m	14.63
	2	1,001-2,000 m	12.93
	3	>2,000 m	72.45
Slope	1	0-8%	0.00
	2	8.01-15%	46.26
	3	15.01-25%	53.74
	4	25.01-40%	0.00
	5	> 40%	0.00
Elevation	1	< 100 m	59.18
	2	100.1-200 m	40.82
	3	200.1-300 m	0.00
	4	>300 m	0.00
NDVI	1	-0.16-0.15	5.10
	2	0.16-0.30	15.99
	3	0.31-0.43	78.91
Soil association	1	Paleudults-Tropudults-Tropaquepts	0.00
	2	Tropaquults-Paleudult-Tropodults	2.04
	3	Tropudults-Dystropepts	32.99
	4	Tropudults-Plinthudults-Paleudulst	64.97
	5	Tropudults-Tropaquepts	0.00

Table 2. Wallow characteristics of the Sumatran rhino habitat in the Kutai Barat forest

No.	Length (m)	Width (m)	Water pH	Distance from the river (m)
1	3.20	2.55	6.33	100
2	4.80	4.70	6.25	125
3	3.50	2.43	6.23	0
4	3.89	2.95	6.15	20
5	2.99	1.90	6.10	30
6	3.20	2.70	6.05	15
7	2.45	1.40	5.64	0
8	1.70	1.40	5.75	6
9	2.50	2.00	5.45	5
10	2.20	2.00	5.10	5
11	3.50	2.70	5.80	4
Mean	3.08	2.43	5.90	28.18
SD	0.861	0.914	0.386	43.030

Table 3. Mineral content of the river, wallow, salt lick

Location	Mineral concentration (mg L^{-1})					
	P	Na	Ca	Fe	Mg	K
River	1.10	3.22	1.40	0.96	1.41	1.06
Wallow	1.80	4.02	2.30	1.76	3.26	1.86
Salt Lick	1.57	2.94	1.28	0.45	2.19	1.15
Mean	1.49	3.39	1.66	1.06	2.29	1.36
SD	0.357	0.560	0.556	0.660	0.929	0.438

Ultisols are soil orders generally found in Kalimantan. Within this order, there is an association of Tropaquults, Paleudult, and Tropodults which can influence habitat selected by rhinos. The result is consistent with the earlier study in Sabah, Malaysia (Kretzschmar et al. 2016). According to Prasetyo et al. (2001), Ultisols are typified by clay texture, low pH, low organic matter and low base saturation, and being aquatic up to 50 m from the surface. The characteristics of Ultisols appear to support wallowing behavior of rhinos as the soil can be converted easily.

Habitat selection of the Sumatran rhinos

A Chi-Square test revealed that there was a significant correlation ($p < 0.05$) between habitat types and the frequency of rhino occurrence in the Kutai Barat forest. It means that rhinos select a particular type of habitat to fulfill their needs. The difference between the observed and expected frequency also supports the finding. Once a significant correlation occurs, habitat selection index could be conducted (Neu et al. 1974). Table 4 and 5 summarize the result of the Chi-Square test and Neu index.

Table 5 clearly shows that the most selected habitat of the Sumatra rhinos was in the secondary forest although in the real condition their habitat is also composed of shrubs. The secondary forest in the study area is a consequence of regular logging activity of timber plantation. Indonesian laws strictly control logging from natural forest. A tree in timber plantation is allowed to cut if it has reach > 50 cm in diameter. Besides, tree cutting should be conducted within a 25-30 years block rotation. Currently, rhino habitat in the Kutai Barat forest is situated in a block that was last logged in the 1990s so that vegetation has well regenerated and transformed into an old secondary forest. Unfortunately, illegal logging that sometimes happens is likely to affect the ongoing regeneration process, resulting in the disturbed secondary forest.

van Strien (1985) explained that the ideal habitat for the Sumatran rhinos is old secondary forest without human interference. Therefore, finding rhinos in the secondary forest surrounded by human activity is challenging. Rhinos are known as a cryptic animal, avoiding human contact (Nardelli 2014). This specific characteristic prevents rhinos to present in open areas like shrubs. However, some studies found that rhinos sometimes appear in open areas for foraging and return to the forest for shelter and protection (van Strien 1974; Kretzschmar et al. 2016). This behavior is in line with our analysis towards habitat selection.

The selection probability towards resources in rhino habitat

To detect the most preferred habitat by the Sumatran rhinos in the Kutai Barat forest, we did logistic regression analysis. Before running the analysis, it is imperative to test multicollinearity among habitat variables. Logistic regression analysis can be executed if tolerance value is > 0.1 and VIF (Variance Inflation Factor) < 10. The analysis will produce a model called Resources Selection Function (RSF), and it then can be applied to assess the probability of habitat used. Our finding revealed that there was no multicollinearity so that logistic regression analysis was applied. The analysis used 37 points of rhino presence and 104 points of absence on a 2 x 2 Km^2 grid. Table 6 and 7 shows the result of the multicollinearity test and logistic regression analysis, respectively.

Based on the stepwise forward run in SPSS (Table 4), we found that slope and wallow had a significant influence on the presence (Y) of the Sumatran rhinos ($p < 0.05$). Therefore, the RSF model was:

$$Y = \frac{\text{Exp}\ (-4.098 + 2.175\text{Slope} + 5.497\text{Wallow})}{1 + \text{Exp}\ (-4.098 + 2.175\ \text{Slope} + 5.497\text{Wallow})}$$

Table 4. A chi-square test for the relationship between habitat types and the occurrence frequency of rhinos

Habitat	Size (Ha)	Proportion	Oi	Ei	Oi-Ei	$(Oi-Ei)^2/Ei$	$(X^2)_{0.05}$
Shrub	8,665.32	0.20	6.00	57.54	-51.54	46.17	
Secondary forest	35,606.71	0.80	288.00	236.46	51.54	11.24	
Total	44272.03	1.00	294.00	236.46	0.00	57.41	3.84

Table 5. Neu selection index of the Sumatran rhino's habitat in the Kutai Barat forest

Habitat	Available habitat		Encountered signs		Neu Index	
	Size (Ha)	Proportion	Recorded	Proportion	Selection (w)	Standardized (B)
Shrub	8,665.32	0.20	6.00	0.02	0.10	0.08
Secondary forest	35,606.71	0.80	288.00	0.98	1.22	0.92
Total	44,272.03	0.40	294.00	1.00	1.32	1.00

Table 6. Multicollinearity test

Model		Unstandardized Coefficients B	Standardized Coefficients Std. Error	Beta	T	Sig.	Collinearity Statistics Tolerance	VIF
1	(Constant)	1.701	.373					
	Roads (X2)	-.046	.045	-.081	-1.021	.310	.602	1.661
	Rivers (X1)	-.165	.044	-.274	-3.753	.000	.705	1.419
	Wallow (X7)	-.293	.050	-.470	-5.856	.000	.584	1.712
	Salt Lick (X8)	-.182	.079	-.159	-2.318	.022	.801	1.249
	Slope (X3)	.061	.055	.085	1.106	.271	.635	1.574
	Elevation (X4)	-.009	.067	-.010	-.130	.897	.700	1.429
	NDVI (X5)	-.040	.054	-.050	-.736	.464	.806	1.241
	Soil (X6)	.085	.043	.129	2.002	.048	.905	1.105

Table 7. Logistic regression analysis

		B	S.E.	Wald	df	Sig.	Exp(B)	95.0% C.I.for EXP(B) Lower	Upper
Step 2[b]	Slope (X3)							1.321	58.685
	Wallow (X7)	5.497	1.324	17.242	1	.000	243.845	27.639	2.151E3
	Constant	-4.098	1.207	11.529	1	.001	.017		

A developed model of RSF fitted for estimating the presence of the Sumatran rhinos in the Kutai Barat forest. A goodness of fit test represented by Hosmer and Lameshow showed a significant value of 0.4447 ($p > 0.05$). A developed model had high accuracy displayed by the value of Area Under Curve (AUC) which was 0.955. The RSF model also suggested that independent variables simultaneously affected the presence of rhinos as shown by the Omnibus test with a value of 0.000 ($p < 0.05$). Furthermore, the value of Cox and Snell R Square and Nagelkerke R Square were 0.514 and 0.719, respectively. Those values informed that independent variables could explain 71.90% of the probability of the rhino presence (Y). It means that other factors explained 28.10% of the rhino presence. Thus, the RSF model was suited to analyze the presence of rhinos.

Unlike linear regression, we could not interpret the result directly. The easiest way to translate the result of regression was through the odds ratio value or Exp (B) and by doing a simulation of the developed model (Lele et al. 2013). According to the RSF model, it could be interpreted that an increase in slope would decrease the probability of the rhino presence as much as 8.803 fold. Meanwhile, for the wallow, an increase in a 1,000 m distance would reduce the likelihood of rhino presence as 243.845 fold. From the simulation of our model, we suggested that the probability of the rhino presence would be higher in slope with a gradient of 8-15% (0.92) and < 1,000 m from the nearest wallow (0.99).

Slope might be a factor restricting natural movement of rhinos. Rhinos are more likely to select habitat with a moderate slope for moving and foraging. It is known that the home range of the Sumatran rhinos was 5-6 Ha day^{-1} (van Strien, 1985). So, to prevent energy loss, moderate slope appears to be preferred. The gradient of slope in the Kutai Barat forest, which is mostly < 25%, seems to support the natural behavior of rhinos. We assume that slope and wallow complement each other. We found it difficult to form a wallow on a steep area since there was no water-bearing capability. Also, wallow construction is influenced by a river which is found frequently in a flat area. In fact, during fieldwork, we found that wallows were often close to rivers with the distance of 28.18 ± 43.030 m.

The Sumatran rhinos exhibited a high frequency of wallowing. This behavior is useful to maintain body temperature and protect rhinos from diseases (van Strien 1974; Ng 2001). Prevention from bathing for an extended period will cause detrimental effects such as inflammation and cracked skin. It even can drive a young rhino to die shortly in a week (van Strien, 1985). Low pH of the water as found in a bathing area is resulted from leaf litter, protecting rhinos from diseases. Based on fieldwork, we argue that wallows were mostly identified near the main rhino trail including the presence signs of rhinos.

For rhinos, wallowing has a crucial role in maintaining body temperature. Wallowing also provides a chance for rhinos to communicate with each other, though rhinos are considered as a solitary animal. Meeting and communication during wallowing are beneficial particularly in a breeding season. According to van Strien (1985), Ng (2001), and Bracke (2011) a wallow was capable of supporting scent marking sexual behavior. The result of analysis on salt mineral concentration suggested that the Sumatran rhinos in the Kutai Barat forest were wallowing in mud containing a high level of salt. There was a correlation between the frequency of wallowing and the need for salt mineral per individual. However, much uncertainty still exists about that correlation so that further research on that issue is urgently needed.

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Evaluation of farmers' knowledge on the rare Abyssinian pea (*Pisum sativum* var. *abyssinicum*) landraces of Ethiopia

BERHANE GEBRESLASSIE GEBREEGZIABHER♥, BERHANU ABRAHA TSEGAY
Department of Biology, College of Science, Bahir Dar University. P.O. Box 76, Bahir Dar, Ethiopia. ♥email: birie2006@gmail.com.

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Abstract. *G*ebreegziabher *BG, Tsegay BA. 2018. Evaluation of farmers' knowledge on the rare Abyssinian pea (*Pisum sativum *var.* abyssinicum*) landraces of Ethiopia. Biodiversitas 19: 1851-1865.* Abyssinian pea (*Pisum sativum var. abyssinicum* A. Braun) is a rare and problematic taxon requiring evaluation of present farmers' local knowledge. Cross-sectional data were collected from 444 respondents and analyzed using SPSS software. Descriptive statistics was used; one way ANOVA for significance test of variance and Exhaustive CHAID growth method for predictions. Prediction results showed that the crop requires about two good rains, Nitisol soils and about 21-30 kg ha^{-1}seeding rate. The flowering to maturity time ranges 1½ to 2½ months depending on the agroecology (highland or lowland), with a yield of about 300-400 kg ha^{-1}on average. The crop distribution is currently limited to three to four districts and sown after other crops are harvested. Major factors hindering its distribution are agro-ecological suitability, lack of intervention and preference of high yielding pea varieties. The crops' inferiority in yield and pest susceptibility is the main reason for less extensive awareness on the crop. Though inferior in yield and susceptible to pest, farmers still prefer to grow the crop because of its marketability for local exchange and consumption. The core production problems currently remarked by farmers are expensive price of the seed to buy and small land holding.

Keywords: Agronomic descriptors, indigenous knowledge, seeding rate, soil type, yield

INTRODUCTION

Abyssinian pea **(***Pisum sativum* var. *abyssinicum* A.Braun), is one among the pulse field crops grown in Ethiopia (CBD 2009). Local farmers have a great belief to express the crop's special taste, marketability, and earliness (Personal communication). Researchers are critically studying it and yet not fully attain factors and genes responsible for the delightful taste, the early flowering and maturity of the crop (Yemane and Skjelvåg 2003; Weeden 2007; Weller et al. 2012; Smýkal et al. 2015; Rubenach et al. 2017). Farmers in Ethiopia appreciate it in different ways; "urban stew", "stew for recipient of hospitality" and "chicken stew of the poor" for its delightful taste. On the other way, it is named as "seed of the well-off", for its marketability and unavailability for the poor. Regarding to its earliness, Abyssinian pea is named as "fetnoderash" (early maturing) referring to its short life cycle. As the studies made so far about the crop are slighter, there is lack of local yield gap estimation method developed to maximize the Abyssinian pea yields (Ittersuma et al. 2013).This is a major problem, particularly to the undeveloped countries like Ethiopia facing challenges to achieve potential yield (Affholder et al. 2013), especially when viewed in the global climate change scenario.

As the crop is less known outside of the tropical and subtropical belts of Africa (Mikić and Mihailović 2014), it is becoming herbarium specimen. It is a germplasm accession included in the 6096 pulse accessions of Ethiopia (CBD 2009). This requires evaluation of the stakeholders' indigenous knowledge so as to re-establish the prospect of food legumes together with the ecological insight to the agricultural system (Tomich et al. 2011). Besides, loss of diversity in farmers' field crops is decreasing and lacks the expected evidences of the threats in the available literatures (FAO 2010). Moreover, a study from Mikić et al. (2013) indicated the narrow distribution of Abyssinian pea that brought a narrow genetic variation with long inherent partial or complete tolerance adaptions for biotic and abiotic stresses particularly of salt (Tsegay and Gebreslassie 2014). Therefore, the aim of the study was mainly to evaluate farmers' knowledge on current trend, agronomic practices and production of the crop so as to lay basis for forthcoming studies on intensification. We believe that this would help in addressing issues of sustainable growth of the crop and obtaining optimum yield in order to tackle the challenges of population growth, food security, and climate change and resource conservation.

MATERIALS AND METHODS

Description of the study areas

The study was conducted in Northeast Ethiopia in Amhara and Tigray regions (Figure 1). It was piloted in six districts that proportionated into three districts per region based on agro-ecology. Each district was well-adjusted into three kebeles (small administrative units of a district existing in Table 2) which represent different agro-ecology. The districts have different geographical and climatic features (Table 1).

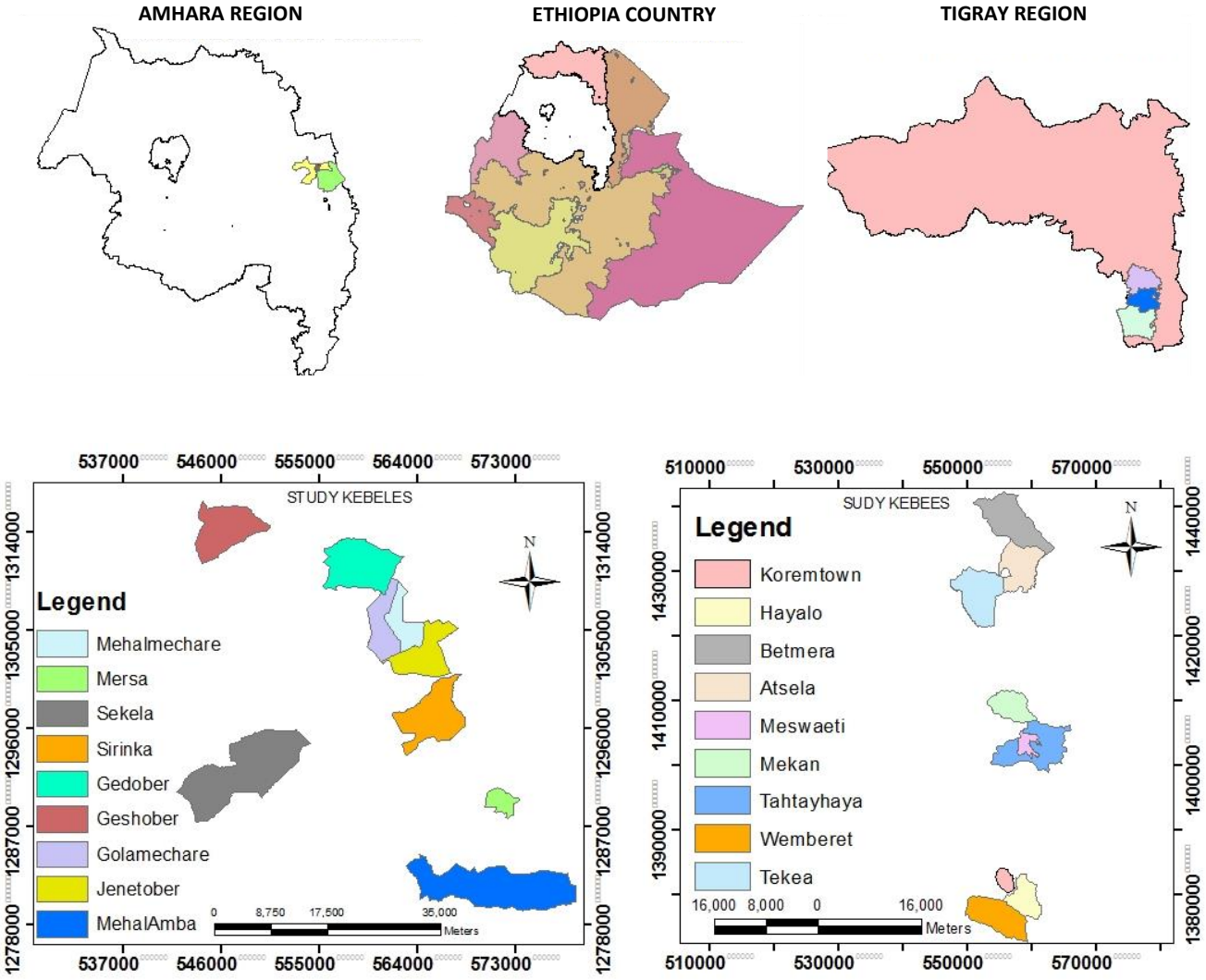

Figure 1. Map of Ethiopia showing location of the study areas within Amhara and Tigray regional states

Description of study areas

Gubalafto and Habru are situated in east plain possessing high potential agricultural value chain and beneficial from the varying topography with suitable climate and agro-ecology for crop production (Save the Children 2013). In Woldia, the study was conducted in kebeles within a radius of 10-15 km. Ofla and Amba-Alaje districts are mostly highland agro-ecological districts (Abrha and Simhadri 2015). They have normally sufficient rainfall and suitable temperatures for rainfed agriculture as indicated in Table 1.

Crop production areas in Ethiopia are shaped by the agro-ecological variability (Hurni 1998; Warner et al. 2015). The study districts from north Wollo are mainly characterized by production of stable crops such as sorghum, maize, teff, chickpea, and coffee and the districts from south Tigray (Table 1) produce stable crops namely wheat, barely, teff, chickpea and maize (Warner et al. 2015, personal observation). All regions produce Abyssinian pea though at different rates.

Table 1. Geographical and climatic features of the study areas

Sampling regions	Districts	Altitude (m asl.)	Mean annual rainfall (mm)	T (°C)
Amhara	Gubalafto	1379-3200	990-1030	21-25 (a)
	Woldia	2112-2218	600-820	10-20 (b)
	Habru	700-3500	300-876	21-32 (c, d)
Tigray	Ofla	2432-2450	350-1200	20-26 (e)
	Endamehoni	1600-3960	600-1000	9-18 (f)
	Amba-Alaje	2445-2480	580-845	14-22 (h)

Note: T^{O} (°C): temperature in degree Celsius, (a) Mengistie and Kidane (2016), (b) Svein and Adal (2002), (c) Damtew (2006), (d) Mekonnen et al. (2014), (e) Admasu et al. (2011), (f) Ebrahim et al. (2015), (g) Gebrewahd et al. (2017).

Data collection design

Cross-sectional data collection design was employed as this is better and more effective for obtaining information about the current status or the immediate past (Kahn 2000; Mengistie and Kidane 2016) of this icon crop (Abyssinian Pea). Personal interviews with oral verbal stimuli were presented and replied by way of oral verbal responses in the first steps of this study. This is in order to collect information that helps to understand the research topic better through information interchanged between the individuals. Pilot study tested questionnaires consisting of series of printed multiple choice questions, to be marked by the informants were distributed and collected. Observations (on residence, market and field) were ended for seed colors, pests and soil conservations (Figures 2 and 5), and focus group discussion with farmers who have the current growing experience of the crop for the last three years were used. Data were collected in 2017 using local language of informants that latter translated to English.

Sample size determination

Districts were selected purposely based on the current production potential of the crop and the climatic and geographic (altitudinal) differences of the districts (Table 1). Moreover, recommendations from agricultural and rural development experts were used.

A total of 6228 Abyssinian pea producer farmers of the six districts found from the pilot survey were proportioned into the representative kebeles using the formula of Yamane (1967). Additionally, a total of 82 agricultural experts with crop specializations from two Agricultural Research Centers (ARCs) were proportioned into their respective districts.

Table 2. Sex of respondents involved in study by districts and kebeles

Study regions	Districts	Kebeles	Male (N)	Female (N)	Total (N)
Amhara	Gubalafto	Sekela	12	10	22
		Geshober	14	8	22
		Gedober	18	4	22
	Woldia	Mehalmechare	13	9	22
		Jenetober	14	8	22
		Gola-mechare	17	5	22
	Habru	Sirinka	18	4	22
		Mersa	20	2	22
		Mehalamba	18	4	22
Tigray	Ofla	Korem-suburb	22	0	22
		Wenberet	15	7	22
		Hayalo	18	4	22
	Endamehoni	Meswati	20	2	22
		Tahtayhaya	18	4	22
		Mekan	20	2	22
	Amba-Alaje	Tekia	17	5	22
		Atsela	20	2	22
Both regions	ASARCs	Betmara	18	4	22
		Alamata-Sirinka	48	0	48
Total			356	88	444

Note: Kebeles (Singular Kebele) are small administrative units of Ethiopia, ASARCs: Alamata and Sirinka Agricultural Research Centers. N: number of respondents.

$$n = \frac{N}{1+N\,(e)^2}$$ (Yamane 1967)

Where, n: the number of required samples of respondents for the districts and ARCs (sample size); N: total farmers of the districts (population size) and Agricultural Experts; e: confidence level (0.05 (95%) level of precision. Accordingly, 396 farmers and 48 agricultural experts respectively with a total of 444 sample respondents were taken (Table 2).

Data analysis

Descriptive statistics, one way ANOVA, and Exhaustive CHAID growth method were used for socioeconomic data analysis, significance test of variance, and prediction of yield and yield related agronomic descriptors, respectively, based on agroecologies using SPSS v.20 computer software to evaluate farmers' knowledge on Abyssinian pea.

RESULTS AND DISCUSSION

Socioeconomic features of respondents

The impact of the different population sectors to the crop diversity, cultivation and production varies among the communal and agro-ecological areas. A great variation was observed on sex of respondents among districts and kebeles; in some of the areas, females take no part. This is particularly observed in Tigray region,Ofla district where 59 (13.3%) of the respondents were men and Korem-suburb kebele where 22 (100%) of them were men. Women living in Amhara region, Gubalfto and Woldia districts are equally concerned as men (Table 2). Therefore, the current study showed gender gap persistence in the crop diversity, cultivation and production knowledge similar to the case stated by Kahn et al. (2000) in the US states where women gaps remain in society's understand of the relation between income inequality, health and agriculture. Similar idea was stated by World Bank 2015 where hitherto women farmers are consistently found to be less productive than male farmers. The study is also consistent with the indication of Blau and Kahn (2000) declaring women as a group tend to work fewer weeks per year and hours per week than men. But this contradictes with the traditional property rights of gender-crop roles within rural societies (FAO 2012). Health wise, this contributes to the nutrition related diseases that affect pregnant and lactating women as stated by FAO (2015).

Most of the respondents were within the age groups of 44-56, 137 (30.8%) of the total participants followed by 31-43, 97 (22%). The knowledge about the crop is less in the potential young respondents (age group 18-30) though the crop is said worth marketable, provide job seeking for trading and income source for local exchange. The informant household family size was 4-5, 145 (32.7%) similar to average size of households by region of an atlas series of Ethiopia (CSA 2013). About 200 (45%) of them cannot read and write and about 64 (14.4%) have education below high school (Table 3). Hence, the production of the

crop is still through traditional means with little literacy and numeracy skills. This is alike the finding of Save the Children (2013).

Survey of indigenous knowledge on Abyssinian pea and factors affecting it

One way ANOVA between groups was used (Tables 4 and 6) to explore the knowledge of respondents based on districts of different agro-ecologies (Table 1) . The results of the one-way between groups analysis of variance with post-hoc tests, example for familiarity to the crop are presented as [F (2, 424): 2.2, p: .002]. Values are presented in percentages and leading questions are nominated as descriptors. Familiarity with the crop, duration, areas of distribution, and reasons for irregular occurrence and less knowledge about the crop and the likes were evaluated. There was a statistically significant difference (at the $p<0.05$) among districts and agro-ecologies for the descriptors [F (2, 424): 2.2, p: .002; F (2, 424): 1.8, p: .019; F (2, 424): 4.8, p: .000; F (2, 424): 2.4, p: . 001, F (2, 424): 1.8, p: .026; F (2, 424): 3.0, p: .000; F (2, 424): 3.4, p: .000; F (2, 424): 1.46, p: 0.054; and F (2,424): 2.7, P: 0.029], respectively.

Respondents from highland areas of both regions knew Abyssinian pea very well even their cultivation practice is not much as such as their familiarity. Possibly due to less cultivation practice in the agro-ecologies, there are some respondents who do not know the crop in the midland and lowland areas of Amhara and lowlands of Tigray regions, respectively. Descriptors for duration of knowledge about Abyssinian pea from the two regions are presented in Table 5. Most of the respondents knew the crop for the last thirty years ago actually even Abyssinian pea is a primitive landrace that displays traits usually associated with initial steps in the domestication process (Weeden 2007). It has domesticated some 4000-5000 years ago in the now Northern highlands of Ethiopia. Edwards et al. (2007) also describe Abyssinian pea as one among the crops with high genetic diversity in Ethiopia because of its origin. The crop cultivation practice has a long history in Tigray region study sites, where farmers had growing for the last thirteen years ago across all agro-ecological areas. This indicates that, familiarity is with the oldest age in Tigray region.

Table 3. Respondents' age group, family size, marital status, and educational background

Age group of respondents	Name of districts and Agricultural Institutes							
	Gubalafto	Habru	Ofla	Woldia	Endamehoni	Amba-alaje	ARARCs	Total
18-30	12 (2.7)	17 (3.8)	15 (3.4)	11 (2.5)	5 (1.1)	8 (1.8)	8 (1.8)	76 (17.1)
31-43	10 (2.3)	21 (4.7)	11 (2.5)	11 (2.5)	20 (4.5)	18 (4.1)	6 (1.4)	97 (22.0)
44-56	20 (4.5)	15 (3.4)	20 (4.5)	17 (3.8)	18 (4.1)	25 (5.6)	22 (4.9)	137 (30.8)
57-69	6 (3.6)	8 (1.8)	13 (2.9)	15 (3.4)	13 (2.9)	6 (1.4)	6 (1.4)	77 (17.3)
70 and >70	8 (1.8)	5 (1.1)	7 (1.6)	12 (2.7)	10 (2.3)	9 (2.0)	6 (1.4)	57 (12.8)
Total	66 *	66*	66*	66*	66*	66*	48**	444 (100)
Family size								
1	2 (0.5)	1 (0.2)	4 (0.9)	0 (0)	0 (0)	0 (0)	2 (0.5)	9 (2.0)
2-3	31 (6.9)	19 (4.3)	16 (3.6)	16 (3.6)	21 (4.7)	12 (2.7)	14 (3.2)	129 (29.0)
4-5	15 (3.4)	30 (6.7)	19 (4.3)	25 (5.6)	19 (4.3)	23 (5.2)	14 (3.2)	145 (32.7)
6-7	16 (3.6)	9 (2.0)	17 (3.8)	18 (4.1)	18 (4.1)	22 (4.9)	0 (0)	100 (22.5)
8-9	2 (0.5)	6 (1.4)	9 (2.0)	5 (1.1)	8 (1.8)	4 (0.9)	16 (3.6)	50 (11.3)
>9	0 (0)	1 (0.2)	1 (0.2)	2 (0.5)	1 (0.2)	4 (0.9)	0 (0)	11 (2.5)
Total	66*	66*	66*	66*	66*	66*	48**	444 (100)
Marital status								
Married	51 (11.4)	54 (12.2)	52 (11.7)	60 (13.5)	60 (13.5)	46 (10.4)	38 (8.6)	361 (81.3)
Single	6 (1.4)	3 (0.6)	4 (0.9)	3 (0.6)	4 (0.9)	18 (4.1)	8 (1.8)	46 (10.4)
Divorced	6 (1.4)	9 (2.0)	4 (0.9)	3 (0.6)	1 (0.2)	2 (0.5)	2 (0.5)	27 (6.1)
Widowed	3 (0.6)	0 (0)	4 (0.9)	0 (0)	1 (0.2)	0 (0)	0 (0)	8 (1.8)
Others	0 (0)	0 (0)	2 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	2 (0.5)
Total	66*	66*	66*	66*	66*	66*	48**	444 (100)
Education background of the respondents								
Cannot read and write	32 (7.2)	33 (7.4)	25 (5.6)	35 (7.8)	43 (9.7)	32 (7.2)	0 (0)	200 (45.0)
Read and write (1-4)	12 (2.7)	10 (2.3)	11 (2.4)	15 (3.4)	1 (0.2)	15 (3.4)	0 (0)	64 (14.4)
Elementary School (5-8)	6 (1.4)	9 (2.0)	4 (0.9)	7 (1.6)	11 (2.4)	9 (2.0)	0 (0)	46 (10.3)
Secondary School (9-10)	2 (0.5)	1 (0.2)	6 (1.4)	2 (0.5)	0 (0)	1 (0.2)	0 (0)	12 (2.7)
Certificate and above	8 (1.8)	8 (1.8)	13 (2.9)	7 (1.6)	7 (1.6)	7 (1.6)	48 (10.8)	98 (22.1)
Religious and adult	6 (1.4)	5 (1.1)	7 (1.6)	0 (0)	2 (0.5)	2 (0.5)	0 (0)	22 (5.0)
Science and religious	0 (0)	0 (0)	(0)	(0)	2 (0.5)	0 (0)	0 (0)	2 (0.5)
Total	66*	66*	66*	66*	66*	66*	48**	444 (100)

Note: the values within parentheses are percentages. *Indicates the percentage (14.9%) of the total sixty six respondents per district. ** Indicates the percentage (10.8%) of the total forty eight agricultural experts from two ASARCs.

Table 4. ANOVA for knowledge about current distribution and reasons for limitation of the crop

Descriptors	Source of variation	Sum of squares	df	Mean square	F	Sig.
Knowledge about Abyssinian pea	Between Groups	1.393	19	.073	2.216	.002
	Within Groups	14.030	424	.033		
	Total	15.423	443			
Duration of familiarity with Abyssinian pea	Between Groups	91.023	19	4.791	1.815	.019
	Within Groups	1119.417	424	2.640		
	Total	1210.439	443			
Abyssinian pea availability in the study districts	Between Groups	135.709	19	7.143	4.772	.000
	Within Groups	634.568	424	1.497		
	Total	770.277	443			
Reasons for Abyssinian pea limitation in sporadic areas	Between Groups	157.372	19	8.283	2.399	.001
	Within Groups	1463.727	424	3.452		
	Total	1621.099	443			
Reasons for Abyssinian pea's less known	Between Groups	159.684	19	8.404	1.750	.026
	Within Groups	2036.091	424	4.802		
	Total	2195.775	443			
Production status of Abyssinian pea	Between Groups	4.707	19	.248	2.983	.000
	Within Groups	35.212	424	.083		
	Total	39.919	443			
If the production of Abyssinian pea decreasing, reasons for reduction	Between Groups	551.756	19	29.040	3.368	.000
	Within Groups	3656.053	424	8.623		
	Total	4207.809	443			
Intervention by agricultural extensions	Between Groups	1.696	19	.089	1.595	.054
	Within Groups	23.727	424	.056		
	Total	25.423	443			
Type/s of intervention	Between Groups	16.860	19	.887	1.731	.029
	Within Groups	217.417	424	.513		
	Total	234.277	443			

Respondents from highland areas of both regions recognized that Abyssinian pea is currently available in about four districts of their neighborhoods. In the midland and lowland agro-ecologies, the crop availability is limited to three districts of the two regions. This showed the sporadic distribution of the crop comparable to the claims of Yemane and Skjelvåg (2003) and Mikić et al. (2013). The main reason for limitation in distribution of the crop is its agroecology preference particularly in the highlands that is not restructured with food system of Ethiopia. This finding matches with the works by Wart et al. (2013) and Gliessman (2016). Lack of intervention for expansion and preference of high yielding other pea varieties in Amhara region districts and the soil type and moisture requirements in the midland and lowland areas of Tigray districts affects the crops distribution. The crops' inferiority in yield and pest susceptibility before people consume it is the main reason for less consideration of farmers about the crop cultivation in all agro-ecologies of Tigray region. Cultural bias against peasant crops and lack of expansion by extension experts to other areas is the reason participants said the crop got least attention in reverse of its importance in the highland and midland areas of Amhara region. Similar to the idea of National Research Council (2008) stating cultural bias against peasant crops is an ultimate calamity because plants that poor people grow are the very type well-suited to feeding the hungriest and most vulnerable sections of society. Now it is grown ordinarily as solitary planting. The crop production result showed that the crop is intensely decreasing and becoming rare. The main cause for reduction at small farmers scale of the crop is expensive price to buy the seed and small land holding of farmers. According to this result, there is no noticeable intervention started (Table 5).

Farmers' agronomic descriptors and use value knowledge on Abyssinian pea

A one-way ANOVA was conducted to explore the knowledge on agronomic performances and use values of Abyssinian pea. There was a statistically significant difference (at $p < 0.05$) for the descriptors across the agro-ecologies as shown in Table 6 [F (2, 424): 2.3, p: .002; F (2, 424): 3.9, p: .000; F (2, 424): 2.0, p: .007; F (2, 424): 11.7, p: .000; F (2, 424): 3.4, p: .000; F (2, 424): 3.3, p: .000; F (2, 424): 10.1, p: .000; F (2, 424): 5.0, p: .000; F (2, 424): 6.0, p: .000; F (2, 424): 5.1, p: .000; F (2, 424): 1.8, p: .025; and F (2, 424): 2.2, p: .003; F (2, 424): 5.0, p: .000; F (2, 424): 6.0, p: .000; F (2, 424): 5.1, p: .000; F (2, 424): 1.8, p: .025; F (2, 424): 2.2, p: .003; F (2, 424): 3.0, p: .000; and F (2, 424): 3.4, p: .000], respectively.

Table 5. Knowledge about current distribution and reasons for limitation of Abyssinian pea in the study area

Descriptors	Tigray			Amhara		
	Highland (N: 74)	Midland (N: 82)	Lowland (N: 66)	Highland (N: 66)	Midland (N: 90)	Lowland (N: 66)
Do know Abyssinian pea?						
Yes	100.0	100.0	92.7	100.0	91.1	97.0
No	0.0	0.0	7.3	0.0	8.9	3.0
%within districts	100	100	100	100	100	100
How long do you know the crop (Duration in years)						
<10yrs ago	2.7	0.0	2.4	0.0	0.0	3.0
10yrs ago	20.3	13.6	9.8	21.2	16.7	12.1
20 yrs ago	16.2	30.3	18.3	16.7	26.7	15.2
30 yrs ago	31.1	40.9	52.4	16.7	26.7	21.2
40 yrs ago	14.9	9.1	12.2	6.1	6.7	13.6
50 yrs ago	9.5	1.5	1.2	15.2	11.1	13.6
60 yrs ago	5.4	4.5	2.4	18.2	7.8	15.2
≥60 yrs ago	0.0	0.0	0.0	6.1	0.0	6.1
I don't know	0.0	0.0	1.0	0.0	4.0	0.0
%within districts	100	100	100	100	100	100
In how many of the the study districts do you know Abyssinian pea availability currently (Distribution knowledge)						
I don't know	0.0	0.0	1.2	0.0	4.0	16.2
1-2 districts	4.5	19.7	7.3	34.8	10.0	19.7
3 districts	14.9	27.3	29.3	25.8	34.4	33.3
4 districts	31.1	22.7	30.5	19.7	17.8	28.8
5 districts	25.7	18.2	15.9	10.6	11.1	12.1
≥6 districts	23.0	12.1	15.9	9.1	22.2	6.1
%within districts	100	100	100	100	100	100
Reasons for irregular pattern in distribution of the crop						
G1	8.1	3.0	2.4	6.1	8.9	4.5
G2	9.5	0.0	4.9	9.1	8.9	12.1
G3	0.0	0.0	7.3	6.1	10.0	15.2
G4	6.8	24.2	25.6	6.1	7.8	13.6
G5	10.8	9.1	12.2	10.6	20.0	21.2
G6	56.8	53.0	39.0	48.5	37.8	22.7
G7	0.0	3.0	0.0	3.0	0.0	0.0
G8	2.7	0.0	3.7	6.1	0.0	10.6
G9	4.7	7.6	3.7	4.5	2.2	0.0
G10	0.0	0.0	1.2	0.0	4.4	0.0
%within districts	100	100	100	100	100	100
Reasons for why the crop got slight attention by participants						
G1*	1.4	6.1	11.0	6.1	13.3	25.8
G2*	2.7	0.0	0.0	0.0	2.2	2.7
G3*	27.8	36.4	23.2	42.4	27.8	19.7
G4*	60.8	43.9	45.1	28.8	18.9	22.7
G5*	0.0	0.0	0.0	3.0	0.0	0.0
G6*	0.0	0.0	1.5	0.0	0.0	1.2
G7*	0.0	4.5	2.4	6.1	7.8	1.5
G8*	20.3	9.1	13.4	13.6	23.3	27.3
G9*	0.0	0.0	3.7	0.0	6.7	1.5
%within districts	100	100	100	100	100	100

Note: G1: soil type requirement, G2: moisture requirement, G3: cultural bias against peasant crops, G4: both soil type and moisture requirements, G5: lack of intervention for the crop expansion and preference of other high yielding varieties, G6: Agro-ecological preferences, G7: combined requirement of soil type, sunlight and moisture, G8: small landholding of farmers and climatic condition susceptibility, G9: susceptibility to pest and birds of the crop, G10: I don't know the reason. G1*: inferiority of this displaced crop by new pea varieties, G2*: misclassification of the crop, G3*: cultural bias against peasant crops and lack of expansion by extension experts to other areas of the crop, G4*: inferiority in yield and pest susceptibility of the crop, G5*: cost effectiveness of the crop, G6*: disappearing in the writing of travellers for scientific communication of the crop, G7*: both cultural bias and inferiority in yield, G8*: Agro-ecological requirements hindering further adaptation of the crop, and G9*: if other specify. N: Number of respondents

Majority of the respondents (of both regions) in the present study cultivate Abyssinian pea keeping the seasonal rainfall. Farmers from Gubalafto highland areas grow Abyssinian pea completely depending on the long rainy season. However, in Tigray region particularly in the lowlands of Endamehoni and midland areas of Amba-Alaje there are irrigation based Abyssinian pea cultivation starting's. Abyssinian pea thrives better in Nitisols in all agro-ecologies in general and in Leptosols in the highland and midland areas of Tigray region in particular, respectively. Sowing time was evaluated to understand the growing seasons and to improve the crop harvest as growing seasons define geographical areas suitable for crops (HarvestChoice 2010). Majority of the respondents in lowland, midland and highland areas of Amhara sow Abyssinian pea the in March and May months (during belg season) after other crops like teff are harvested followed by the long rainy season. Farmers in the Ofla highland areas sow the crop starting from July the half up to August the first with a single plough. The sowing time of the crop in the midland and lowland areas of Tigray is both during belg season and the long rainy season, although very few respondents experienced different sowing times (Table 7).

Respondents were also enquired the seeding rate for Abyssinian pea they used during sowing. Majority of the respondents use a seeding rate of 21-30 kg ha^{-1} even it varies enormously within the agro-ecologies. The variation may be due to planting date, soil type, relative humidity, temperature and the like factors the agro-ecologies possess. Still the seeding rate of the crop is below the seeding rate stated by Winch (2006) for early variety, with small seed that is planted in good time on infertile soil in a dry region may need a seeding rate of about 50 kg ha^{-1}. The crop start flowering one month after planting but florescence best in one month and fifteen days after planting in all the agro-ecologies (Table 7) viewing its early flowering phenotypic traits. This may be probably due to the crop ELF3 gene, a key prehistoric adaptation to shorter growing seasons stated by Rubenach et al. (2017) for some pea varieties. According to the respondents data the crop matures at $2^{1/2}$ months in average. The maximum yield farmers obtain from the 21-30 kg ha^{-1} is 300 kg ha^{-1} that can be enhanced to 400 kg ha^{-1} at the seeding rate of 41-50 kg ha^{-1} (Figure 10).

Table 6. ANOVA for agronomic performances descriptors of Abyssinian pea

Agronomic descriptors	Source of variation	Sum of squares	df	Mean Square	F	Sig.
Abyssinian pea dependency on rain or using other means	Between Groups	6.858	19	.361	2.294	.002
	Within Groups	66.727	424	.157		
	Total	73.586	443			
Abyssinian pea soil preference	Between Groups	345.896	19	18.205	3.900	.000
	Within Groups	1979.402	424	4.668		
	Total	2325.297	443			
Habit of using Abyssinian pea in intercropping and crop rotation	Between Groups	3.488	19	.184	2.023	.007
	Within Groups	38.485	424	.091		
	Total	41.973	443			
Crops intercropped or rotated with Abyssinian pea	Between Groups	807.477	19	42.499	11.650	.000
	Within Groups	1546.758	424	3.648		
	Total	2354.234	443			
Abyssinian pea sowing time (seasons)	Between Groups	241.356	19	12.703	7.404	.000
	Within Groups	727.455	424	1.716		
	Total	968.811	443			
Abyssinian pea's land ploughing number including the final sowing time	Between Groups	23.634	19	1.244	3.307	.000
	Within Groups	159.508	424	.376		
	Total	183.142	443			
Abyssinian pea rainfall requirement starting from sowing to maturity	Between Groups	52.100	19	2.742	10.060	.000
	Within Groups	115.576	424	.273		
	Total	167.676	443			
Seeding rate (Kg/ha)	Between Groups	128.927	19	6.786	4.943	.000
	Within Groups	582.053	424	1.373		
	Total	710.980	443			
Abyssinian pea flowering time (Number of months)	Between Groups	170.863	19	8.993	5.984	.000
	Within Groups	637.144	424	1.503		
	Total	808.007	443			
Time of maturity (months)	Between Groups	70.312	19	3.701	5.138	.000
	Within Groups	305.364	424	.720		
	Total	375.676	443			
Abyssinian pea yield in (100 Kg/ha)	Between Groups	53.996	19	2.842	1.763	.025
	Within Groups	683.644	424	1.612		
	Total	737.640	443			
Abyssinian pea conservation practice started	Between Groups	1.039	19	.055	2.181	.003
	Within Groups	10.636	424	.025		
	Total	11.676	443			
Abyssinian pea use related traits	Between Groups	105.837	19	5.570	2.764	.000
	Within Groups	854.386	424	2.015		
	Total	960.223	443			
Abyssinian pea part/s used for forage	Between Groups	505.395	19	26.600	25.353	000
	Within Groups	444.848	424	1.049		
	Total	950.243	443			
Animals Abyssinian pea straw preference compared to other peas	Between Groups	144.698	19	7.616	7.624	000
	Within Groups	423.545	424	.999		
	Total	568.243	443			
Browser animals prefer Abyssinian pea straw better	Between Groups	709.775	19	37.357	11.234	000
	Within Groups	1409.871	424	3.325		
	Total	2119.646	443			
Abyssinian pea medicinal values	Between Groups	71.770	19	3.777	7.991	000
	Within Groups	200.417	424	.473		
	Total	272.187	443			
Parts used for disease cure	Between Groups	693.293	19	36.489	6.100	000
	Within Groups	2536.455	424	5.982		
	Total	3229.748	443			
Rate of Abyssinian pea usability	Between Groups	64.973	19	3.420	7.436	000
	Within Groups	195.000	424	.460		
	Total	259.973	443			
Way of using Abyssinian pea	Between Groups	39.873	19	2.099	2.843	000
	Within Groups	312.962	424	.738		
	Total	352.836	443			
Abyssinian pea storage mechanism	Between Groups	241.724	19	12.722	5.559	000
	Within Groups	970.303	424	2.288		
	Total	1212.027	443			

Table 7. Knowledge about agronomic performances descriptors of Abyssinian pea

Descriptors	Tigray			Amhara		
	Highland (N: 74)	Midland (N: 82)	Lowland (N: 66)	Highland (N: 66)	Midland (N: 90)	Lowland (N: 66)
Is cultivation of Abyssinian pea dependent on seasonal rain?						
Yes	79.7	80.3	79.3	100	85.6	97.0
No	17.6	19.7	19.5	0.0	8.9	3.0
I don't know	2.7	0.0	1.2	0.0	5.6	0.0
%within districts	100	100	100	100	100	100
Soil type preference of Abyssinian pea						
Vertisol	18.9	19.7	14.6	4.5	21.1	9.1
Nitisols	25.7	36.4	36.6	65.2	32.2	42.4
Lithic leptosols	5.4	0.0	4.9	0.0	2.2	10.6
Cambisols	8.1	1.5	4.9	1.5	2.2	3.0
Leptosols	31.1	40.9	36.6	16.7	25.6	25.8
Regosols	5.4	1.5	1.2	0.0	13.3	4.5
all soil types	5.4	0.0	1.2	12.1	0.0	4.5
I don't know	0.0	0.0	0.0	0.0	3.3	0.0
%within districts	100	100	100	100	100	100
Sowing time of Abyssinian pea						
Long rainy season (June-August)	28.4	28.8	12.2	40.9	30.0	25.8
After crops harvest (belg) e.g Teff	16.2	0.0	9.8	54.5	37.8	57.6
Both during belg and June-August	2.7	48.5	58.5	0.0	15.6	13.6
Late (July half-first August)	52.7	22.7	18.3	4.5	12.2	3.0
If other specify	0.0	0.0	1.2	0.0	4.4	0.0
%within districts	100	100	100	100	100	100
The numbers of plough of land for Abyssinian pea cultivation						
I don't know	0.0	0.0	1.1	0.0	4.4	0.0
One time	70.3	72.7	59.8	65.1	70.0	63.6
Two times	25.7	21.2	29.3	25.8	15.6	30.3
Three to four times	4.1	6.1	9.8	9.1	10.0	6.1
%within districts	100	100	100	100	100	100
Seeding rate respondents experiencing						
10-15 kg ha^{-1}	9.5	25.8	3.7	3.0	3.3	0.0
15-20 kg ha^{-1}	12.2	3.0	17.1	9.1	13.3	6.1
21-30 kg ha^{-1}	41.9	19.7	39.0	40.9	33.3	40.9
31-40 kg ha^{-1}	25.7	18.2	13.4	37.9	21.1	28.8
41-50 kg ha^{-1}	10.8	25.8	23.2	9.1	21.1	19.7
60-70 kg ha^{-1}	0.0	7.6	2.4	0.0	3.3	1.5
I don't know	0.0	0.0	1.2	0.0	4.4	3.0
%within districts	100	100	100	100	100	100
The time required for flowering						
1 month	24.3	24.2	11.0	0.0	4.4	4.5
1 month & 15 days	59.5	56.1	52.4	83.3	73.3	45.5
2 months&15 days	2.7	7.6	20.7	0.0	7.8	18.2
2 months	13.5	6.1	11.0	15.2	10.0	25.8
40 days	0.0	6.1	3.7	1.5	0.0	6.1
If other specify	0.0	0.0	1.2	0.0	4.4	0.0
% within districts	100	100	100	100	100	100
Abyssinian pea maturity time						
2 months	23.0	21.2	20.7	48.5	22.2	51.5
2 months&15 days	31.5	56.1	34.1	48.5	57.8	27.3
3 months	39.2	19.7	39.0	3.0	13.3	21.2
3months &15 days	0.0	3.0	4.9	0.0	0.0	0.0
≤49 days	2.7	0.0	0.0	0.0	2.2	0.0
If other specify	0.0	0.0	1.2	0.0	4.4	0.0
%within districts	100	100	100	100	100	100
Yield in (100 kg ha^{-1})						
200 kg ha^{-1}	16.2	6.1	8.5	9.1	7.8	3.0
300 kg ha^{-1}	43.2	48.5	41.5	45.5	37.8	47.0
400 kg ha^{-1}	25.7	27.3	29.3	30.3	35.6	31.8
500 kg ha^{-1}	12.2	18.2	15.9	12.1	17.8	12.1
600 kg ha^{-1}	2.7	0.0	3.7	3.0	0.0	6.1
If other specify	0.0	0.0	1.2	0.0	0.0	0.0
I don't know	0.0	0.0	0.0	0.0	1.1	0.0
%within districts	100	100	100	100	100	100

Figure 2. Seed size, color (A-F), and resistance (D-F) to pea weevil (*Bruchus Pisorum* L.) differences of Abyssinian pea landraces where creamy seeds are bored more by pea weevil larvae feed on the seed during storage (F)

The price of Abyssinian pea (53 Ethiopian birr per kilogram) was more than twice better than the price of common pea (24 birr) during the market survey time in all the agro-ecologies nearby markets analogous to the assertion by Yemane and Skjelvåg (2003). Besides, delicious tastes followed by expensive seed price are the best use value related descriptors that come to the mind of Ethiopian farmers when asked about Abyssinian pea landraces. Beyond the forage value for donkeys and horses > cattle > sheep and goats), respectively (Table S1), 90.9% of the interviewed farmers from Tahtay-haya (Figure 1 and Table 2), the lowland area from Endamehoni districts claimed the medicinal value of the crop straw as the best cure of their animals' neck wounding by bat during the rainy season. It is also given as food for patients prescribed not eating some foods because of stomach ulcer in the highland areas of Tigray and all the agro-ecologies of Amhara region. The crop is currently fairly usable and mainly for earning income for local exchange of other staple crops, the very type well-suited to feeding the hungriest vulnerable farmers as it grows earlier. From the different storage mechanisms, the most common respondents practicing is dressing seeds using chemicals particularly of malathion after harvest and before storage (Table S2) in all the agro-ecologies to protect from pea weevil (Figure 2.D and 2.F). During consumption the chemically dressed seeds are washed very well.

Morphological and physiological variations among the Abyssinian pea landraces

Differences in seed's size, seed coat color and resistance to the pea seed weevil of the crop farmers' landraces were observed during the study time (Figure 2). This could be due to the variance in their adaptation and interaction to different environments. This is similar to the finding of Teshome (2015) on pea genotypes. Studies from Elzebroek and Wind (2008) and Pavek (2012) confirm the difference used as selection criteria of the various types of peas available by breeders. Pea seeds difference in resistance to pea weevil larvae boring is due to difference in color (Figure 2.F; Teshome 2015**)** and nutritional content (Winch 2006).

The Abyssinian pea (English) is locally named Dekoko (Tigrigna), Agerie Ater (Amharic) correspondingly. The English and Amharic terms describe its origin and the Tigrigna term defines its small seeds.The first ranked morphological descriptors most farmers used for selection of Abyssinian pea from other local peas were earliness > grain coverage (germination rate) > seedling vigor > leaf greenness > plant height (short and vigorous) > pods per plant > branches per plant > seeds per pod (Figure 3).

Abyssinian pea yield and yield related traits prediction using Exhaustive CHAID growth method

The respondents from each district define each descriptor according to their knowledge. Therefore, the independent (predictor) variable that has the strongest interaction with the dependent variable for each agroecology should better be chosen using Exhaustive CHAID growing method. Exhaustive CHAID examines all possible splits for each predictor by merging categories of each predictor if they are not significantly different with respect to the dependent variable. The green ones in the tree diagrams (Figures 4, 6, 7, 8, 9 and 10) indicate the predicted categories having strongest interaction with the descriptors determining the yield and yield related factors of the crop.

Prediction 1. Soil type preference of the crop

Abyssinian pea thrives better in Nitisol soil followed by Leptosol and Vertisol soils (Figure 4), respectively. Farmers from Amba-Alaje and Endamehoni areas grow the crop in Leptosols. The existence of more than half of all Nitisols in tropical Africa; in the highlands (>1000 m asl.) and 12.5% of the Ethiopia highlands where field pea is dominant pulse (IUSS Working Group WRB 2015; Keneni et al. 2013) give an impression of the crop adaption for this soil. Abyssinian pea has excellent root protuberance in Nitisol soils (Figure 5.A). Farmers traditionally conserve soil using different mechanism. A new and unfamiliar soil conservation practices using USAID white sacks (Figure 5.B-D) were observed during the study time.

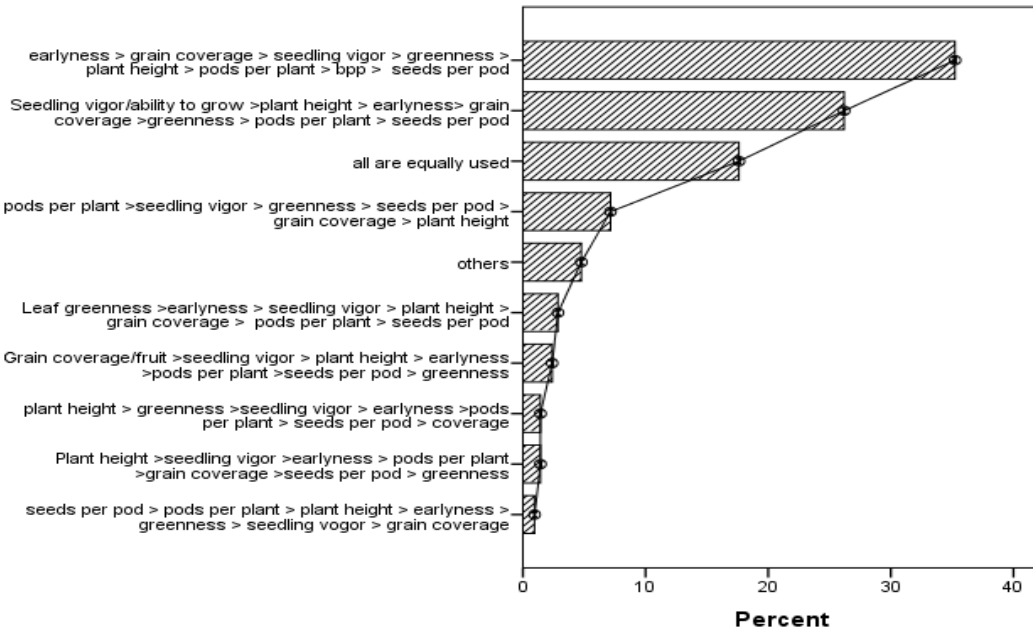

Figure 3. Farmer's morphological selection criteria of Abyssinian pea from other local peas. bpp: branches per plant

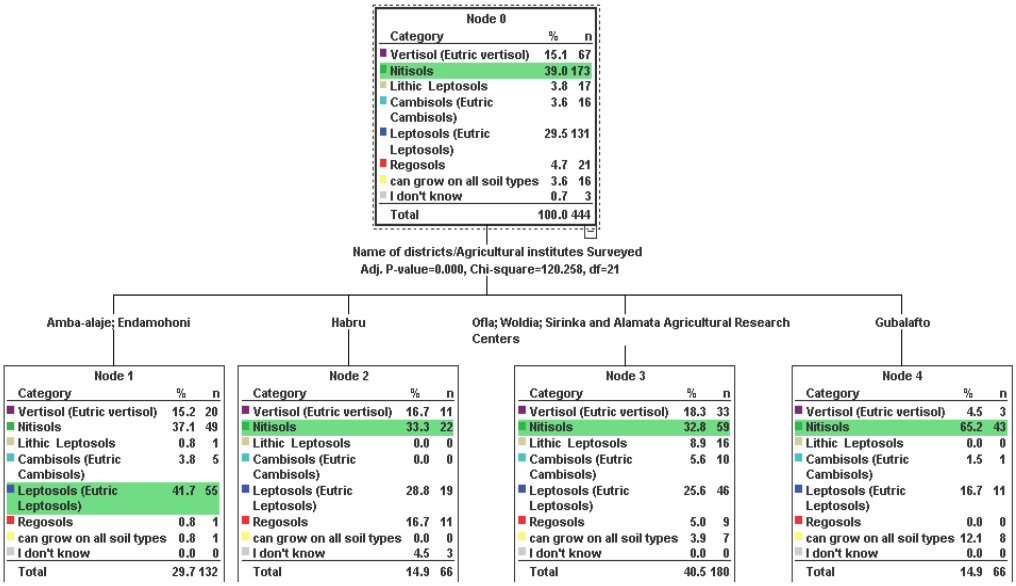

Figure 4. Tree diagram of Abyssinian pea soil type preference (Risk estimate: 0.597, SE: 0.023)

Figure 5. A. The direct physical effect of the soil type on the Abyssinian pea excellent root protuberance efficieny grown in Nitisol soil in Gedober kebele, North Wollo (Photo by BG, October 2017); B-D. Traditional soil conservation practices using USAID white sacks in Mekan kebele, Endamehoni district, South Tigray, where (B). Sunken white sack for soil conservation, (C). Side view of the sunken white sack, and (D). Soil protecting ability of white sack (Photo by BG. March 2017)

Prediction 2. Rainfall requirement of the crop

Abyssinian pea can perform good yield with about two good rain in all soil types and agroecologies (Figure 6). This is mostly common to highland areas that have higher humidity because peas have the ability to benefit from some rain at flowering and seed set with 70% relative humidity (Winch 2006).

Prediction 3. Seeding rate of the crop

Results from the tree diagram showed respondents knowledge as significant predictor of Abyssinian pea seeding rate. The overall category showed that, the common seeding rate (kg ha^{-1}) is 21-30 kg ha^{-1}, followed by 31-40 kg $ha^{-1}$and 41-50 kg ha^{-1}, respectively. The overall significant predictor does not represent all districts as blanket, because they have different agro-ecologies like altitude (Table 1 and Hurni 1998). In Ethiopia the best altitudes for pea ranges 1500-2200 m asl. with rainfall less than 600 mm, and 2200-2300 m asl. with rainfall more than 600 mm per year (Winch 2006). Respondents from Amba-Alaje midland district and ASARCs experience a seeding rate of 41-50 kg ha^{-1}. Of the districts in this category, 36% articulated this seeding rate (Figure 7), lesser than the typical seeding rate for smaller seeded pea varieties (Winch 2006).

The forecaster seeding rate for Endamehoni and Woldia lowland areas, and Habru is 21-30 kg ha^{-1}, where 86% of the respondents shared. Moreover, the seeding rate for the highland areas of Ofla and Gubalafto is 21-30 kg ha^{-1}, followed by 31-40 kg ha^{-1} (Figure 7). This is comparable to Pavek (2012) who revealed seeding rates vary with cultivar, soil type, seed size, climate, disease pressure and seeding method for *Pisum sativum*.

Prediction 4. Flowering time of the crop

It is predicted that, Abyssinian pea require about one month and fifteen days for flowering (Figure 8). This is particularly common for farmers' landraces from Gubalafto where about 55 (83.3%), Habru and ASARCs about 85 (74.6%), Amba-Alaje and Ofla about 74 (56.1%), respectively. For Endamehoni and Woldia lowland agro-ecologies, this is not the significant predictor where about 61 (46.2%) of the total 132 informants approved. This is similar to the findings of Weeden (2007), Weller et al. (2012) and Rubenach et al. (2017) stating Abyssinian pea flower in short days.

Prediction 5. Maturity time of the crop

Abyssinian pea matures in about two months and fifteen days (Figure 9) earlier (Weller et al. 2012; Rubenach et al. 2017). Early maturing helps peas for better seed set during dry season (Winch 2006). This is similar to the growth period for green seed or pods, even there is variation depending on farmers' cultivar, climatic conditions, and planting date of the agro-ecologies (Winch 2006).

Prediction 6. Yield (100 kg ha^{-1}) of the Crop

Yield per hectare of Abyssinian pea was predicted. The overall category indicated, the common yield in kilograms per hectare (100 kg ha^{-1}) for Abyssinian pea is 300 kg ha^{-1} for most districts. Still the production is below the good average yield compared to green peas pods which is 6.5-7 MT ha^{-1} (Winch 2006). This could be because of the small seed rate. Better seed rates can yield up to about 400 kg ha^{-1} of Abyssinian pea (Figure 10). This seeks urgent intervention to fill yield gap (Ittersuma et al. 2013). Little productions were observed during the study period in some sporadic areas of Endamehoni lowlands, and Ofla and Gubalafto high land areas which are belg season productive districts (Hurni 1998; Abrha and Simhadri 2015).

In conclusion, the results showed that, Abyssinian pea productivity is socioeconomically influenced by gender, family size, age, education, small landholding, and expensive price for poor of the seed. Abyssinian pea production was observed to be higher by men and educated respondents. The knowledge on agronomic descriptors like soil preferences, rainfall requirement, seed rate, flowering time, maturity period and yield and affecting factors like the reasons for limitation of the crop on some sporadic areas varies across agro-ecologies. Morphological and physiological variations among the landraces' seeds were observed. Currently, the crop productivity is highly decreasing because of combination of expensive price to purchase for poor farmers and their small land holdings. Evaluated predictors like the crop rain fall requirement, soil type preference, seed rate, flowering and maturity time, and yield showed that the landraces adapted differently to the agro-ecologies. Therefore, improve farmers' educational status and awareness on agronomic descriptors would enhance the production of the crop.

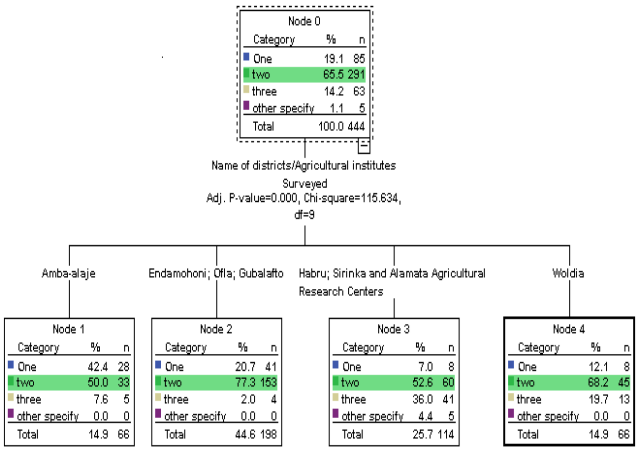

Figure 6. Abyssinian pea rainfall requirement created using the Exhaustive CHAID growth method (Risk estimate: 0.035, SE: 0.023).

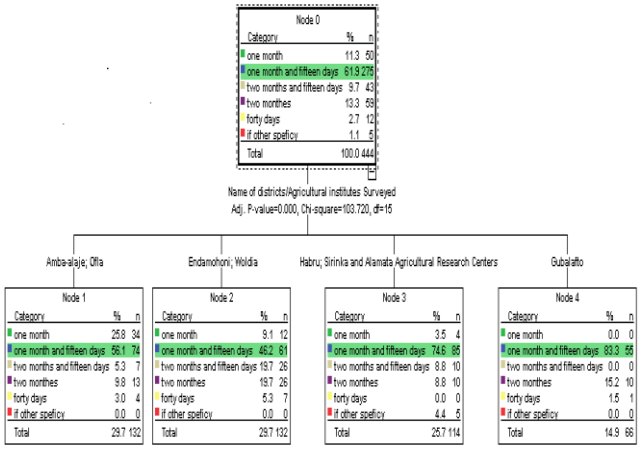

Figure 8. Abyssinian pea flowering time (Risk estimate: 0.060, SE: 0.003)

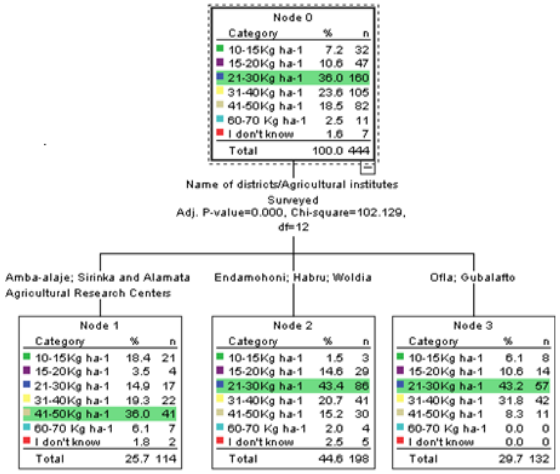

Figure 7. Abyssinian pea seeding rate (kg ha-1) with (Risk estimate: 0.059, SE: 0.002).

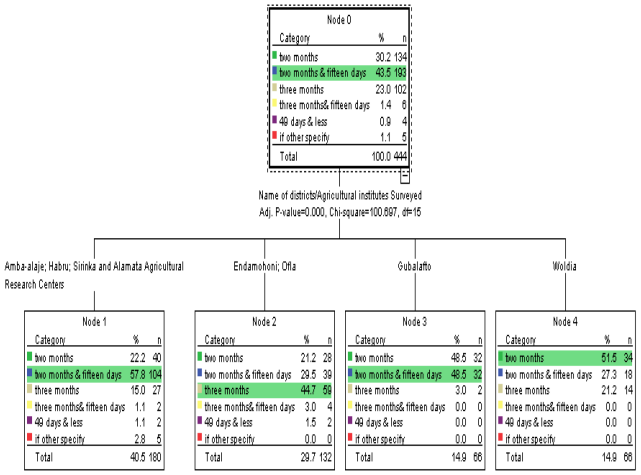

Figure 9. Abyssinian pea maturity time (Risk estimate: 0.060, SE: 0.005)

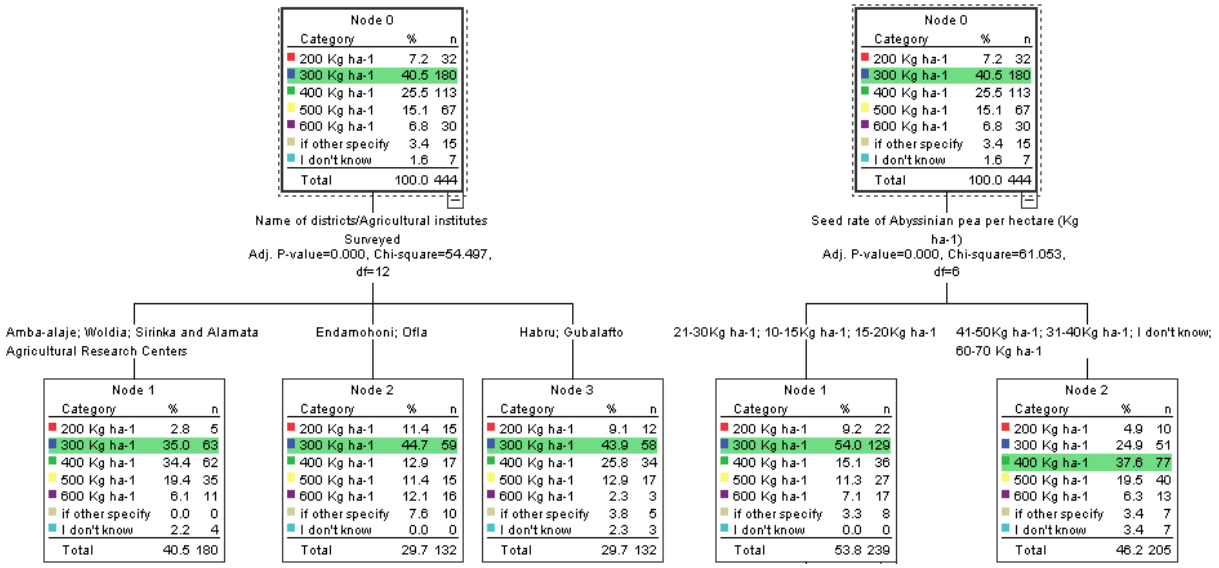

Figure 10. Abyssinian pea yield (kg ha^{-1}) as affected by agroecology (*left*) and seed rates (*right*)

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Table S1. Use and use related traits of Abyssinian pea

Descriptors	Tigray			Amhara		
	Highland (N: 74)	Midland (N: 82)	Lowland (N: 66)	Highland (N: 66)	Midland (N: 90)	Lowland (N: 66)
Market value of Abyssinian pea compared to white pea and common field pea						
Better	100.0	100.0	98.8	100.0	95.6	97.0
Less	0.0	0.0	0.0	0.0	0.0	0.0
I don't know	0.0	0.0	1.2	0.0	4.4	3.0
%within districts	100	100	100	100	100	100
Use related and unique traits of the crop respondents know						
G1UR	33.8	22.7	35.4	27.3	38.9	30.3
G2UR	16.2	15.2	26.8	16.7	17.8	15.2
G3UR	2.7	13.6	7.3	13.6	8.9	7.6
G4UR	39.2	43.9	26.8	39.4	27.8	42.4
G5UR	5.4	1.5	1.2	3.0	1.1	0.0
G6UR	2.7	1.5	1.2	0.0	2.2	4.5
Others	0.0	1.5	1.2	0.0	3.3	0.0
%within districts	100	100	100	100	100	100
Part/s of Abyssinian pea commonly utilized as source of food						
Only seed	18.9	22.7	26.8	28.8	58.9	65.2
Seed & mature	81.1	74.2	72.0	71.2	36.7	31.8
Only the matured	0.0	0.0	0.0	0.0	0.0	3.0
If other specify	0.0	3.0	1.2	0.0	4.4	0.0
%within districts	100	100	100	100	100	100
Part/s of Abyssinian pea commonly utilized as source of forage						
Mature>seed>straw	1.4	19.7	1.2	3.0	2.2	1.5
Straw > mature	1.4	51.5	34.1	9.1	21.1	6.1
Mature >straw	20.3	7.6	20.7	0.0	4.4	1.5
Only mature	58.1	12.1	12.2	54.5	28.9	39.7
Only straw	10.8	9.1	22.0	18.2	31.1	48.5
%within districts	100	100	100	100	100	100
Browser animals Abyssinian pea straw preferences rank						
G1BAP	0.0	0.0	2.0	0.0	0.0	0.0
G2BAP	8.1	0.0	0.0	0.0	0.0	0.0
G3BAP	50.0	24.2	37.8	37.9	33.3	21.2
G4BAP	0.0	60.6	22.0	0.0	12.2	12.2
G5BAP	0.0	0.0	1.2	3.0	1.1	7.6
G6BAP	0.0	0.0	0.0	6.1	0.0	0.0
G7BAP	23.0	7.6	13.4	27.3	13.3	47.0
G8BAP	18.9	1.5	13.4	25.8	17.8	10.6
%within districts	100	100	100	100	100	100

Note: G1UR: Seed expensive price > good taste > low yield, G2UR: good yield > Seed expensive price > good taste, G3UR: good biomass > seed expensive price >good taste, G4UR: good taste > seed expensive price > good biomass, G5UR: straw quality > medicinal value >good biomass, G6UR: earliness > expensive price > good taste >good yield. G1BAP: cattle's > sheep and goats > donkeys and horses, G2BAP: sheep and goats > cattle's > donkeys and horses, G3BAP: donkeys and horses > cattle's > sheep and goats, G4BAP: donkeys and horses > sheep and goats >cattle's, G5BAP: sheep and goats >donkeys and horses > cattle's, G6BAP: cattle's > donkeys and horses > sheep and goats, G7BAP: donkeys and horses > cattle, G8BAP: I don't know. N: number of respondents

Table S2. Current usability, medicinal value and storage mechanisms of Abyssinian pea

Descriptors	Tigray			Amhara		
	Highland (N: 74)	**Midland (N: 82)**	**Lowland (N: 66)**	**Highland (N: 66)**	**Midland (N: 90)**	**Lowland (N: 66)**
Current usability rate of Abyssinian pea by respondents						
Very usable	10.8	1.5	4.9	6.1	13.3	4.5
Most usable	4.1	4.5	12.2	1.5	20.0	15.2
Fairly usable	73.0	86.4	46.3	62.8	56.7	72.7
Unusable	12.2	7.6	35.4	24.4	5.6	7.6
I don't know	0.0	0.0	1.2	0.0	4.4	0.0
%within districts	100	100	100	100	100	100
For what purpose farmers are cultivating Abyssinian pea (Abyssinian pea ways of using)						
Consumption	18.9	34.4	37.8	16.7	34.4	16.7
Earning income	60.8	43.9	32.9	63.6	38.9	47.0
For both	12.2	9.1	8.5	18.2	20.0	31.8
Not growing	8.1	10.6	20.7	1.5	6.7	4.5
%within districts	100	100	100	100	100	100
Does Abyssinian pea have medicinal values?						
yes	70.3	28.8	53.7	68.2	58.9	57.6
No	21.6	23.2	60.6	30.3	27.8	33.3
I don't know	8.1	10.6	23.3	1.5	13.3	9.1
%within districts	100	100	100	100	100	100
Part/s used as medicinal values of Abyssinian pea and for what disease						
Seed/stomach ulcer	70.3	25.8	14.6	68.2	54.4	45.5
Straw/night bird	0.0	43.9	63.6	0.0	4.4	6.1
Leaf/michi	0.0	0.0	0.0	0.0	4.0	6.1
Others	0.0	2.3	0.0	6.0	7.8	13.6
I don't know	29.7	39.0	10.8	31.8	36.6	42.5
%within districts	100	100	100	100	100	100
Storage mechanisms farmers used for Abyssinian pea						
G1SMA	0.0	6.1	6.1	0.0	2.2	0.0
G2SMA	1.4	6.1	0.0	0.0	3.3	0.0
G3SMA	59.5	71.2	45.1	81.8	63.3	48.5
G4SMA	5.4	0.0	13.4	1.5	0.0	0.0
G5SMA	8.1	13.6	12.2	15.2	22.2	43.9
G6SMA	8.1	0.0	4.9	0.0	0.0	0.0
G7SMA	0.0	0.0	2.4	0.0	0.0	0.0
G8SMA	17.5	3.0	15.9	1.5	8.9	7.6
%within districts	100	100	100	100	100	100

Note: G1SMA: drying very well the storage material and storage material selection, G2SMA: changing of the storage materials per two weeks, G3SMA: dressing seeds prior to storage using chemicals such as malathion, G4SMA: by mixing with pest less susceptible crops like teff, G5SMA: dressing chemicals for weevils and using traps for rodents, G7SMA: Both drying well the storage materials and dressing seeds using chemicals, G8SMA: use trap and cat for rodents. N: number of respondents

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Short Communication: Decreased populations of *Scutellaria discolor* and *Plectranthus galeatus* (Lamiaceae) on Mount Gede, West Java, Indonesian and its surrounding

SUDARMONO
Centre for Plant Conservation Botanic Gardens (Bogor Botanic Gardens), Indonesian Institute of Sciences. Jl. Ir. H. Juanda No. 13 Bogor 16122, West Java, indonesia. Tel./fax.: +62-251-8322-187, ♥email: s_darmono@yahoo.com

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Abstract. *Sudarmono. 2018. Decreased populations of* Scutellaria discolor *and* Plectranthus galeatus *(Lamiaceae) on Mount Gede, West Java, Indonesian and its surrounding. Biodiversitas 19: 1866-1870. Scutellaria discolor* Colebr. and *Plectranthus galeatus* Vahl. are herbs belonging to mint family (Lamiaceae) which have the potential to be used for medicinal purposes. However, their population is gradually declining. The population of the two species are now only found in the mountains or highlands or the edges of the forests that are still protected, i.e. Mount Gede and Telaga Warna protected forest. This research aimed to know the condition of parent and seedling population of *Scutellaria discolor* Colebr and *Plectranthus galeatus* Vahl. on Mount Gede and Telaga Warna vicinities, West Java Province, Indonesia. The research method used was parallel transect lines. Association of the existing plants in the vicinity of the area were also observed. In Mount Gede at altitudes about 1200 m above sea level there are 203 seedlings and 167 parents of *S. discolor* species, while there are 69 seedlings and 11 parents of *P. galeatus*. This is higher than the populations of 1100 m altitude, i.e. there are 76 seedlings and 45 parents of *S. discolor* and 12 seedlings and 9 parents of *P galeatus*. In Telaga Warna, *S. discolor* exist only at altitudes about 1500 m asl., i.e.. 47 seedlings and 52 parents. While for *P. galeatus*, there are 37 seedlings and 31 parents at altitudes about 1500 m. At altitude below 1400 m, there are 18 seedlings and 8 parents, this is very rare. Populations of *S. discolor* and *P. galeatus* in Mount Gede at an altitude between 1100-1300 m asl is a balance between the seedlings and its parent population which is the same for seedling populations of *S. discolor* in Telaga Warna at an altitude of 1400-1500 m asl, but for *P. galeatus* seedling number decreases from an altitude of 1400 to 1500 m asl.

Keywords: Lamiaceae, Mount Gede, Telaga Warna, West Java

INTRODUCTION

Scutellaria discolor Colebr. and *Plectranthus galeatus* Vahl. are members of mint family (Family Lamiaceae) (Figure 1). These herbaceous terrestrial plants grow on Mount Gede and Telaga Warna Hill (commonly called Telaga Warna) of West Java Province at an altitude of 500-3,200 m, along with the grass, in shady and moist places in wet forest, forest paths, wet rock in the ravine, in the Mandalawangi forest, and dipterocarp plateau at about 1,700 m above sea level (asl). *S. discolor* is locally called Hamru Lemah, which is used as traditional medicinal herb for aphrodisiac treatment (Burkill 1966). This plant has the characteristics of small herb, having height of 20-50 cm, but rarely reach 100 cm. Compound flowers are located at the end of the stem, like a bunch of simple inflorescence of 10-15 cm long. The color of the flowers are blue crown, pale blue, light purple and dark purple. The flowers bloom sequentially in acropetal succession. Flowering occurs from January to December. *P. galeatus* or jawer kotok is an important medicinal plant. Some diseases like piles, diabetes, constipation, boils, abscesses and irregular menstruation can be treated with jawer kotok (Burkill 1966). Jawer kotok is a shrub, herbaceous and creeper with erect stem height ranging from 30 cm to 150 cm. Leaves are heart shaped and on each edge, the leaf is adorned by oblong or oblong-thin contours which are continuous and supported by the petiole. The flowers appear at the top of the inflorescences shaft rod-shaped composite strands and verticillaster. Jawer kotok can flourish in the lowlands up to an altitude of 1,500 meters asl. (asl). This plant is very diverse in kinds and colors of the leaves. Jawer kotok is commonly found as wild plants around rivers or rice fields and the edges of rural roads.

Both species are found on Mount Gede and Telaga Warna at altitudes above 1000 m asl. Mount Gede is one of the tropical mountain ecosystems in West Java Province with height range between 400 m up to 2210 m asl (Van Steenis 2007). Mount Gede is located inside Mount Gede National Park where a 400 km^2 conservation area is located in the province of West Java. It is located near Telaga Warna Ecoturism lake, not far from the location of Mount Gede-Mount Pangrango National Park (Wijaya 1999). Although many plant species with small ranges are classified as endangered or threatened at the federal or state level, our species selection process did not consider current listed status as a criterion; rather, we consider range size as an important correlation of future risk in the face of climate

Figure 1. *Scutellaria discolor* Colebr. (*left*) and *Plectranthus galeatus* Vahl. (*right*), members of mint family (Lamiaceae).

change, regardless of species' current legal status. We review what is known about the long-term, large-scale range dynamics of forest herbs in response to past climate change and present a new biogeographic analysis investigating how contemporary distribution and diversity patterns among a subset of rare forest herbs may relate to these past climate dynamics. We also discuss how forest herb species may be affected by contemporary climate change and consider options for species conservation.

This research study was aimed at understanding the status of plants *Scutellaria discolor* Colebr. and *Plectranthus galeatus* Vahl. (Figure 1) as well as their parent and seedling populations on Mount Gede and Telaga Warna, West Java Province, Indonesia.

MATERIALS AND METHODS

Study area

Population research was carried out in the Mount Gede-Pangrango National Park of Cibodas, Cianjur, West Java, Indonesia and an ecotourism area of Telaga Warna Nature Recreation Park (Telaga Warna NRP), Cianjur, West Java. Research was carried out for a month, i.e. in June 2016.

Data collection

Plot was built for size of 20 m x 20 m. At least three plots were built if both species of the Mint family existed. The largest of the research area was about a hectare (10000 m^2). The seedling stage count was conducted for plants having height less than 5 cm for *Scutellaria discolor* and less than 25 cm for *Plectranthus galeatus*. The parents' stage count was conducted for plants having height more than 5 cm for *S. discolor* and more than 25 cm for *P. galeatus.* The height of the plot above sea level is also carried out data collection on every 100 meters of elevation, starting from 1100 m above sea level. At Mount Gede, sampling was conducted by making observations based on altitude more than 1000 m asl as well as at Telaga Warna NRP. Data sampling was carried out with parallel systematic vegetation sampling methods (Cropper 1993) which fitted cut contours, in a number of plots that are built with rectilinear plot nesting along transect technique. Plant associations analysis of identified specimens was carried out in the Herbarium Bogoriense (BO), Research Centre for Biology, Cibinong, Bogor, Indonesia.

RESULTS AND DISCUSSION

Plant association

The national park consists of twin volcanoes: Gede 2,958 m asl. and Pangrango 3,019 m asl. The two summits are connected by a high saddle known as Kandang Badak, 2,400 m asl. The mountain slopes are very steep and are cut into rapidly flowing stream, which carve deep valleys and long ridges. For those fortunate enough to stand on the summit of Mount Gede in clear conditions the view is spectacular. The sub-montane ecosystem is characterized by many large, tall trees like jamuju (*Dacrycarpus imbricatus*) and puspa (*Schima wallichii*). The sub-alpine ecosystem, meanwhile, is characterized by grassy meadows of *Isachne pangerangensis*, edelweiss flower (*Anaphalis javanica*), violet (*Viola pilosa*), and sentigi (*Vaccinium varingiaefolium*). Sub-montane zone in the area of Mount Gede is dominated by *Altingia excelsa, Castanopsis javanica* and *Lithocarpus indutus* stands (Sunaryo & Rugayah 1992). In this area, it is generally found where trees are large with a diameter of 50 cm, with a dense canopy cover,, low light intensity and higher humidity. According to Wiharto (2009), tree stands having high important value index (IVI) on the Northern Slope of Mount Gede, West Java, i.e. *Villebrunea rubescens* (45.15%), *Altingia excelsa* (39.77%), *Schima walichii* (21.92%), and others, which are common plant species in the northern slopes that have a diameter below 30 cm. Meanwhile, the tree that has a diameter of 51 cm is dominated by *Altingia excelsa* and *Schima walichii*. Next research study was conducted in Telaga Warna, Sub-district Cisarua, District Bogor, West Java Province, Indonesia (6o702'S, 106o996'E). This study area is a Nature Reserve and Nature Recreational Park (Taman Wisata Alam). The Nature Reserve is a conservation area for 549.66 ha tropical rainforest with high plant diversity. The reserve has a hilly terrain with an altitude that ranges from 1097 m-1600 m above the sea level. Area of the Nature Recreational Park is about 5 ha. There is a lake in the middle of the Nature Recreational Park. The lake is surrounded by a steep cliff. In Telaga Warna, vegetation is almost the same, but there is a Telecommunication Tower on the hill which makes the surrounding area open. According to Nila et al. (2014), vegetation composition at Telaga Warna, is *Villebrunea rubescens, Caryota mitis, Lithocarpus sundaicus, Saurauia distasosa, Schima wallichii, Blumea lacera, Turpinia sphaerocarpa, Ficus ribes, Ficus fistulosa, Fagraea ceilanica, Musa acuminata, Ficus* sp., *Schefflera scandens, Homalanthus populneus, Poikilospermum suaveolens, Chromolaena odorata,*

Persicaria chinensis, Laportea stimulans, Datura metel, Castanea argentea, Vaccinium korthalsii, Tetrastigma laevigatum, Syzygium laxiflorum, Clidemia herta, Elaeocarpus floribundus and *Castanea javanica.* At this location, the most common stand and the dominant species is *Villebrunea rubescens* with the largest diameter class of less than 20 cm. In general, the succession has not yet reached a climax. According to Rahayu et al (2011), habitat of *Hoya purpureofusca* in the sub montane zone of Cibodas is dominated by *Altingia excelsa, Castanopsis javanica* and *Lithocarpus indutus* stands.

Based on the association tests conducted, three species (*Antidesma tetrandrum, Pinanga coronata,* and *Castanopsis javanica*) were associated with *Saurauia bracteosa*. Significantly, while *Altingia excelsa* and *A. tetrandrum* were associated with *Symplocos costata*, as they had association indices of 0.3, based on Jaccard Index (Wihermanto 2004). Both locations, namely Mount Gede and Telaga Warna have a composition of similar plants, as stated by Whitten, et al. (1996). The implications of the association of plant species with large diameter timber is that they provide shade so that availability of sunlight that is required by shrub species is reduced. Shrub plants prefer the conditions that are exposed by the sun's radiation.. Associations consisting of large trees will reduce the growth of low canopy trees. Associations consisting of large trees will reduce the growth of low canopy trees. The association of translucent canopy trees will play a role in the growth of each plant. Association is very influential on the succession. Herbaceous plants will be grown on the slopes of the area of forest edge and spreading through the rain water and river water. Among the plant species characteristic of Temperate Decidious Forest, forest herbs may be especially vulnerable to climate change for several reasons, i.e., many forest herbs have biological and ecological traits that may limit the rate at which they are capable of migrating in response to changing climate (e.g., species with seed dispersal mechanisms adapted primarily to local movement rather than long-distance dispersal; Van der Veken et al. 2007)

Mount Gede location

Scutellaria discolor Colebr.

At an altitude of 1200 m asl., 167 individuals parent population of *S. discolor* and 203 seedlings were found within 20x20 m^2 plot. However, at an altitude of 1100 m asl., *S. discolor* parent population consisted of s 45 individuals and seedling population consisted of 76 individuals. Condition at altitudes above 1100 m is highly expected to remain in a balanced state of parents and seedling, considering that the distance between the two plots is only 5 meters or tend to be flat so that seed dispersal is adjacent to its parent (Figure 2). However, at altitudes below 1100 m asl, only 21 and 24 individuals of *S. discolor* parent (1120 m and 1138 m asl respectively) were found. Seedling can still be expected to regenerate future populations (van Steenis 2007). Conditions on *S. discolor* is still good to keep a balance between the parents and seedlings.

Plectranthus galeatus Vahl.

On observation of *P. galeatus* in Mt. Gede, the parent population number is found to be lower than that of the seedling. At an altitude of 1200 m asl., there are 69seedlings, while at an altitude of 1100 m there are 12 seedlings and at altitude 1300 m asl there are 14 seedlings (Figure 2). Substitution regeneration in *P. galeatus* also still going well because the number of seedlings is far more than its parent.

The tree with the diameter below 30 cm in five main tree species in the north part of the Gede Mountain totalling 61.7% (66 trees) showed the process of the plant succession proceeding smoothly (Sudarmono 2011). However the existence of the old tree or trees with the diameter more than 50 cm totaling 26% (28 trees) that was dominated by two species, namely *Altingia excelsa* and *Schima walichii*. Both of those trees tend to cover of understorey vegetation. These vegetation could not grow well without sunlight for photosynthesis. Therefore growing patterns can be followed by prunning the canopy so that the vegetation under the canopy-shaded getting sunlight. Wijaya (1999) recorded 789 trees or density of 394 trees/ha in an area of 2 hectares with a size 20 x 1000 m^2 in the same area. ^{2}. So for a duration of 10 years (1999-2011) in the north slope of the Gede Mountain had a two-fold density and the diversity increased with the increasing width of the observation plot.

Telaga Warna location

Scutellaria discolor Colebr.

Seedling of *S. discolor* (204 individuals) in Telaga Warna was found to be more than that of the parent (95 indiv.) at 1600 m asl. (47 indiv.; Figure 3). However seedling at 1500 m asl. was almost same to its parents (52 indiv.). This showed that the process of regeneration of populations of *S. discolor* in Telaga Warna is still good. In the future the parent population shows the population of *S. discolor* in Telaga Warna is getting fewer and its seedling are increasingly less resistant to life. The parent population at Telaga Warna shows that the population of S. discolor displays a declinng trend in the future with its seeds being less resistant to environmental factors.

Plectranthus galeatus Vahl.

Regarding the species of *P. galeatus* in Telaga Warna at altitude below 1500 m asl., the seedlings (37 indiv.) are found to be more than the parents' number (31 indiv.) also at 1400 m asl., seedlings and parents are 18 indiv. and 8 indiv. respectively, but at 1600 m asl., the parents' number (59 indiv.) is more than that of the seedlings (34 indiv.; Figure 3). This shows habitat imbalance between parent and seedling populations at altitudes about 1600 m asl.

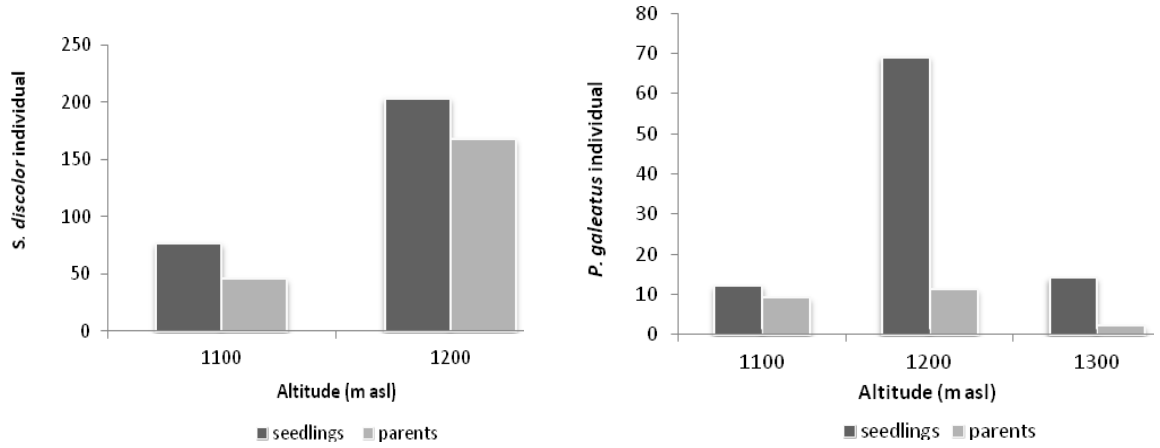

Figure 2. Individual number of *Scutellaria discolor* (*left*) and *Plectranthus galeatus* (*right*) at Mount Gede, West Java, Indonesia

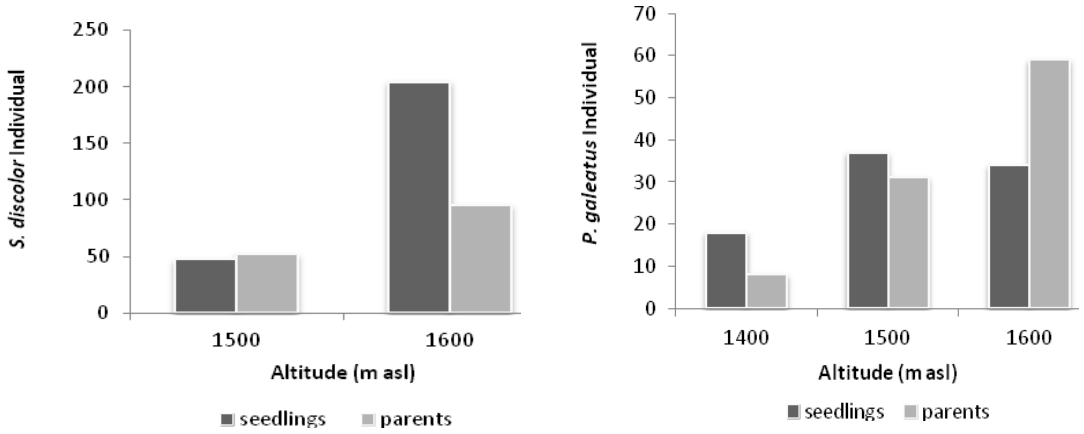

Figure 3. Individual number of *Scutellaria discolor* (*left*) and *Plectranthus galeatus* (*right*) at Telaga Warna, West Java, Indonesia

Harmer et al. (2005) studied the number of oak and ash seedlings and concluded that it was positively related to the number and proximity of parent trees. There were no consistent relationships between decreases in the sizes of the seedling populations and the type, amount and height of vegetation. The size of seedling populations generally declined with time with annual reductions varying from 0 to 90% depending on species and year; for most of the study, oak and ash populations fell by 40-50% each year. The study of *Impatien capensis* by Waller (1985) indicated that the importance of the initial variables (seed weight, seed type, and parent) increased with decreasing average plant size. The increased risk of extinction for small-ranged species can be traced back to a number of ecological and biogeographical factors. For example, macroecological studies have frequently detected a positive correlation between range size and local abundance, such that small-ranged species are often characterized by lower abundances and smaller population sizes than widespread species (Gaston 2003), a result that has been apparent in several plant-focused studies. This characteristic, combined with the geographic clustering of populations, may expose small-ranged species to greater risk of extinction due simply to stochastic population processes or to chance regional events (e.g., drought, introduction of novel pathogens; (Gaston 2003). In addition to risk factors that may be inherently linked to small range size, modern climate change poses a significant new threat to many small-ranged, endemic species (Thomas et al. 2004, 2011). Specifically, substantial geographic disjunctions are likely to develop between the locations of many small-ranged species' current ranges and the locations of climatically similar areas in the future (Thomas et al. 2004; Schwartz et al. 2006). Such disjunctions between present and future habitat areas are less likely for widespread species, where at least some portions of these broadly distributed species' ranges are likely to remain climatically suitable to the future, buffering against climate-driven threats (Thomas et al. 2004; Schwartz et al. 2006). Without successful long-distance dispersal to track shifting climate zones as they move poleward, populations of small-ranged species may soon be exposed to novel climatic regimes that fall outside

the range of climatic conditions they currently exist under; for some species this is likely to result in population declines or extinction (Thomas et al. 2004).

To conclude, populations of *Scutellaria discolor* and *Plectranthus galeatus* in Mount Gede at an altitude from 1100 m-1300 m asl. is a balance between the seedlings and its parent population. Likewise, populations of *S. discolor* in Telaga Warna is still going well between parent and seedling at an altitude of 1600 m asl., but for the species of *P. galeatus* (altitude 1400 to 1500 m asl), parents' number decreases.

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Latent variable models for multi-species counts modeling in ecology

RIKI HERLIANSYAH[1,♥], IRMA FITRIA[2]

[1]Department of Mathematics, Institut Teknologi Kalimantan. Kampus ITK Karang Joang, Balikpapan 76127, East Kalimantan, Indonesia
Tel.: +62-542-8530801, Fax.: +62-542-8530800, ♥email: rherliansyah@itk.ac.id

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Abstract. *Herliansyah R, Fitria I. 2018. Latent variable models for multi-species counts modeling in ecology. Biodiversitas 19: 1871-1876.* High-dimensional multi-species counts are often collected in ecology to understand the spatial distribution over different locations and to study effects of environmental changes. Modeling multivariate abundance is challenging as we need to consider the possibility of interactions across species. Latent variable models are the recent popular approaches in statistical ecology to address such issue that has a similar framework to ordinary regression models. In this paper, we employed the poisson distribution for modeling count responses and a negative binomial distribution for more frequent zeros in observations. The implementation of a latent variable model, Generalized Linear Latent Variable Models (GLLVMs), was demonstrated on multi-species counts of endemic bird species collected in 37 different sites in Central Kalimantan, Indonesia. The main objectives were to study the effect of logging activities on abundance of endemic species and their interactions and to observe the habitat preference of certain species. Our study found that out of four endemic species, Alophoixus bres and Eurylaimus javanicus species were significantly affected by logging activities. The sign of parameters was negative indicating the logging activities in 1989 and 1993 bring significantly negative impacts on those species. The interaction created among species was strongly negative for major endemic species especially Alophoixus bres and Eurylaimus javanicus that prefer living in primary forest than in logging areas.

Keywords: Multi-species counts, latent variable, endemic species

INTRODUCTION

Studying a spatial distribution of a group of species and their interactions with the ecosystem becomes the main objective in species modeling. Various researches regarding species richness and biodiversity, in Indonesia especially, have been widely carried out to understand the behavior of species towards changes in environment. Janiawati et al. (2016), for instances, studied the relationship between environmental characters and 21 species of reptiles in Gianyar Regency, Bali; Pritchett et al. (2016) analyzed 20 species of rattan palms to observe the behavior of rattan species towards edaphic niches; Kaban et al. (2017) recorded 40 bird species Gunung Walat, West java, Indonesia to explain the response of birds to various plantation forests; Kurniawan et al. (2018) studied Arthropod community of Semedi Show Cave in Gunungsewu Karst Area, Pacitan, East Java, Indonesia. These researches successfully collected groups of species (multivariate counts) during the observations but statistical tools used were limited to the analysis in univariate cases or to simple descriptive statistics to explain the data. Working on multivariate cases, however, where large groups of species are collected is still minor and difficult to do. In order to do this, a joint statistical model is necessarily required as we need to induce the correlations across species into the model; interactions among species are not independent.

In statistical ecology, one model that could explain multivariate inference and has been rapidly expanded in a wide range of applications is latent variable models. Recent applications of latent variable models on species modeling can be seen in Warton et al. (2015), Thorson et al. (2016), Ovaskainen et al. (2016) and Caraka et al (2018). Latent variables are used in the model to explain the unobserved quantities in environment and to incorporate the interactions across species. In this paper, we introduce a latent variable model, Generalized Linear Latent Variable Models (GLLVMs), for modeling multivariate count data. GLLVMs is an extension of Generalized Linear Models, ordinary regression models, that similarly aims to study the effect of explanatory variables and can also be used for species ordination (Hui et al. 2015). The distributional choices for multivariate count responses considered in this paper for modeling were Poisson and negative binomial distributions. These distributions have been shown to fit count data types better for GLLVMs (Warton 2005).

The main challenge in most latent variable models that it is complicated to use especially for non-statistical background users since the marginal likelihood function is not straightforward, involving the integration on latent variables. Hence, certain approaches are required to approximate the function that cannot be straightforwardly used in practice. Laplace approximation has been popularly employed to estimate parameters of GLLVMs (Huber et al. 2004; Niku et al. 2017). Hui et al. (2016) proposed a variational approximation that produced similar outcomes in terms of accuracy and computation to Laplace approximation. A recent study by Herliansyah et al. (2017) regarding how to improve computational issues of GLLVMs using Template Model Builder (TMB) leads to unexpected outcomes. This research later was used to

create a new package designed especially for fitting GLLVMs either using Laplace or variational approximations for various choice of distributions and easy to implement.

To demonstrate the application of GLLVMs, we used multivariate endemic bird species collected in three different habitat structures in Central Kalimantan, Indonesia (Cleary et al. 2005). Our objectives were to study the effect of logging activities in 1989 and in 1993 on bird abundance, to explain the interaction among endemic species (whether the relationship is random or associated), to show spatial distributions of endemic species over three habitats (species ordination) and to obtain species clustering. The rest of this paper is structured as follows. Section 2, we provide a brief description of data used for modeling followed the idea of GLLVMs in next section. Section 3 presents the application of GLLVMs under assumptions of poisson and negative distributions on responses with the last section containing discussions and conclusions.

MATERIALS AND METHODS

Data of endemic species

Indonesia is a country that has a higher number of endemic birds than any other country. This condition is supported by the size, tropical climate, and archipelagic of Indonesia. The data of endemic species used in this paper refers to Cleary et al. (2005) that collected data from Indonesia. Based on that paper, the sampling was collected in the area that represents the natural vegetation and the regional topography of the inland, upstream region in Kalimantan. Specifically, the data was obtained in 300,000 ha Kayu Mas logging, near Sangai, Central Kalimantan, from June until October in 1997 and 1998 respectively. Thirty-seven samples were collected from habitat classes, unlogged primary forest, and forest logged in 1997 and 1993-1994. The total area was about 196 km2.

The study found over 170 different bird species observed in three different habitat classes where seven of them were endemic species as presented in Table 1. In this paper, we consider four of seven endemic species used for modeling species with sums of more than 10. Due to a large number of zeros from the remaining three species, we decided to exclude them from the model as it would affect the estimation. Finally, habitat classes, primary and logged forests, are the only explanatory variables used in the model. See Cleary et al. (2005) for more details.

Statistical analysis

Generalized linear latent variable models (GLLVMs) is a statistical model that can be thought as an extended version of Generalized linear model (GLM) with the addition of random effects (Moustaki 1996) and often be used for species ordination. The random effects in GLLVMs are described as hidden variables or unobserved environmental factors and used to induce correlations across species. Let Y_{ij} is count responses with $i = 1,2,\dots,n$ being the site where data are collected and $j = 1,2,\dots,p$ being the number of species. The functional relationship between mean responses and the linear predictor, η_{ij} is defined by

$$\mathrm{E}(Y_{ij}) = \mu_{ij} = g^{-1}(\eta_{ij})$$

With $g(.)$ is a link function. Linear components of the predictor are similar to GLM structures with the inclusion of multivariate random effects as follows:

$$\eta_{ij} = \alpha_i + \boldsymbol{\beta}_{0j} + \boldsymbol{x}_i'\boldsymbol{\beta}_j + \boldsymbol{u}_i'\boldsymbol{\lambda}_j$$

Where: α_i represents the site variation or row effect treated as fixed parameters, β_j contains a matrix of the regression coefficient to corresponding independent variables, $\boldsymbol{x}_i'$. In many papers, the distributional choice of latent variables, $\boldsymbol{u}_i$, is a normal distribution with mean zero and constant variance and assumed to be independent of each other. These latent variables are often used for species ordination, to show distributions of species across sampling sites. The term $\boldsymbol{u}_i'\boldsymbol{\lambda}_j$ is a random component that has the variance-covariance matrix $\boldsymbol{\Sigma}$ controlling the correlations across species:

$$\boldsymbol{\Sigma} = \boldsymbol{\lambda}_j'\boldsymbol{\lambda}_j$$

Where the number of latent variables is less than the number of species, $q<p$. Loading factors λ_j represents parameters connecting the unobserved environmental variables to responses.

Table 1. Data of endemic species collected at thirty-seven sites from three different habitat classes in Central Kalimantan, Indonesia with references to Cleary et al. (2005)

Species/habitats	Primary forest (P)	Logged forest in 1989 (L89)	Logged forest in 1993 (L93)	Total
Alcedo euryzona	0	0	1	1
Alophoixus bres	85	21	35	142
Chloropsis cochinchinensis	41	46	66	160
Eurylaimus javanicus	17	8	7	32
Hemicircus concretus	2	0	1	3
Meiglyptes tristis	5	7	6	18
Pellorneum capistratum	8	2	0	10

In GLLVMs, we also need to choose the link function and distributions for responses. In this paper, we only consider poisson and negative binomial distributions for modeling multi-species counts. A negative binomial distribution was proven to be more appropriate choices than poisson and zero inflated distributions for more frequent zeros in data (Warton 2005). The choice of link function can be based on selected distributions for responses. See details in Skrondal and Rabe-Hesketh (2004). For poisson and negative binomial distributions, the obvious choice for a link function is the log link. Hence, the relationship between mean responses and a linear predictor can be rewritten as

$$\mu_{ij} = \exp\left(\alpha_i + \boldsymbol{\beta}_{0j} + \boldsymbol{x}_i'\boldsymbol{\beta}_j + \boldsymbol{u}_i'\boldsymbol{\lambda}_j\right)$$

Where, for the poisson regression the variance is equal to its mean, μ_{ij}. For a negative binomial model, the variance is equal to $\mu_{ij} + \phi_j\mu_{ij}^2$ with ϕ_j being the overdispersion parameters.

To assess the adequacy of models, residuals of the model are often presented. The similarity between GLLVMs and GLMs leads to the same issue in assessing goodness of fit. For most cases in GLMs, the model does not have a constant variance and a zero mean especially when overdispersion occurs. Pearson and deviance residuals are two common used residuals for diagnostic checking. For overdispersion cases, however, both residuals are often not normally distributed (Dunn and Smyth 1996). Hence, in this paper we use Dunn-Smyth or quantile residuals for assessing models. Residuals are defined as follows (Hui et al 2015):

$$r_{ij} = \Phi^{-1}\left(z_{ij}F_{ij}(y_{ij}) + (1 - z_{ij})F_{ij}^{-}(y_{ij})\right)$$

Where: $\Phi(.)$ and $F_{ij}(.)$ are the cumulative density functions of a standard normal distribution and responses, y_{ij} and z_{ij} being generated from a standard uniform distribution.

To fit the model, we use a new created package in R, gllvm(), designed especially for fitting GLLVMs with the help of Template Model Builder (TMB). See details in Kristensen et al. (2015) for more information about TMB. This package can be found at https: //cran.r-project.org/package=gllvm. The choice of distributions for fitting GLLVMs available in this package are a binomial distribution for binary responses, poisson, negative binomial and zero inflated poisson distributions for count data with two optional approaches, Laplace and variational approximations.

RESULTS AND DISCUSSION

In this section, we fitted GLLVMs on endemic bird data to study the species distribution over habitat classes, the environmental effects, species interaction, species prediction and species clustering. To begin with, we present descriptive statistics of data in the following figure. Distributions of abundance of endemic species over three habitat structures showed in Figure 1.A are roughly identical each other with slightly higher mean and more outliers in primary forest. Each species was distributed variously across sampling sites given in Figure 1.B. *Meiglyptes tristis* and *Eurylaimus javanicus* were found to be less abundant than other two species with average numbers of individual close to zero.

To fit GLLVMs, we assumed poisson and negative binomial distributions for responses and a fixed number of latent variables, q = 2 for ordination purposes while it can also be selected based on either information criteria, AIC or BIC, or coverage probabilities. We used habitat structures as explanatory variables defined as follow: D_1 (logged forest in 1989 = 1; otherwise = 0) and D_2 (logged forest in 1993 = 1; otherwise = 0) with primary forest as the reference variable (P = 0). Predicted values of abundance across sites, $\hat{y}_i = \sum_{j=1}^{p}\hat{y}_{ij}$, were computed and compared to actual counts given in Figure 2. Both models seem fit data very well where predicted values lie closely to actual counts. There is almost no distinction between poisson and negative binomial models. Either poisson or negative binomial models can be selected in this case for modeling; diagnostic checking on residuals, however, can be used for model selections.

In this paper we use Dunn-Smyth residuals for assessing the adequacy of models as explained in previous section. A good model is supposed to be independently and normally distributed as in GLM. As we can observe, residual models for a negative binomial model are randomly spread over linear predictors, no clear pattern, while a poisson model produce fan-shaped pattern as an indication of overdisversion (Hui et al. 2015). Both residuals, a negative binomial and a poisson regressions lie closer to a normal distribution line with no major outlier is found in the model. In conclusion, a negative binomial model is a more appropriate model to use for further analysis; testing our hypothesis about logging effects on abundance and species ordination as we observe no sign for a lack of fitting.

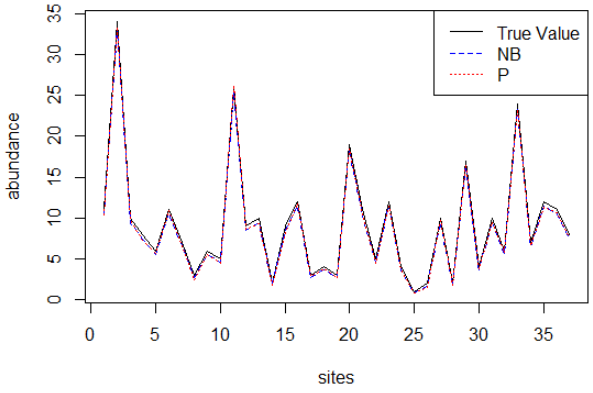
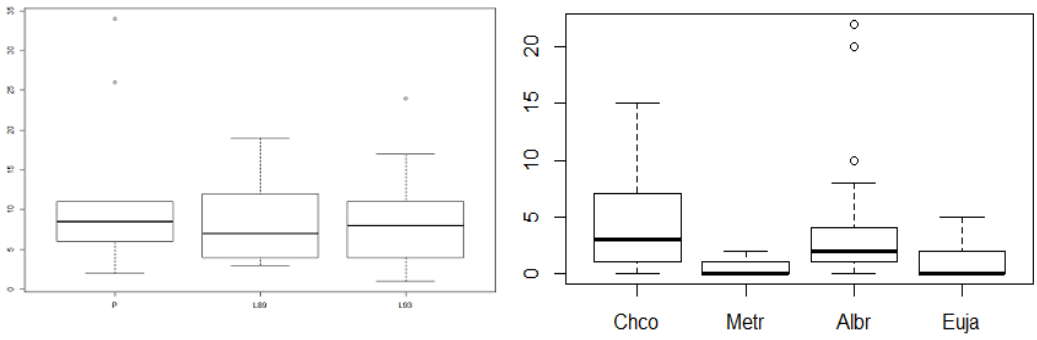


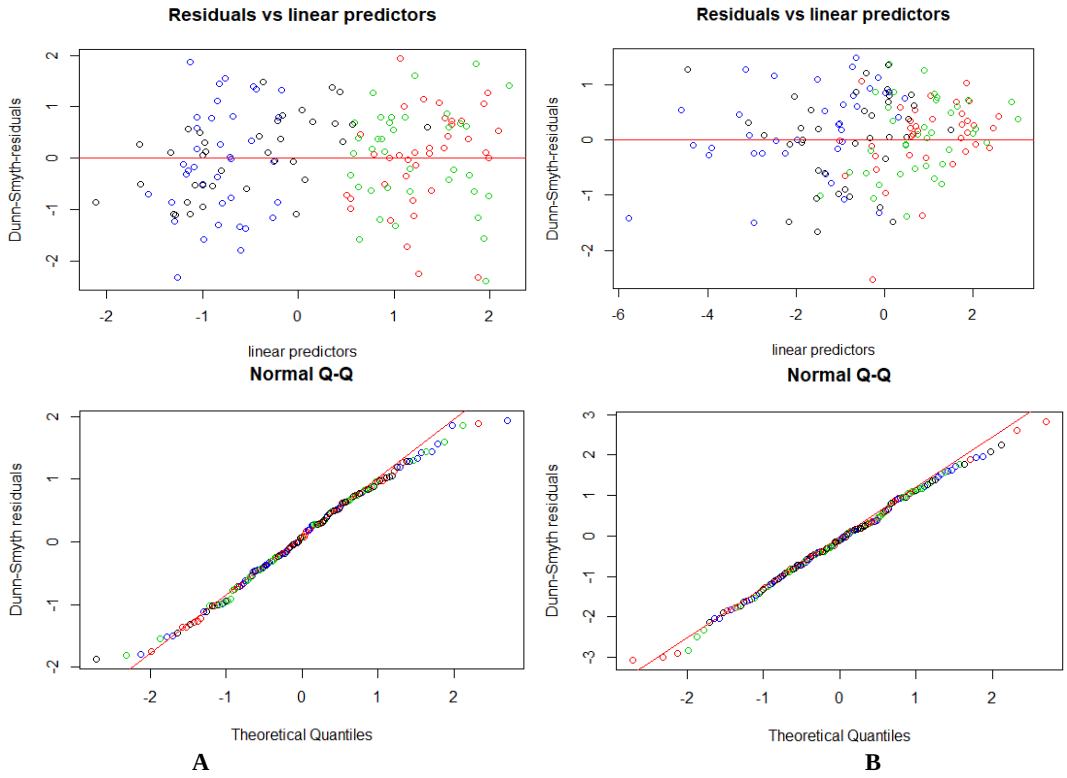
Figure 2. Comparisons of observed values and predicted values of poisson and negative binomial regression models

To answer our questions in introduction regarding the logging effects on abundance of endemic bird in Central Kalimantan, we run a hypothesis testing for two dummy variables defined before. Figure 4 displays parameters of four endemic species with 95% confidence interval for poisson and negative binomial regression models. If 95% confidence intervals contain zero then the corresponding parameter is not statistically significant. As we can observe from the following figure, two models give the same conclusion about species that were significantly affected by logging activities. For a dummy variable, D_1, we found that *Alophoixus bres* was the only species receiving a significant effect from logging activities in 1989. The parameter lies in the negative area indicating that logged forest in 1989 brought negatively a significant impact on abundance of *Alophoixus bres*. Logged forest in 1993 also affected the same species as in 1989, *Alophoixus bres*. This species received a negative effect on its abundance, while remaining species seem not too much different over three habitat structures.



A **B**

Figure 1. Distributions of endemic species for (A) different habitats: Primary forest, logged forests in 1989 and 1993 respectively (B) each species



A **B**

Figure 3. Diagnostic checking on residuals model: (A) Dunn-Smyth residuals of a negative binomial model, (B) Dunn-Smyth residuals of a poisson model

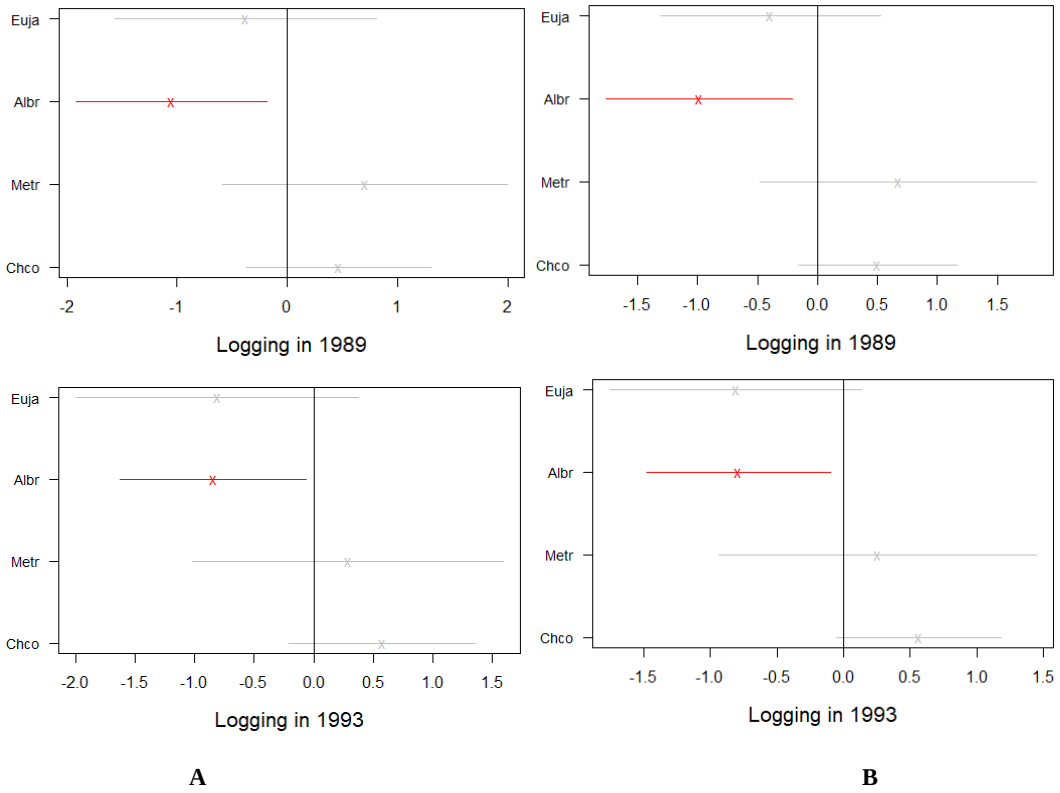


Figure 4. Estimation of parameters and their 95% confidence intervals: (A) a negative binomial regression and (B) a poisson regression.

Finally, GLLVMs also can be used for species ordination like those in multidimensional scaling and principal component analysis and for explaining interactions among species. Estimated latent variables were used to create ordination species while the correlation matrix describing association among species was computed through variance-covariance matrix using loading factors, $\hat{\boldsymbol{\Sigma}} = \hat{\boldsymbol{\lambda}}'\hat{\boldsymbol{\lambda}}$. To create ordination plot, we used an unconstrained model, a model without explanatory variables, and loading factors used to create correlation matrix were loading factors after controlling habitat structures effects, a model with D_1 and D_2. Figure 5.A shows the distributions of endemic specie over three different locations. As we can see, distributions of endemic species in logged forests in 1989 and in 1993 were roughly similar over different sites. In primary forest, however, some sites were located further from logged forest indicating a different pattern of species distribution from those two habitats. For instance, *Eurylaimus javanicus* was found to be more abundant in one site of primary forest while *Meiglyptes tristis* was observed more in one logged forest in 1989 sites. Most species seem to be strongly associated with more negative interactions. Positive correlations indicate endemic species tend to be close to each other, their existence is positively relied on each other while negative correlations imply endemic species prefer to stay further from one to another. Figure 5.C presents species clustering of endemic species in Central Kalimantan. This clustering was measured based on the Euclidean distance

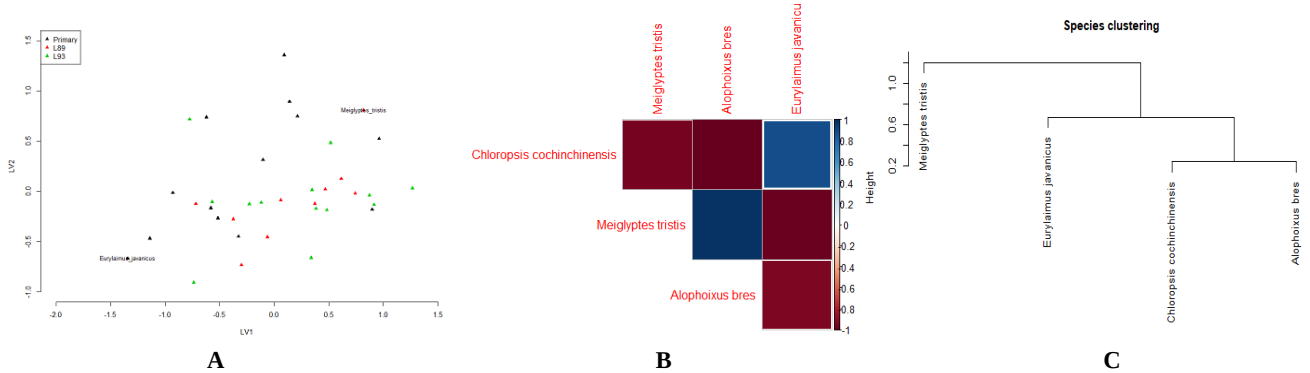


Figure 5. Ordination species using latent variables (A), correlation matrix (B) and species clustering (C) using loading factors for a negative binomial model

More information can be extracted using GLLVMs from data compared to univariate cases e.g., species distributions, effects of environmental changes on abundance, species interactions, the prediction and the species clustering. In this paper, we only use four endemic species as responses for a demonstration while in practice it could handle larger numbers of species. Our results show that a negative binomial seems fit the data better, satisfying normal assumption and more random than a poisson model even though they lead to the same inferences. Logging activities have been shown to negatively affected abundance of some endemic species where most species tended not to interact each other. Remaining endemic species, however, gave a positive response to logging activities regarding their insignificant parameters. We also describe the relationship between mean responses and only one independent variable, habitat structures while more environmental factors can be included into to the model to analyze either qualitative or quantitative types. Further works in species modeling, GLLVMs can be extended to explain the association between species traits and environmental variables also known as a forth corner model where we included species traits into the model and their interactions with environments as explanatory variables (Brown et al. 2014).

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Molecular identification of coffee (*Coffea arabica*) pollinator insects in North Sumatra, Indonesia based on designed COI primers

AIDA FITRIANI SITOMPUL[1,♥], ELIDA HAFNI SIREGAR[1], DEWI IMELDA ROESMA[2,♥♥], DAHELMI[2], EKO PRASETYA[1,♥♥♥]

[1]Departement of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Medan. Jl. Willem Iskandar, Pasar V, Medan Estate, Medan 20221, North Sumatra, Indonesia. Tel.: +62-61-6613365, Fax.: +62-61-6614002, ♥email: aiesitompul@yahoo.co.id, ♥♥♥ ekoprasetya.biologi@gmail.com
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Andalas. Kampus Unand Limau Manih, Padang 26253, West Sumatra, Indonesia. Tel.: +62-75177427, Fax.: +62-75171343, ♥♥email: dewi_roesma@yahoo.com

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Abstract. *Sitompul FA, Siregar EH, Roesma DI, Dahelmi, Prasetya E. 2018. Molecular identification of coffee (*Coffea arabica*) pollinator insects in North Sumatra, Indonesia based on designed COI primers. Biodiversitas 19: 1877-1883.* Coffee (*Coffea arabica* L.) is one of the most important economic commodities in the province of North Sumatra, Indonesia. Insects associated with pollination of *C. arabica* are one of the key factors for successful cultivation of *C. arabica*, but, the research regarding of these was still limited. The population of coffee plant is scattered across the highlands of Indonesia and the pollination of *C. arabica* is strongly believed linked to a diverse group of pollinating insects. However, lack of taxonomic identification of insects pollinating these plants has become one of constraints to succeed the cultivation of *C. Arabica*. This study aimed to analyze types and variations of pollinating insects of *C. arabica* in the province of North Sumatra, Indonesia, using DNA barcoding. DNA barcoding is now considered an alternative method of molecular identification. Sixteen of *C. arabica* flower visitors were captured in different planting location in North Sumatra province. Using *mtDNA* markers, the *cytochrome oxidase subunit sequence I* (COI), about 12 pollinator insect species were identified based on the COI sequence i.e *Amegilla cingulata, Apis dorsata, Apis cerana, Trigona chanchamayoensis, Idiella divisa, Dolichopodidae* sp., *Allactoneura* sp., *Stomorhina discolor, Phytomia erratica,* Rhiniidae sp., *Melipona bicolor,* and Hymenoptera sp.

Keywords: *Coffea arabica, cytochrome oxidase subunit I,* mtDNA, North Sumatra, pollinator insects

INTRODUCTION

Indonesia is the fourth largest producer of coffee in the world from 2016 to 2017 with the production output reaching 11.49 million/kg (ICO 2017). The production of coffee in Indonesia are located in several islands with suitable appropriate climatic conditions (Schroth et al. 2015). of which Sumatra Island produced coffee by 74.2% of all national production of which provinces of South Sumatra contributes 21.4%, Lampung contributes 12.6%, Nanggroe Aceh Darusallam is 8.7%, Bengkulu is around 7.4%, and East Java is around 7.2% (Wahyudi et al. 2012). North Sumatra Province is one of the central coffee plantations in Sumatra Island.

Coffee is a member of Rubiaceae family, Gentianales order and *Coffea* genus with more than 124 member species (Davis et al. 2011). Commercial coffee production is dominated by two main species i.e., *Coffea arabica* L. and *Coffea canephora* Pierre ex A. Froehener (Tran et al. 2017) with 66% and 34% of the coffee market's production (Camargo 2009).

Coffea arabica is the most well-known coffee in the market which is widely produced (DaMatta et al. 2007). *Coffea arabica* has a high quality in terms of flavor and aroma (Flament 2002). *Coffea arabica* comes from the rainforest in the Ethiopian highlands (Monaco 1968), growing at the altitude of 1000-2800 m above sea level (Schmitt 2006), with 1200-1800 mm of rainfall and temperatures ranging between 18-20ºC (Alégre 1959). Flowering period of *C. arabica* varies in different regions but it generally occurs between January to April (Ngo et al. 2011). The flowers wilt within 1-2 days after pollination (Aga 2005) and continue with fruit development in 7-10 months (Eira et al. 2006). Most literature state that the percentage of flowers can develop into fruits ranging from 20 to 40% (Free 1993).

Almost 90% of flowering plants heavily rely on pollinator insects (Klein et al. 2007). The productivity of majority of cross-pollinated crops can be increased by improving the pollination process with the help of pollinator insects (Klein et al. 2007; Abrol 2012). The diversity and abundance of pollinator insects can increase the number and quality of fruits (Garibaldi et al. 2011).

The success of pollination processes is strongly influenced by the diversity of pollinating insects (Mayer et al. 2011). Lack of information about identification of pollinating insects is an obstacle in conducting this research (FAO 2009). DNA barcoding using mtDNA as a marker can be used to identify species diversity (Hebert and Gregory 2005; Floyd et al. 2009). This becomes a solution for the identification of pollinator insects in agriculture (FAO 2009). This study aimed to identify pollinator insects of *C. arabica* in the province of North Sumatra, Indonesia, using DNA barcoding.

MATERIALS AND METHODS

Study area

Insect pollinator samples were obtained from three districts in the province of North Sumatra, Indonesia i.e North Tapanuli, South Tapanuli, and Dairi. All three districts are the center of coffee cultivation in North Sumatra (Figure 1, Table 1). The used samples were fresh samples of pollinating insects obtained from coffee planting in North Sumatra. Sampling of pollinator insects on coffee flower planting was done on sunny days with the scan sampling method (Bookhout 1996).

Procedure

Genomic DNA extraction

DNA was extracted using a Purelink ™ kit Genomic DNA Mini Kit. A total of 25 mg of insect tissue was taken into the micro centrifuge tube by adding 180 µL purelink genomic digestion buffer and 20 µL K proteinase. It was then incubated for 2 hours at 55 ºC temperature and centrifuged for 3 minutes at 10,000 x g speed. After the supernatant was transferred to a new microcentrifuge tube, 20 µL of RNAse A was added and incubated at 27ºC for 2 minutes. A total of 200 µL of purelink genomic lysis and 200 µL of ethanol 96% were added to the microcentrifuge tube and then homogenized. The solution sample was transferred into a spin column and centrifuged for 1 minute at a rate of 10,000 x g. DNA was washed using wash buffer I and wash wafer II, followed by centrifugation for 1 minute at 10,000 x g. DNA was eluted using 100 µL of purelink genomic elution buffer.

Amplification of mtDNA COI sequence

COI (*cytochrome oxidase subunit I*) sequences were amplified using a pair of forward primers LCO1490 5'-GGT-CAA-CAA-ATC-ATA-AAG-ATA-TTG-G-3' and reverse primer HCO2198 5'- TAA-ACT-TCA-GGG-TGA-CCA-AAA-AAT-CA-3' (Folmer et al. 1994). Total volume of PCR (polymerase chain reaction) reaction was 25 µL (2.5 µL DNA template; 2.5 µL primer forward; 2.5 µL primer reverse; 5 µL distilled water; 12.5 µL PCR master mix (MyTaq™ DNA Polymerases). The reaction was run in PCR with PCR condition as follows: pre-denaturation at temperature of 97ºC for 5 minutes followed by 40 cycles with denaturation reaction conditions at temperature of 94ºC for 1 minute 30 seconds, annealing at temperature of 52ºC for 1 minute, and extension at 72ºC

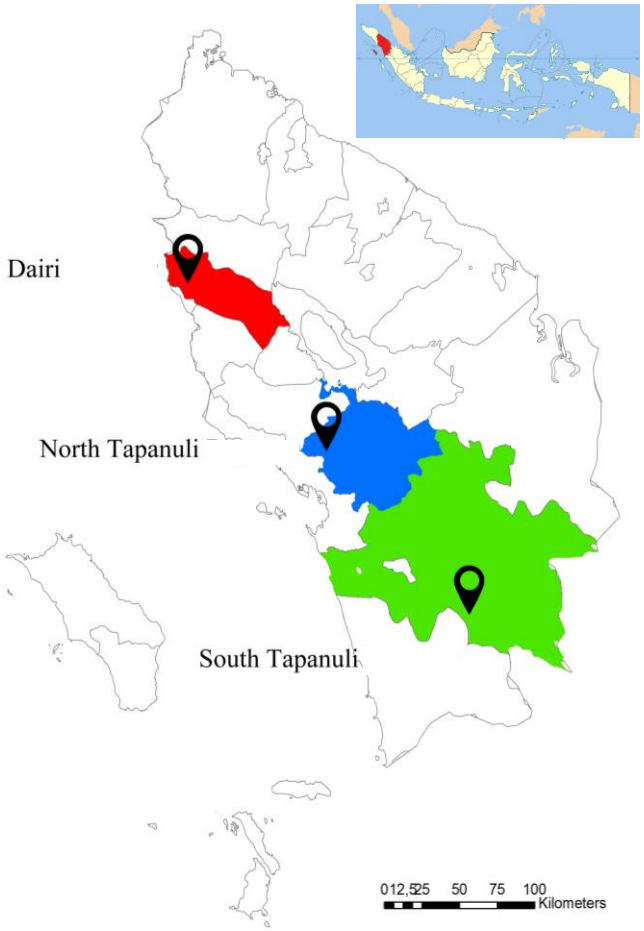

Figure 1. Sampling location in North Sumatera Province, Indonesia

Table 1. Samples of pollinator insects used for analysis

Sample	District	Sub-district	Village	Elevation (m asl.)	Latitude	Longitude
Sample 4	Dairi	Parbuluan	Lae Hole II	1321	N 02^0 41' 27.9"	E 098^0 24' 2.06"
Sample 53	Dairi	Parbuluan	Lae Hole II	1321	N 02^0 41' 27.9"	E 098^0 24' 2.06"
Sample 85	Dairi	Siempat Nempu Hulu	Lae Nuaha	1056	N 02^0 46' 1.04"	E 098^0 18' 25.06"
Sample 95	Dairi	Siempat Nempu Hulu	Lae Nuaha	1056	N 02^0 46' 1.04"	E 098^0 18' 25.06"
Sample 314	North Tapanuli	Pangaribuan	Rahut Bosi Onan	1280	N 01^0 58' 9.2"	E 099^0 11' 25.7"
Sample 335	North Tapanuli	Pangaribuan	Batu Nadua	1180	N 01^0 58' 49.7"	E 099^0 11' 8.7"
Sample 336	North Tapanuli	Pangaribuan	Batu Nadua	1180	N 01^0 58' 49.7"	E 099^0 11' 8.7"
Sample 337	North Tapanuli	Pangaribuan	Batu Nadua	1180	N 01^0 58' 49.7"	E 099^0 11' 8.7"
Sample 338	North Tapanuli	Pangaribuan	Batu Nadua	1180	N 01^0 58' 49.7"	E 099^0 11' 8.7"
Sample 340	North Tapanuli	Pangaribuan	Rahut Bosi Onan	1280	N 01^0 58' 09.2"	E 099^0 11' 25.7"
Sample 341	North Tapanuli	Pangaribuan	Sidori	1180	N 01^0 58' 49.7"	E 099^0 11' 8.7"
Sample 348	North Tapanuli	Pangaribuan	Sidori	1180	N 01^0 58' 49.7"	E 099^0 11' 8.7"
Sample 399	South Tapanuli	Mamancar	Aek Sabaon Julu	981	N 01^0 30' 31'0"	E 099^0 13' 35.3"
Sample 445	South Tapanuli	Dolok	Siranap	903	N 01^0 31' 32.8"	E 099^0 12' 22.5"
Sample 454	South Tapanuli	Dolok	Siranap	903	N 01^0 31' 32.8"	E 099^0 12' 22.5"
Sample 461	South Tapanuli	Dolok	Siranap	903	N 01^0 31' 32.8"	E 099^0 12' 22.5"

temperature for 1 minute, then PCR process ended with post extension at 72°C temperature for 5 minutes. The PCR product was visualized using 1% agarose gel added with 4 µL SYBR® Safe DNA Gel Stain. A total of 6 µL of PCR products were added with 1 µL of loading dye in running with a 100 bp DNA ladder marker using an electrophoresis engine with a mobile phase of 1X TBE buffer at 45 volts for 30 minutes. Visualization of ribbon emerging by gel documentation (Biostep). The PCR product with the next visible band was sent to 1st Base DNA Sequencing Service for sequencing.

Data analysis

Sequence data that has been obtained was edited using BioEdit ver program. 7.0.1 to combine the results of the forward and reverse primary sequences. The combination of mtDNA sequence of COI data was analyzed by sequencing homology using mega BLAST program which can be accessed at the National Center for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences homology analysis was performed by comparing COI sequence of pollinator insects samples with NCBI GenBank Database. Phenogram analysis was performed using the MEGA 7 program.

RESULTS AND DISCUSSION

Sample collection

Samples were obtained from 3 coffee planting centers in North Sumatra province. A total of 60 pollinator insect samples were used in the analysis and only 16 samples were successfully amplified using COI primers. Four samples were obtained from Dairi District, 8 samples were obtained from North Tapanuli District, and 4 samples were obtained from South Tapanuli District. A total of 16 samples were used in the present study.

Alignment of sequences

A total of 799 bp of the alignment result of the sequencing product was observed successfully. From these data, there were 155 conservative characters, 619 character variable sites, and 496 characters of informative parsimony. This suggests that the mtDNA COI sequence on insects is less conservative and has a high degree of variability. The mean frequency of nucleotides in the COI sequence in the sample was 39.0% (T), 16.3% (C), 30.4% (A), and 14.3% (G) (Table 2). In Table 3, each entry showed the probability of substitution from one line to another. Different transitional substitution levels were shown in bold and transverse substitutions were shown in italics. This sequence was rich in A/T (69.4%), while G/C was 30.6%. The approximate transition/transversion bias (R) was 0.88. The substitution pattern and approximate rate were based on the 2-parameter model kimura (Kimura 1980).

Sequencing analysis of PCR amplified region

The sequencing results of the PCR amplification product of 16 pollinating insects were presented in Table 4. The nucleic acid sequence of samples was analyzed using the BLASTN program to determine the identity of pollinator insects. The BLASTN analysis showed that sequences of 20 isolate insect insects had 79% -99% similarity with 12 insect species, *Amegilla cingulata, Apis dorsata, Apis cerana, Trigona chanchamayoensis, Idiella divisa,* Dolichopodidae sp., *Allactoneura* sp., *Stomorhina discolor, Phytomia erratica,* Rhiniidae sp., *Melipona bicolor,* and Hymenoptera sp. Pearson (2003) stated that two genes or homologous DNA fragments having similarity with range of 80% or 25% of amino acid sequences between two genes or DNA fragments mostly having identical species performances.

Phenogram construction analysis

The entire sequence of individual sequenced results was analyzed and phenogram was made by Neighbor-Joining (NJ) method to see differences in genetic distance and analyze similarities between samples. Phenogram analysis was performed to compare the results of alignment of nucleotide sequences of pollinator insect samples with some homologous sequences (selected from *GenBank*) as reference using MEGA 7 program. The alignment results showed COI sequence in the sample and the references used has a high level of variety. This was indicated by the least conservation sequence (306/1050) and the high value of the site variable (688/1050). Each sample clumped together with a homologous reference sequence (Figure 2).

Table 2. Variation in length, AT content and GC content on COI sequences on Insect

Sample	T	C	A	G	Total	%AT	%GC
Sample 4	43.2	15.0	27.6	14.3	526.0	70.7	29.3
Sample 53	40.8	15.0	32.7	11.5	642.0	73.5	26.5
Sample 85	41.3	14.5	33.8	10.4	671.0	75.1	24.9
Sample 95	47.1	11.6	31.7	9.6	622.0	78.8	21.2
Sample 314	36.6	17.2	29.3	16.9	656.0	65.9	34.1
Sample 335	31.6	19.6	30.4	18.4	648.0	62.0	38.0
Sample 336	34.5	18.2	29.2	18.1	565.0	63.7	36.3
Sample 337	35.1	18.1	29.4	17.4	609.0	64.5	35.5
Sample 338	39.2	14.1	30.6	16.1	633.0	69.8	30.2
Sample 399	40.7	14.9	32.6	11.8	610.0	73.3	26.7
Sample 340	38.8	14.7	29.7	16.8	585.0	68.5	31.5
Sample 341	38.3	14.7	30.2	16.8	572.0	68.5	31.5
Sample 348	40.4	13.7	30.3	15.5	611.0	70.7	29.3
Sample 445	41.4	15.2	32.8	10.6	592.0	74.2	25.8
Sample 454	40.7	18.3	27.6	13.5	624.0	68.3	31.7
Sample 461	41.8	14.6	32.6	11.0	610.0	74.4	25.6
Average	39.0	16.3	30.4	14.3	603.3	69.4	30.6

Table 3. Maximum composite probability estimate of the pattern of nucleotide substitution

	A	T	C	G
A	-	*9.24*	*3.90*	**6.06**
T	*7.31*	-	**9.84**	*3.36*
G	*7.31*	**23.29**	-	*3.36*
C	**13.19**	*9.24*	*3.90*	-

Table 4. Results of sequencing and BLASTN analysis on 16 pollinator insects on coffee

Sample number	Location (District)	Fragmen size (bp)	Identification result	Query cover	Identical
4	Dairi	526	*Amegilla cingulata* (KY842920.1)	100%	96%
53	Dairi	642	*Apis cerana* (KU963187.1)	100%	99%
85	Dairi	674	*Apis dorsata* (KU752355.1)	99%	98%
95	Dairi	623	*Trigona chanchamayoensis* (KC853315.1)	100%	99%
314	North Tapanuli	656	*Idiella divisa* (KY031806)	94%	99%
335	North Tapanuli	648	Dolichopodidae sp. (KY833537.1)	98%	86%
336	North Tapanuli	565	Dolichopodidae sp. (KY833537.1)	97%	97%
337	North Tapanuli	609	Dolichopodidae sp. (KY833537.1)	99%	97%
338	North Tapanuli	633	*Allactoneura* sp. (KT175590.1)	98%	99%
340	North Tapanuli	585	*Stomorhina discolor* (KY031819.1)	98%	98%
341	North Tapanuli	572	*Phytomia erratica* (KR831181.1)	99%	95%
348	North Tapanuli	611	Rhiniidae sp. (KX054613.1)	100%	99%
399	South Tapanuli	610	Hymenoptera sp. (KF200070.1)	100%	98%
445	South Tapanuli	593	Hymenoptera sp. (KF200070.1)	98%	99%
454	South Tapanuli	626	*Melipona bicolor* (KC853346.1)	93%	79%
461	South Tapanuli	610	Hymenoptera sp. (KF200070.1)	99%	98%

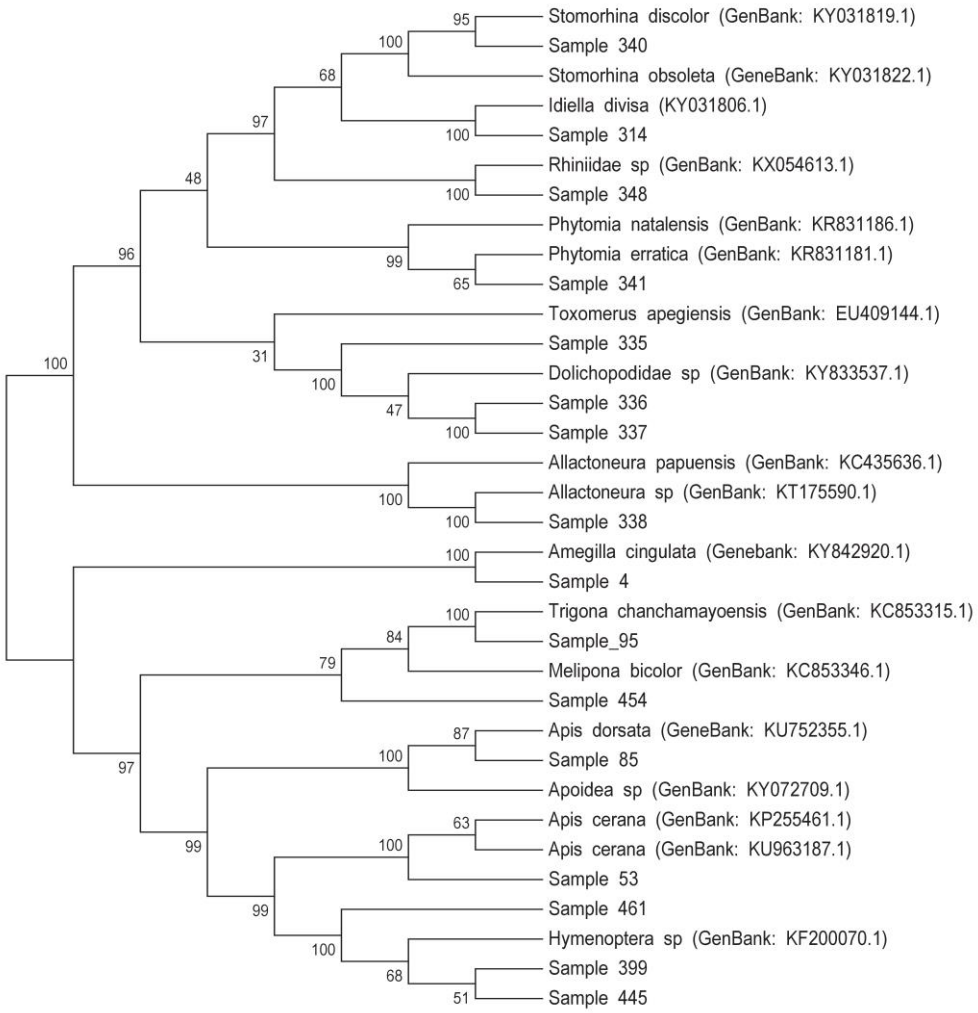

Figure 2. Phylogenetic sequences of insect pollinators in coffee plants. Twelve species were identified using COI sequences. Identified insects include *Amegilla cingulata* (4), *Apis cerana* (53), *Apis dorsata* (85), *Trigona chanchamayoensis* (95), *Idiella divisa* (314), Dolichopodidae sp. (335, 336, 337), *Allactoneura* sp. (338), *Stomorhina discolor* (340), *Phytomia erratica* (341), Rhiniidae sp. (348), Hymenoptera sp. (399, 445, 461), and *Melipona bicolor* (454). The numbers on the branches show the confidence level of the branch separation

Discussion

Wind is an important source of pollination process for 18% of species in the Angiosperm family (Culley et al. 2002). Le Pelley (1973) noted that based on the grain structure of pollen on *C. arabica*, winds cannot carry pollen moving away and cross-fertilization is mostly associated with insects. Bees have long been known for its important role in pollination of *C. arabica* (Ricketts 2004). Klein et al. (2003) stated that social bees more often visit *C. arabica* in Indonesia with the most frequent visitors are *Apis nigrocincta, Apis dorsata,* and *Apis cerana*. Flowering of single coffee flower outside flowering events is still accompanied by the existence of solitary insects (*Megachile frontalis* and *Amegilla* sp.) (Ngo et al. 2011). Solitary bees are considered to be more efficient as pollinators than social bees although the rate of social bees' visit to coffee flowers is higher (Willmer and Stone 1989; Klein et al. 2003). Amaral (1972) observed that the honeybee is the main visitor of the coffee flower on *C. arabica* compared to *Trigona* and *Partamona* bees and wood bees (*Xylocopa* sp.), which are the least frequent visitors. Roubik (2002) reported that besides *Apis mellifera* which visits flowers of *C. Arabica, Trigona* spp., *Melipona* spp., *Bombus* spp., and *Centris* sp. were also visitors.

The application of a long-term agroforestry system has created a specific habitat for coffee cultivation, including Berytidae, Calliphoridae, Dolichopodidae, Dytiscidae, and Histeridae, which were only found in coffee plantations (Kinasih et al. 2016). This study also identified several species that do not have references as pollinator insects in coffee plants such as *Idiella divisa, Allactoneura* sp., *Stomorhina discolor, Phytomia erratica,* and Rhiniidae sp. *Idiella divisa,* which were found across Indonesia (Sulawesi), Vietnam (The Catalogue of Life Partnership 2017), India, and Taiwan (Yang et al. 2014). *Stomorhina discolor* are spread across Bangladesh, Australia, Indonesia, Pakistan, China, and Malaysia (Ratnasingham and Hebert 2007). *Rhiniidae* is known as pollinator on bitter ground, *Momordica charantia* Linn (Subhakar et al. 2013). *Allactonoura* is known to only be found in Asia, northern Australia New Guinea, a series of islands in the Indian Ocean, and East Africa (Zaitzev 1981).

Pollinator Insects of coffee plants have been shown to increase crop productivity (Klein et al. 2003). The pollination process by insects benefits the major crops by up to 75%, representing 35% of the world's crop production (Klein et al. 2007). Flowering coffee plants that are pollinated by insects have 15.85% higher fruit set than their own self-pollinated flowers (Ngo et al. 2011).

Earlier works on using COI sequences to identify the pollinator insects are scanty. Our present study confirms that DNA barcoding based on COI sequences can be applied for taxonomic identification of pollinating insect species in coffee plants. The aim of our study was to ensure that molecular identification methods could be applied to pollinating insects of coffee plants in the province of North Sumatra, Indonesia. Sometimes, the morphological markers used in insect identification showed similarities so that it confused to determine the species of insects. Molecular markers can facilitate the identification of insects in shorter period of time, making this method more efficient. DNA barcode is able to facilitate integrative approaches in species identification but it still involves a classical taxonomic approach (Imtiaz et al. 2017).

Subunit of *cytochrome oxidase subunit I* (COI) has been used extensively by molecular biologists around the world to identify insect species (Jalali et al. 2015). COI barcoding sequences can be used to identify insect species at all stages of development (Armstrong and Ball 2005; Ball and Armstrong 2006). COI sequences are not only widely used in Diptera (Alessandrini et al. 2008), but also in Coleoptera identification (Paul et al. 2009; Fang 2009). In East Asia, mtDNA including COI (Kim et al. 2000) and COII (Suzuki et al. 2004) has been used in identifying evolutionary relationships and biogeography in some Coleoptera families. COI barcoding sequences have been used to identify species in a number of studies (Harvey et al. 2003).

DNA barcoding has been applied to identify the Coleoptera order (Greenstone et al. 2005), Hymenoptera (Fisher and Smith 2008), Lepidoptera (Hajibabaei et al. 2006), Orthoptera (Trewick 2007), and Diptera (Burns et al. 2008). DNA barcoding of COI sequences could be used to identify six orders of insects (Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, and Orthoptera) with significant variation among orders on Neighbor-Joining Tree analysis with the highest identification proportions in Hymenoptera and Orthoptera and the lowest one in Diptera (Virgilio et al. 2010). In the present study, two insect orders had been identified, namely Diptera order (*Idiella divisa, Dolichopodidae* sp., *Allactoneura* sp, *Stomorhina discolor, Phytomia erratica,* and *Rhiniidae* sp.) and Hymenoptera (*Amegilla cingulata, Apis cerana, Apis dorsata, Trigona chanchamayoensis, Hymenoptera* sp., and *Melipona bicolor*).

In this study, COI sequence analysis showed that there were various types of pollinating insects in coffee. Pollinator insects of coffee plants are identified using *mtDNA* markers, the COI sequence, i.e., *Amegilla cingulata, Apis dorsata, Apis cerana, Trigona chanchamayoensis, Idiella divisa, Dolichopodidae* sp., *Allactoneura* sp., *Stomorhina discolor, Phytomia erratica,* Rhiniidae sp., *Melipona bicolor,* and Hymenoptera sp. DNA barcoding using COI sequences could be an effective screening method for pollinating insects. Research on the identification of pollinating insects in coffee plants can improve the yield productivity by providing information about insects that are efficient for coffee pollination. The coffee conservation and management strategy focus on conservation of important pollinating insect communities. This research can be used to know the identification of the pollinating insect communities, which are beneficial to coffee plants.

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Growth characteristics and copper accumulation of bacterial consortium *Acinetobacter* sp. and *Cupriavidus* sp. isolated from a wastewater treatment plant

WAHYU IRAWATI[1,♥], TRIWIBOWO YUWONO[2,♥♥], NOMMENSEN PANGIHUTAN OMPUSUNGGU[1]

[1]Department of Biology Education, Universitas Pelita Harapan, M.H. Thamrin Boulevard 1100, Lippo Karawaci, Tangerang 15811, Banten, Indonesia. Tel./fax.: +62-21-5460910, ♥email: w.irawati3@gmail.com

[2]Department of Agricultural Microbiology, Faculty of Agriculture, Universitas Gadjah Mada. Bulaksumur, Sleman 55281, Yogyakarta, Indonesia. Tel./fax.: +62-274-563062, ♥♥email: triwibowo_y@faperta.ugm.ac.id

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Abstract. *Irawati W, Yuwono T, Ompusunggu NP. 2018. Growth characteristics and copper accumulation of bacterial consortium Acinetobacter* sp. *and Cupriavidus* sp. *isolated from a wastewater treatment plant. Biodiversitas 19: 1884-1890.* Pollutant treatments are part of the human calling, as the crown of creation, to subdue, preserve, and cultivate the earth in bringing goodness for all creatures. Bioremediation of copper using indigenous bacteria is well known as the best water treatment for polluted environment recovery. *Acinetobacter* sp. and *Cupriavidus* sp. are indigenous bacteria isolated from industrial sewage in Indonesia. Bioremediation in environment is a process involving community of bacterial consortium for heavy metal or any other polluting materials accumulation. The purposes of this research were: (i) to characterize growth of *Acinetobacter* sp. and *Cupriavidus* sp. consortia in sewage medium, enrichment medium, and medium supplemented with copper, (ii) to establish the potency of bacterial consortia to accumulate copper. The growth of bacteria was observed based on cell turbidity using spectrophotometer at wavelength of 600 nm. Cells pellet was destructed by nitric acid at 100ºC and copper concentration was analyzed by atomic absorption spectrophotometer as copper accumulation value. The results showed that the growth of bacterial consortia in medium containing copper was better than that of single bacterium. The best bacterial consortium was the mixture of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2. The use of sewage as cultivation medium decreased bacterial growth by up to 25% but still resulted in the same level of logarithmic phase in enrichment medium. The highest accumulation capability was of a consortium of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 at a level of 6.45 g/mg copper/g cells dry weight, suggesting that 5.09% of copper were accumulated by cells. It was concluded that the best composition of consortia in growth and copper accumulation capability was the mixture of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2. These results may be due to the fact that both bacteria belong to the same genus that allowed them for synergistic interactions.

Keywords: *Acinetobacter*, accumulation, copper, *Cupriavidus*, growth

INTRODUCTION

God created all things in the earth, visible and invisible realms, gigantic and microscopic creatures – and God has put all creation on the earth under human care. So the concern and the engagement in the bacterial research are a part of human calling to have a deeper understanding on this matter for better caring of the earth and all the life in. Bacteria are living systems that demonstrate high adaptability to stress conditions. They can easily experience environmental changes by altering their genetic systems, transfer of genetic elements, and many other mechanisms to maintain the structure and function of the ecosystem (Ryan et al. 2009). Bacteria are known to survive in all condition of environments. They possess unique features such as small size, high surface area to volume ratio, and adaptability. With their features, bacteria enable to utilize organic pollutants as a carbon source and degrade it into non-toxic products. On the other hand, they develop resistance mechanism to inorganic pollutants such as heavy metals by efflux the metals outside the cells, transformation to less toxic, or accumulation them inside the cells (Williams et al. 2012).

Heavy metals contamination, as an impact of industrial activities, is an important issue in Indonesia. The disposal of heavy metals into aquatic environments become a serious problem because they are non-biodegradable and persistent in the environment (Sen et al. 2016). Metal pollution problem can become a threat to the ecosystem and human health. The most common metal pollutant in the environment is copper (Das et al. 2016). The persistent environmental problem could be solved by some technologies to remediate the environment. Recent developments of novel technologies involve multidisciplinary approaches by utilizing microorganisms for enhanced bioremediation capability (Niti et al. 2013). Bioremediation using indigenous bacteria is a promising method for heavy metals removal because of its capabilities to accumulate them inside the cells (Irawati et al. 2017a). Bioremediation is technology for controlling pollution by using biological system to catalyze the biodegradation or transformation processes of various toxic chemicals to less harmful forms. This natural process of bioremediation includes bioengineering and the capabilities of intrinsic microorganisms to clean the environment as an effective alternative to conventional remediation methods (Saranraj

and Stella 2012). The use of bacteria as bioremediation agent to treat copper-contaminated water requires the assessment of the effect of different copper concentrations on cell growth. Bacterial consortia are commonly applicated in biological wastewater treatment because bacterial communities can easily adapt to environmental changes. Meanwhile, pure cultures bacteria are difficult to thrive in environment (Carpio et al. 2014). Bacteria in natural environments commonly exist as communities of multiple species. The Bacteria communities demonstrated more varied and complicated tasks than single bacterial species. (Brune and Bayer 2012). *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4 were indigenous copper-resistant bacteria isolated from wastewater treatment plant in Surabaya, Indonesia. The purposes of this research were to study: (i) the growth characteristics of *Acinetobacter* sp. and *Cupriavidus* sp. as a pure culture and consortium in medium containing appropriate concentration of copper and in medium supplemented with wastewater both incubated at room temperature and 37^{O}C, and (ii) the potency of the pure culture and bacterial consortia in accumulating copper.

MATERIALS AND METHODS

Bacterial consortia

Three high copper-resistant bacteria used for mixing bacterial consortia were *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4 isolated from a wastewater treatment plant, in Rungkut-Surabaya. Bacterial consortia were formulated by mixing equal proportions of pure bacterial cultures. Consortium 1, 2, 3, and 4 consisted of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2; *Acinetobacter* sp. IrC1 and *Cupriavidus* sp. IrC4, *Acinetobacter* sp. IrC2 and *Cupriavidus* sp. IrC4; *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4, respectively. The pure culture of each bacterial strain was used as a control. The consortia also were formulated by mixing three of the pure bacterial strains in a consortium (Irawati et al. 2018).

Medium preparation and bacterial growth

Bacteria were grown in Luria Bertani (LB) agar containing the following (per liter of aquadest): tryptone 10 g, yeast extract 5 g, NaCl 10 g, and glucose 0.1 g. Medium containing copper was made by addition of 1 M $CuSO_4$ to the autoclaved media. LB agar was made with addition of 2% pure agar. Sewage-supplemented medium was made by using sewage from Cisadane River to replace distilled water as the solvent. The medium was autoclaved at 121°C, 1 atm, for 15 minutes before being used as growth medium (Irawati et al. 2016).

Bacterial culture of 1500 µL volume with the optical density of 0.6 (Colony 100 Forming Unit = 1.2 x10^9) was used for inoculating 50 mL LB medium. Cells were grown in LB medium supplemented with copper and incubated at 37°C and room temperature. Growth was monitored by measuring optical density at 600 nm using a spectrophotometer. Cells cultivated in medium without copper also were observed as a control (Irawati et al. 2018).

Copper accumulation

Cells were grown in medium containing various copper concentrations and incubated at 37°C or room temperature with shaking at 120 rpm. The cells were centrifuged at 5000xg for 20 min at 4°C to separate it into supernatant as growth medium and pellets as bacterial cells. Cells pellets were washed several times with copper-free phosphate buffer. Then, the cells were added by distilled water and were digested with HNO_3 at 100°C for measuring the ability of microorganisms in accumulating copper. The cells dry weight from the same culture was also determined. The copper content was determined by using an Atomic Absorption Spectrophotometer at 324.9 nm. All the experiments were done in triplicate to ascertain the accuracy of the results (Irawati et al. 2017a).

RESULTS AND DISCUSSION

Growth characteristics of bacteria as a pure culture and as a consortium

The ability of pure culture and bacterial consortia to grow in high concentration of copper were shown in Figure 1A. It showed that bacterial growth as a consortium was better than as a pure culture in medium containing 3 mM of copper. The pure culture demonstrated a lag phase longer than the bacterial consortia. Rolfe (2012) reported that heavy metals concentration impacted to the lag phase of growth of bacteria. Bacterial growth undergoes lag, logarithmic, stationary, and death phase. The lag phase involves slow growth and a period of acclimating which the bacteria is adjusting to a new condition in order to successfully grow. Irawati et al. (2017b) stated during the lag phase, the bacteria synthesized some proteins due to the resistance mechanism to face the toxicity of copper. After finishing the lag phase period, the bacteria continued the growth by entering the logarithmic phase.

The best bacterial growth as a pure culture was *Acinetobacter* sp. IrC1 followed by *Cupriavidus* sp. IrC4 despite the fact that it demonstrated a long lag phase for 12 hours (Figure 1A). *Acinetobacter* sp. IrC2 did not show the activity of growth during observation time. Surprisingly, it showed good growth when it was grown with *Acinetobacter* sp. IrC1 as C1C2 and when it was combined with *Acinetobacter* sp. IrC1 and *Cupriavidus* sp. IrC4 as C1C2C4. The lag phase of *Acinetobacter* sp. IrC2 as a consortium was shorter (4 hours) than the pure culture. The best bacterial consortia growth was the consortium of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 followed by the consortium of *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4. Figure 2B showed the similar result that the best growth of bacterial consortia in medium without copper also was consortium of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2.

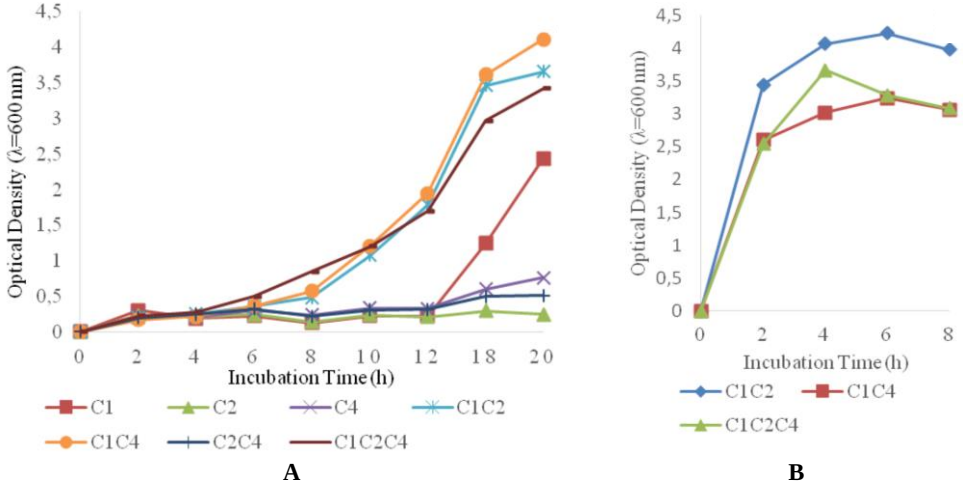

Figure 1. The comparison of bacterial growth both as a pure culture and consortia. A = Pure culture and bacterial consortia in enrichment medium containing 3 mM of copper. B = Bacterial consortia in enrichment medium without copper. C1 = *Acinetobacter* sp. IrC1, C2 = *Acinetobacter* sp. IrC2, C4 = *Cupriavidus* sp. IrC4

This result suggests that the bacteria were more resistant to copper as a mixture of culture than as a pure culture. This result was similar to the previous study conducted by Latorre et al. (2016) which demonstrated that consortium had a greater capacity to resist copper compared to pure culture. Ilhan-Sungur et al. (2017) reported that the combination of different bacteria species is the important thing in this consortium. Each species of bacterium performed differences in metabolic processes that support the whole processes of bioremediation. Sannasi et al. (2006) stated the survival and stability of bacteria are better when they are present as a mixed culture. This is because each strain has significant differences both physiologically and metabolically and the varied responses and resistance that exhibited by each of them towards different metals. This condition would generate a dynamic, well-adapted and more resilient population through exchange of genetic material between the strains present to overcome toxicity of heavy metals. According to Subashchandrabose et al. (2011), bacterial consortium may provide robustness to environmental fluctuations, ability to share metabolites, and resistance to stress condition.

Growth characteristics of bacterial consortium with appropriate formulation

To obtain a better growth, three bacteria were combined in one consortium with appropriate composition of culture and cultivated them in a medium supplemented with copper. Figure 2 shows the best growth of consortium was *Acinetobacte*r sp. IrC1; *Acinetobacte*r sp. IrC2; *Cupriavidus* sp. IrC4 with composition of culture of 2: 2: 0. In the other word, the best consortia was *Acinetobacte*r sp. IrC1 and *Acinetobacte*r sp. IrC2 with the formulation of 2: 2. The other composition of bacterial consortia, however, also showed comparable growth. This result was different from the previous study in Figure 1 that concluded the best consortium when bacterial consortia are grown with formulation of 1: 1 was *Acinetobacte*r sp. IrC1: *Acinetobacte*r sp. IrC2. It suggested that volume of bacterial culture influenced the activity of growth of bacterial consortia. Each volume of bacterial culture in formulation of 1: 1 and 2: 2 were 1500 µL and 300 µL, respectively.

Growth characteristic of bacterial consortia in enrichment medium supplemented with wastewater

Bioremediation occurs in nature by indigenous bacterial communities living in a contaminated area. Bioremediation using bacteria is usually applied in bioreactor filled with wastewater. Therefore, it was of interest to determine the best bacteria for bioremediation that result in good growth under this condition. *Acinetobacte*r sp. IrC1, *Acinetobacte*r sp. IrC2, and *Cupriavidus* sp. IrC4 are well-known indigenous bacteria isolated from wastewater treatment plant in Surabaya (Irawati et al. 2012). Before using this bacterial consortium for bioremediation agent, it was of importance to establish the influence of wastewater as cultivation medium for cultivation of *Acinetobacte*r sp. IrC1, *Acinetobacte*r sp. IrC2, and *Cupriavidus* sp. IrC4 (Figure 3).

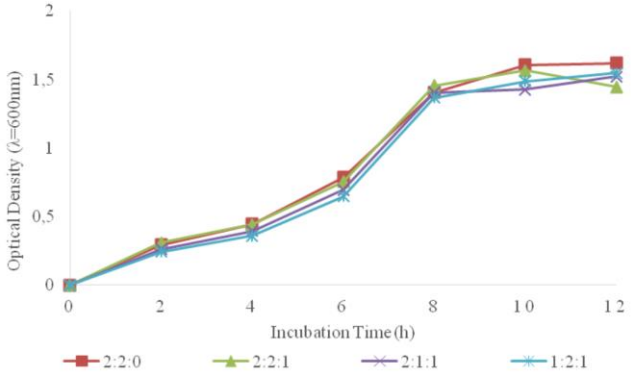

Figure 2. Characteristics of growth of bacterial consortia with appropriate culture composition in enrichment medium supplemented with 3 mM of copper. C1= *Acinetobacter* sp. IrC1, C2= *Acinetobacter* sp. IrC2, C4= *Cupriavidus* sp. IrC4.

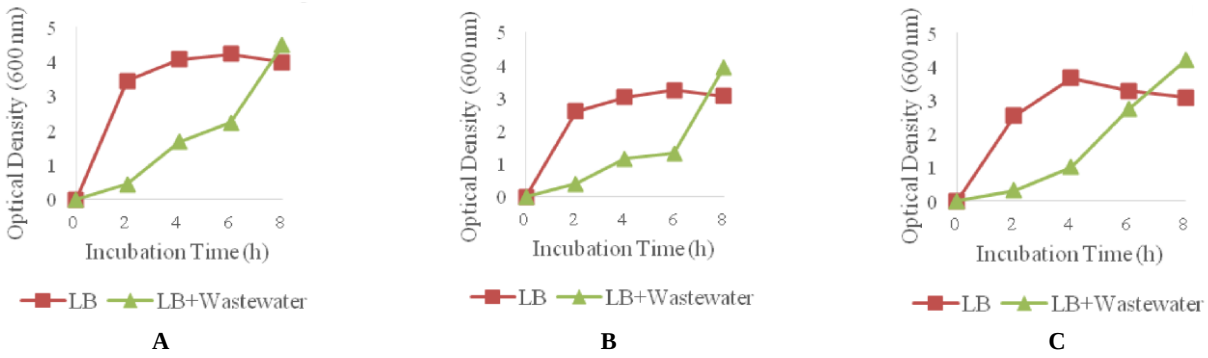

Figure 3. The influence of sewage as cultivation medium for the growth of bacterial consortia. A= *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2. B= *Acinetobacter* sp. IrC1 and *Cupriavidus* sp. IrC4. C= *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4.

Figure 3 shows that the growth activity of bacterial consortia decreased when they were grown in wastewater medium. The results indicated that wastewater medium inhibited bacterial growth. The peak of logaritmic phase of bacterial consortia in an enrichment medium was reached faster than that of in an enrichment medium supplemented with wastewater. Bacterial consortia in an enrichment medium achieved the peak of logarithmic phase at the second of incubation time. Meanwhile, it occurred at the eighth of incubation time in medium supplemented with wastewater. Inhibition of growth in wastewater medium might be due to the fact that Cisadane River as a wastewater medium containing toxic effluent. Rochyatun (2006) reported that Cisadane River was contaminated by lead, zinc, copper, and cadmium.

Growth characteristic of bacterial consortia at room temperature and medium supplemented with copper

Bioremediation in nature usually occurs at room temperature, therefore it was of interest to establish the growth of bacterial consortia in medium containing 2 mM and 3 mM of copper incubated at room temperature. Figure 4 shows that room temperature did not inhibit the growth of bacterial consortia, but the inhibition occurs when they were grown in medium supplemented with 2 mM of copper. On the other hand, the bacterial consortia did not show growth activities when it was grown in medium containing 3 mM of copper.

It was possible that the bacterial consortia could not survive in the elevated concentration of copper when it was cultivated not at the temperature optimum. Temperature is the important factor that influences metabolism activities. Previous study demonstrated that optimum temperature of *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4 was 37ºC (Irawati et al. 2012). Metal concentration above threshold levels impacted functional activities completely inhibit various metabolic activities of bacteria. At the higher concentration, bacteria develop resistance mechanism to overcome in stress condition through its intrinsic properties such as metal bioaccumulation. Such property is important to improve the overall efficiency of treatment process in bioremediation (Ahemad et al. 2009; Habi and Daba, 2009; Rodrigues, 2011). Bioaccumulation mechanism includes precipitation, intracellular accumulation and oxidation or reduction. These processes are often associated with an active defense system and require longer response time due to the gradual transportation and accumulation within the cytoplasm after binding metals inside the cell (Unz and Shuttleworth, 1996).

The potency of copper accumulation by bacterial consortia and pure culture bacteria

The potency of bacteria as a consortium and as a pure culture for copper accumulation in medium containing 2 mM and 3 mM of copper in incubation time of 37ºC were shown in Figure 5. Figure 5 shows that in medium supplemented with 2 mM of copper, there were no significant differences between copper accumulation by the pure culture of *Cupriavidus* sp. IrC4 with the consortium of *Acinetobacte*r sp. IrC1 and *Acinetobacte*r sp. IrC2, by the average of 2.88 mg/g dry weight of cells. Similar results were also observed between the pure culture of *Acinetobacte*r sp. IrC2 with the consortia of *Acinetobacte*r sp. IrC1 and *Cupriavidus* sp. IrC4 by the average of 2.77 mg/g dry weight of cells. On the other hand, in medium supplemented with 3 mM of copper, the amount of copper accumulated by the consortia bacteria was higher than by the pure culture bacteria except the consortium of *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4.

The highest copper accumulation in medium supplemented with 2 mM was the consortium of *Acinetobacte*r sp. IrC1 and *Acinetobacte*r sp. IrC2 with the total amount of 2.87. mg/g dry weight of cells. Meanwhile, the consortia of *Acinetobacte*r sp. IrC1 and *Cupriavidus* sp. IrC4 was the highest copper accumulator in medium containing 3 mM with the total amount of 3.49 mg/g dry weight of cells. It indicated that in medium containing 2 mM, the bacterial consortia only require one genus to respond copper which is the genus Acinetobacter. Whereas, in higher concentration, the consortia require the other genus that was more resistant than the genus Acinetobacter. Previous study demonstrated that *Cupriavidus* sp. IrC4 was more resistant than *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 (Irawati et al. 2012).

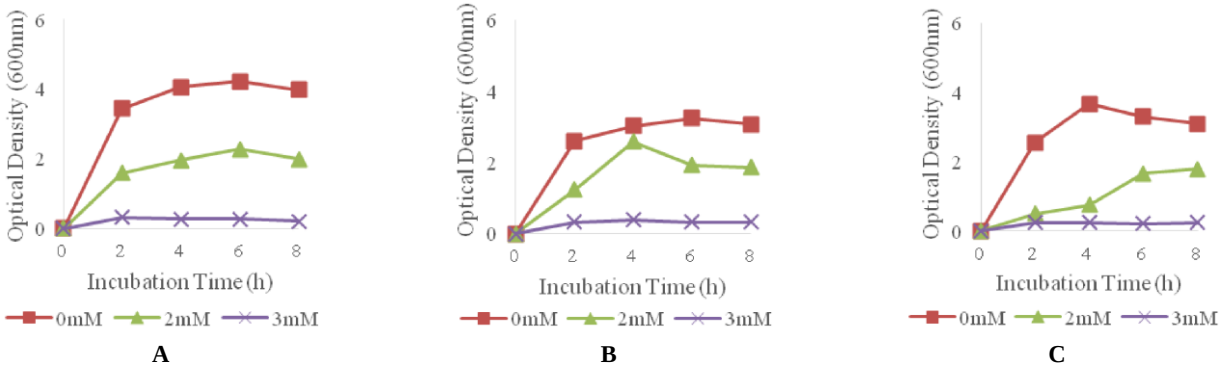

Figure 4. The influence of copper concentration and room temperature of incubation on bacterial consortia growth. A= *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2. B= *Acinetobacter* sp. IrC1 and *Cupriavidus* sp. IrC4. C= *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4

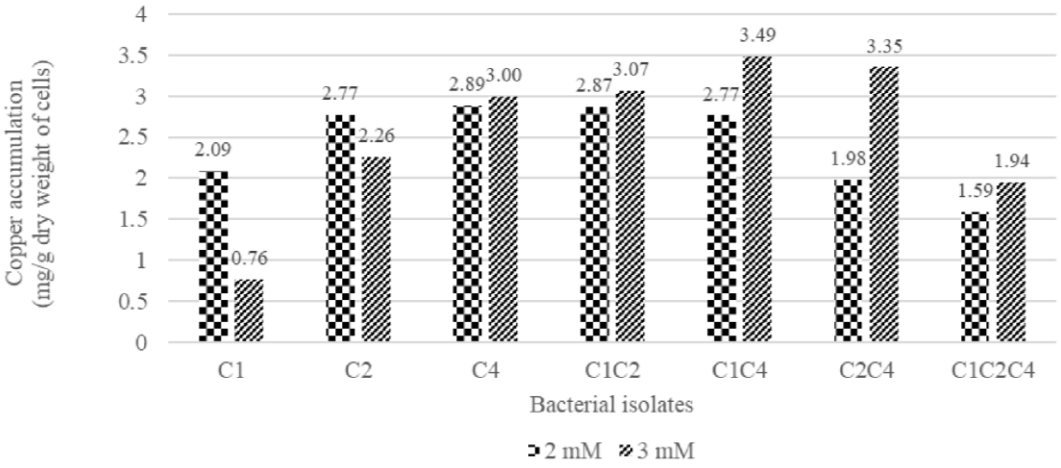

Figure 5. The potency of bacteria as a consortium and as a pure culture for copper accumulation in medium containing 2 mM and 3 mM of copper, and incubated at 37ºC. C1= *Acinetobacter* sp. IrC1, C2= *Acinetobacter* sp. IrC2, C4= *Cupriavidus* sp. IrC4

The potency of copper accumulation incubated at room temperature

Comparison of copper accumulation by bacterial consortia incubated at 37C and room temperature was shown in Figure 6. It is quite clear that the potency of bacterial consortia for copper accumulation incubated at room temperature was higher than that of at 37˚C. Under both incubation treatment at room temperature and at 37˚C, it was observed that the highest copper accumulation was obtained from the consortia of *Acinetobacte*r sp. IrC1 and *Acinetobacte*r sp. IrC2 followed by the consortia of *Acinetobacte*r sp. IrC1and *Cupriavidus* sp. IrC4, then the consortia of *Acinetobacte*r sp. IrC1, *Acinetobacte*r sp. IrC2, and *Cupriavidus* sp. IrC4. The consortia of *Acinetobacte*r sp. IrC1 and *Acinetobacter* sp. IrC2 was the highest copper accumulator when it was incubated at room temperature and at 37ºC with a total of 6.45 mg and 2.87 mg/g dry weight of cells. At room temperature, the bacterial consortia accumulate copper in medium containing 2 mM of copper higher than that of in 3 mM of copper. It might be due to the toxicity of copper in higher concentration would inhibit the growth and the ability in accumulating copper.

The potency of copper accumulation with appropriate culture formulation

Bioremediation in nature occurs not only by single bacteria but by some bacteria forming a community with appropriate formulation. In this study, each pure culture of *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, *Cupriavidus* sp. IrC4 was combined with appropriate formulation of culture as follows: 2: 2: 0; 2: 2: 1; 2: 1: 1; 1: 2: 1. Figure 7 shows the potency of copper accumulation of consortia bacteria with appropriate culture formulation in medium supplemented with 3 mM of copper.

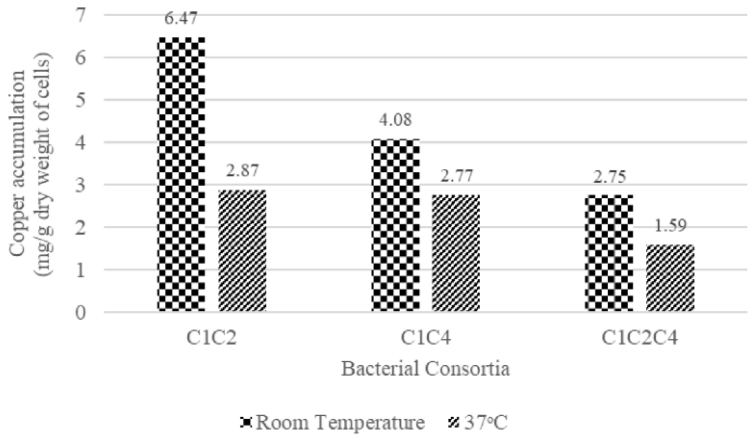

Figure 6. Comparison of copper accumulation in medium supplemented with 2 mM of copper and incubated at 37ºC and room temperature. C1= *Acinetobacter* sp. IrC1, C2= *Acinetobacter* sp. IrC2, C4= *Cupriavidus* sp. IrC4

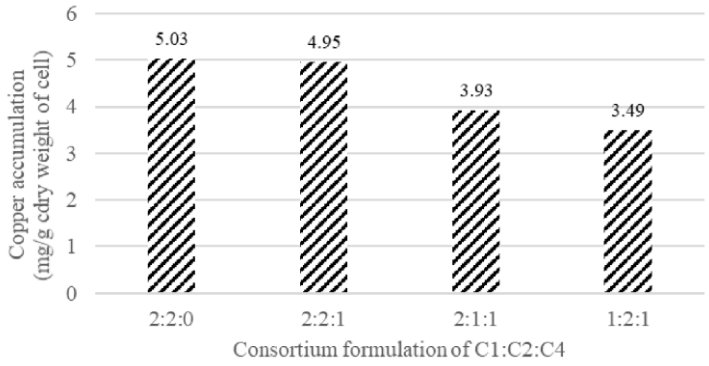

Figure 7. Copper accumulation of bacterial consortia with appropriate culture composition in medium supplemented with 3 mM of copper

Figure 7 shows that the highest copper accumulation was obtained from the consortia of *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, *Cupriavidus* sp. IrC4 with the formulation of 2: 2: 0 followed by 2: 2: 1 with a total of 5.03 mg and 4.95 mg/g dry weight of cells, respectively. This result is consistent with previous study that the best growth and copper accumulation was consortium of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2. It might be due to the fact that both bacteria belong to the same genus that allowed them for synergistic interactions to enhance bioremediation processes. Ilyas et al. (2014) reported that bacterial consortia are important aspect that influences efficiency of heavy metal removal. According to Subashchandrabose et al. (2011), each bacterium in a consortium excreted organic matter base on synergistic relationship between the two species bacteria. The construction of consortia with desired partners serves a dual mission of pollutant removal and commercial production of microbial metabolites. Bacterial metabolites and its production improve the efficiency of bioremediation processes more than individual microorganism.

In conclusion, bacterial consortium formulation is an important aspect that influences the growth and bioaccumulation efficiency of copper. It has successfully formulated bacterial consortium considering to the ability to grow and accumulate copper. *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 was the best consortium demonstrating resistance to copper and ability to accumulate copper. The highest number of copper accumulation by this consortium was 6.45 mg/g dry weight of cells when it was grown on to medium supplemented with 2 mM $CuSO_4$ and incubated at room temperature. Olajire and Essien (2014) stated bioremediation required the cooperation of more than one single species of bacteria forming a consortium. The consortium is composed of many different bacterial species with broad enzymatic capacities that are required to increase the rate of heavy metal bioremediation. On the other hand, single bacteria can metabolize only a limited substrate. Bioremediation by bacterial communities depends on the composition of the community and its adaptive response to the presence of heavy metals. According to Hays et al. (2015), cultures that

consist of multiple bacterial species contain wide range of genes and metabolic capabilities in comparison to monocultures. Thus, bacterial consortia can more successfully be applied to overcome environmental problem by remediation than single bacteria.

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The potential risk of tree regeneration failure in species-rich Taba Penanjung lowland rainforest, Bengkulu, Indonesia

AGUS SUSATYA
Department of Forestry, Faculty of Agriculture, Universitas Bengkulu. Jl. WR Supratman, Kota Bengkulu 38371A, Bengkulu, Indonesia. Tel./fax. +62-736-21170, email: agussusatya@unib.ac.id

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Abstract. *Susatya A. 2018. The potential risk of tree regeneration failure in species-rich Taba Penanjung lowland rainforest, Bengkulu, Indonesia. Biodiversitas 19: 1891-1901.* Tropical lowland rain forest is recognized by its high species richness with very few trees per species. It is also known for having tendency to outcrossing of its species with different floral sexualities, which requires the synchronization between flowering of its trees and the presence of pollinators. Such ecological attributes raise possible constraints for the forest trees to regenerate. The objective of the study was to assess the potential risk of failed regeneration for each tree species of the forest. Each of species with dbh of more than 5 cm in a one-ha plot was collected, identified, and its ecological criteria, including rarity, floral sexuality, seed size, and flowering phenology were determined. The potential risk of the failure of regeneration was calculated by summing all scores from Analytical Hierarchical Process of the criteria. The results indicated that the forest consisted of 118 species belonging to 69 genera and 37 families. Rare species accounted to 52.10% of the total species. Of the 118 species, the potential medium risk category contributed to 38.14%, and more than 33% were grouped into very high and high risk or were more prone to failed regeneration in the future. All rare dioecious species were categorized into very high and high risks. Only 21 species (17.79%) are listed in 2017's IUCN red list. Among unevaluated species, 22 and 13 species were respectively included in very high and high potential risk categories. The results revealed more detailed potential risk of failed regeneration of tree species, and can serve as basic information to develop proper conservation management.

Keywords: Bengkulu, dioecy, hermaphrodite, phenology, rarity, rainforest, risk regeneration

INTRODUCTION

Tropical rainforests and their intangible functions are getting more important in recent years due to their vital roles in providing life-support and ecological services such as carbon sequestration, water provision, and prevention of global warming and its negative effects. However, their existences are constantly being threatened by economic as well as human population pressures. Indonesian lowland rain forests especially in Sumatra and Kalimantan Islands, have been undergoing rapid deforestation and degradation as the results of conversions into mainly oil palm plantations as well as into industrial plantation forests which have simpler stand structure. In addition to those external factors, the forests inherently have their own ecological attributes that potentially constrain their abilities to regenerate. In species-rich tropical rain forests, each of their tree species generally consists of very few individual trees (Whitmore 1983; Sakai et al. 1999; Sakai 2001).

Susatya (2007, 2010) studying three different tropical rainforests discovered the similarity of their structural patterns, namely, they were composed of many species, but with very few individual trees. The very low density of tree species appears to be more prevalent to the climax tree species than the pioneer ones (Susatya 2010). Furthermore, unlike pioneer species that have good capabilities to explore wide ecological ranges, the climax tree species have been known to adapt to more limited ecological ranges as well as more stable environments, and have difficulty to grow under warmer and drier environments (Whitmore 1983). Therefore, rapid environmental changes induced by both climate change and forest degradation will pose constraints for climax tree species to regenerate. Moreover, according to floral sexuality, Bawa et al. (1985) shows that dioecy is common among tropical tree species, which requires at least two different individual trees to perform sexual regeneration.

In the tropical forest, even hermaphrodite species tend to be not self-compatible, and consequently have to do outcrossing (Bawa et al.1985). Both phenomena require flower synchronization among trees of the same species in order that pollination process can occur. Even if this takes place, then pollination process is still difficult, because different flowering trees can be distant from each other (Whitmore 1983). In the tropical rainforest of Malaysia Peninsular, for example, it requires 32 ha to find two trees of the same species (Poore 1968). Therefore tropical trees must adapt to the rapid environmental alterations; otherwise, they may be threatened by those changes and may face difficulties in regeneration because of their own reproductive biology. The focus of the study was to determine the potential risk of failed regeneration of tree species based on their floral sexualities, tree density, flowering patterns, and seed sizes.

MATERIALS AND METHODS

The research site was located at Taba Penanjung Area within Bukit Daun Protection Forest (*Hutan Lindung Bukit Daun*), Bengkulu Province, Indonesia (Figure 1). This lowland rainforest was well protected and minor illegal logging in the form of cutting small pole (< 10 cm dbh) for social purposes infrequently occurred. Records at Talang Pauh climate station showed that in the last decade, Taba Penanjung area received the annual rainfall around 2848 mm, with no monthly rainfall less than 100 mm. November, December, and January respectively received, 533, 420, 304 mm rainfall, higher than that of the other months. August was known to receive the lowest monthly rainfall (BPS Kab. Bengkulu Tengah 2012). Unusual low monthly rainfall occurred in 1991 and 1994, when September and October got only 3 mm. The monthly relative humidity was 83%, and reached as high as 87.7%, but dropped as low as 75.96%. The average monthly temperature was 26.2 ^{0}C, and reached its respective maximum and minimum at 29 ^{0}C in August, and 23 ^{0}C in October (Susatya 2007). Basic floristic data were collected from a one-hectare plot in 2015. All trees with dbh of > 5 cm were tagged, their diameters measured, and their herbarium specimens collected. Species identification was carried out in the Herbarium of Universitas Bengkulu (HUB). Species nomenclature followed by Turner (1995). In the case of the absence of tree's reproductive aspects such as floral sexuality, flowering phenology, and seed size for each species, I relied on the available secondary information including Soerianegara and Lemmens (1994), Lemmens et al. (1995), Sosef et al. (1998), and Plants of Southeast Asia (www.asianplant.net) to collect those data.

To determine the potential risk of failed regeneration (PRR), I applied Analytic Hierarchy Process (AHP) developed by Saaty (1980), and adopted a similar approach from Oktariadi (2009), who used AHP to develop the risk ranking of tsunami in Southern Java. The method of AHP was selected because PRR was calculated by summing the score of different criteria or biological aspects such as density, floral sexuality, flowering phenology, and seed size. AHP is widely used for selecting alternatives from different criteria in different hierarchies. AHP transforms qualitative data into the quantitative ones through pairwise comparisons by experts (Saaty, 1980). Each comparison was conducted to assign a value between two criteria according to their relative importance (Table 1).

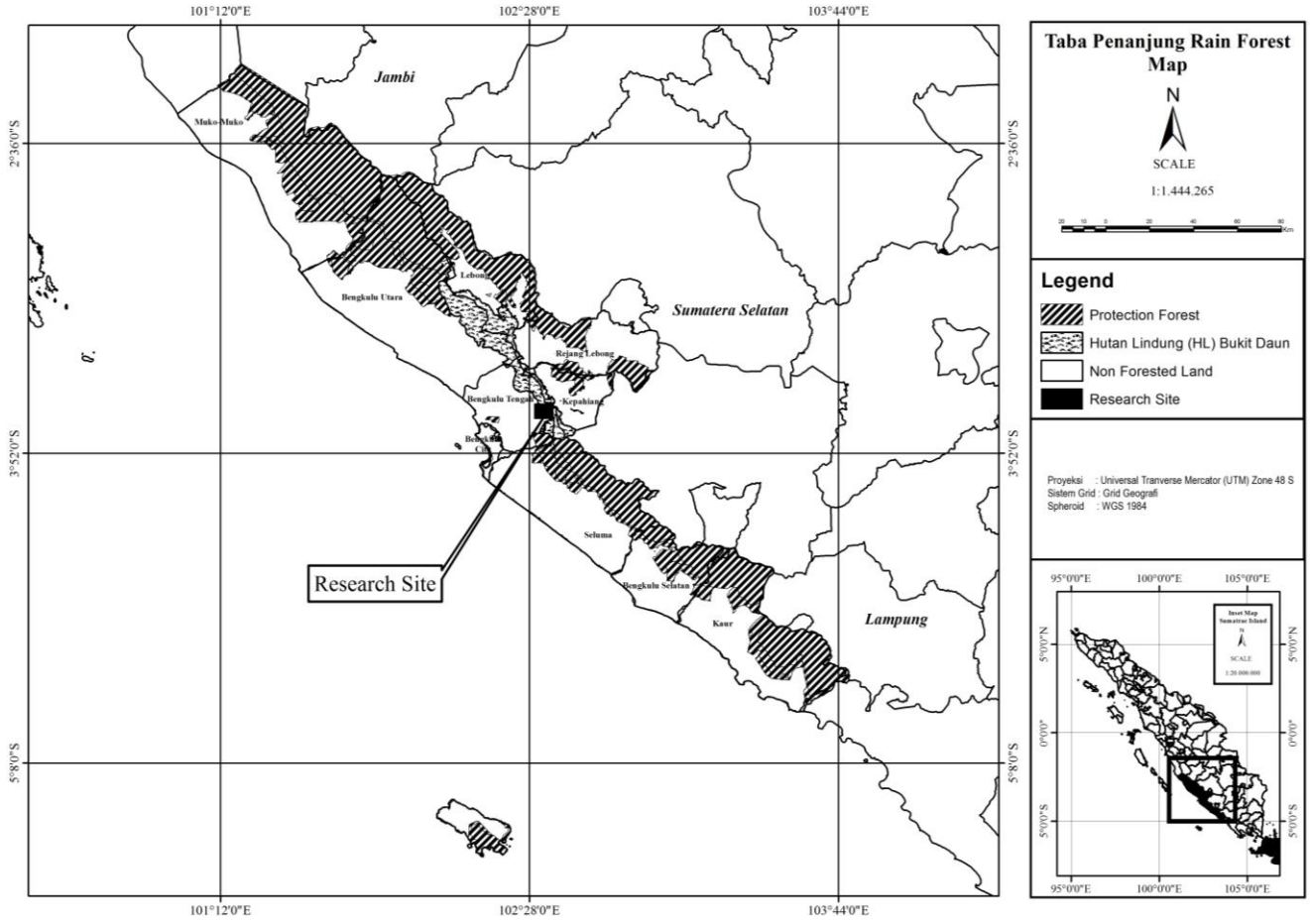

Figure 1. Study site in Taba Penanjung Area within Bukit Daun Protection Forest, Bengkulu Province, Indonesia

Table 1. Assigned values of pair-wise comparisons

Assigned value	Definition	Explanation judgment
1	Equally important	Two criteria or subcriteria are equally important to influence the potential risk of regeneration failure.
3	Moderately more important	One criterion or subcriterion is moderately more important to influence the potential risk of regeneration failure.
5	Much more important	One criterion or subcriterion is much more important to influence the potential risk of regeneration failure.
7	Very much more important	One criterion or subcriterion is very more important to influence the potential risk of regeneration failure.
9	Extremely more important	One criterion or subcriterion is extremely more important to influence the potential risk of regeneration failure.
2, 4, 6, 8	Intermediate judgment value	Values between two consecutive judgments

For the purpose of the study, two hierarchies were established. The first hierarchy consisted of main criteria such as species sexuality (S_i), flowering phenology (P_i), seed size (Z_i), and rarity (R_i) or the number of individual trees per species per ha. Meanwhile, the second hierarchy was subcriteria within sexuality, phenology, seed size, and rarity. Subcriteria of floral sexuality (S_j) included hermaphrodite (S_1), monoecious (S_2), dioecious (S_3), while subcriteria of the flowering phenology (P_j) consisted of once (P_1), twice (P_2), throughout year (P_3), and supra annual (P_4). We defined supra annual category as tree species that performs flowering every more than 1 year, while throughout year was tree species that flowers more or less continuously within a year. The subcriteria of seed size (Z_j) was categorized and developed following Chacon et al. (1998). It consisted of very small (0-4 mm), small (4-8 mm), medium (8-12 mm), large (12-16 mm), and very large (> 16 mm), and was respectively coded as Z_1, Z_2, Z_3, Z_4, and Z_5. Subcriteria of the rarity (R_j) consisted of R_1, R_2, R_3, R_4, R_5, R_6, and R_7, which was defined by species with 1, 2, 3, 4, 5-6, > 7 trees per ha, respectively. Three senior ecologists within the Department of Forestry, University of Bengkulu were selected as experts to carry out pairwise comparisons between two criteria or subcriteria. The results of pairwise comparisons were used to construct the matrix of the value judgments, which was further analyzed to find the matrix of the priority rank (eigenvalue). Each eigenvalue of criteria or subcriteria reflected the score of their relative importance in determining the potential risk of failed regeneration.

At each hierarchy level, the consistencies of all scores were checked by comparing their calculated consistency ratios with Saaty's consistency ratio table. If there were inconsistencies in their judgments, all processes of pairwise comparison and analysis were repeated (Saaty 1980, Saaty 2008). Potential risk of failed regeneration, then, was calculated by summing the score of criteria and subcriteria of each species $_i$ ($S_i S_j + P_i P_j + Z_i Z_j + R_i R_j$) x 100. Five categories of the potential risks consisting of very high, high, medium, low, and very low were developed. A species was included in either very high, high, medium, low or very low risks, if it had respectively a total score of PRR between 30.93-36.44, 25.42-30.93, 19.91-25.42, 14.41-19.92, and 8.89-14.40. Potential risk was developed to indicate the relative sensitivity of a species to regeneration failure. It was aimed to extend the interpretation of species threats and the modifications of the systems in determining extinction risk in IUCN at local level. A species with very high-risk category implies that over the time, this species is expected to be more sensitive to the regeneration failure than those in lower risk categories. Any species with very high and high risks will have respectively very high and high probability of regeneration failure in the near future.

RESULTS AND DISCUSSION

Taba Penanjung lowland rainforest consisted of 118 species belonging to 69 genera and 37 families (Supplement). The forest structure was composed mostly of species with a single tree per ha that contributed to 60 species or 50.85% of the total species (Figure 2.A). Only four species, namely *Microcos laurifolia, Croton argyratus, Elateriospernum tapos,* and *Endospermum diadenum,* had more than 10 trees/ha. *Elateriospernum tapos* (Euphorbiaceae) had the highest density per ha with 31 trees. Following to Ng's category (1978) on species rarity, who defined that any species with a single tree is categorized as rare species, then the forest structure is unproportionally composed of rare species. This rarity was also shown at both genus and family levels in that 8 families or 21.62% of the total families and 20 genera or 28.98% of the total were respectively represented by a single tree. Therefore, any loss of an individual tree of the rare species can result in the loss of species, genus, and family. The unproportional number of rare species composing forest structure appears to be common in Bengkulu such as in Tambang Sawah lower montane forest of Kerinci-Seblat National Park (Susatya 2010), and Talang Tais secondary lowland rainforest (Susatya 2007). Euphorbiaceae was the most diverse family with 11 genera

and 23 species, followed subsequently by Moraceae with 3 genera and 13 species, and Meliaceae with 3 genera and 10 species. It seems that the abundant species of Euphorbiaceae is one of the characters of the floristic composition of Sumatra lowland forest. This is also observed elsewhere in West Sumatra (Hadi et al. 2009, Kohyama et al. 1989). Interestingly, the rare species was also common among the most diverse families. Among 23 species of Euphorbiaceae, 11 species (47.82%) were rare species. Meanwhile, the families of Moraceae and Meliaceae respectively had 33.33% and 40% of their species categorized as rare. Species characterized by very few individual trees per ha potentially faces more difficult to maintain its population.

According to floral sexuality, hermaphrodite species were the most prevalent, contributing to 41.53% of the total species (49 species). Meanwhile, monoecious and dioecious species respectively consisted of 33.05% (39 species), and 25.42% (30 species) (Figure 2.B). Monoecious and dioecious species generally account to 4% and 6% respectively, but the later appears to be more prevalent in the tropics than in temperate regions (Renner 2014). The number of monoecious and dioecious species of Taba Penanjung lowland rain forest was higher than that of Costa Rica wet premontane forest (Breanne 2017) as well as of Central Kalimantan, Indonesia (Brearley et al. 2007). Monoecious and dioecious species of Costa Rica premontane respectively account to 13.10% and 13.70% (Breanne 2017), while the similar categories of Central Kalimantan respectively contribute to 14.70% and 23.49% of the total species (Brearley et al. 2007). Special to dioecious species, its number appears to be more similar than those found at both Sarawak (Ashton 1969) and Pasoh forests (Kochummen et al. 1991).

Dioecious species at both sites respectively account to 26% and 28%. The number of monoecious and dioecious species of the site altogether accounted up to 58,47% of the total species (69 species). This shows that more than half of the total species have to perform outcrossing in order that pollination can occur. Such a process requires both the synchronization of flowering phenology and the presence of pollinators, which could lead to uncertainty on seed production and tree regeneration. The uncertainty is even greater because the sex ratio of dioecious species is male-favored (Queenborough et al. 2009; Gao et al. 2012), and male flowers bloom earlier than their female opposites at certain species (Queenborough et al. 2013).

With regard to tree regeneration, dioecious species have advantages because they tend to yield large seeds, containing more energy (Varmosi et al. 2008), which increase the probability of seedling survivorship. However, dioecious species also face the difficult regeneration, because they have only half of their adult trees to produce seeds (Renner 2014). Dioecious tree species also tend to generate high seed density around their female parent trees, which further lead to high seed predation. It is speculated that the more distant the individual dioecious trees grow from their female parent trees, the higher probability of their seed survivorships and the better seedling recruitment they have due to predation avoidance (Abebbe 2008).

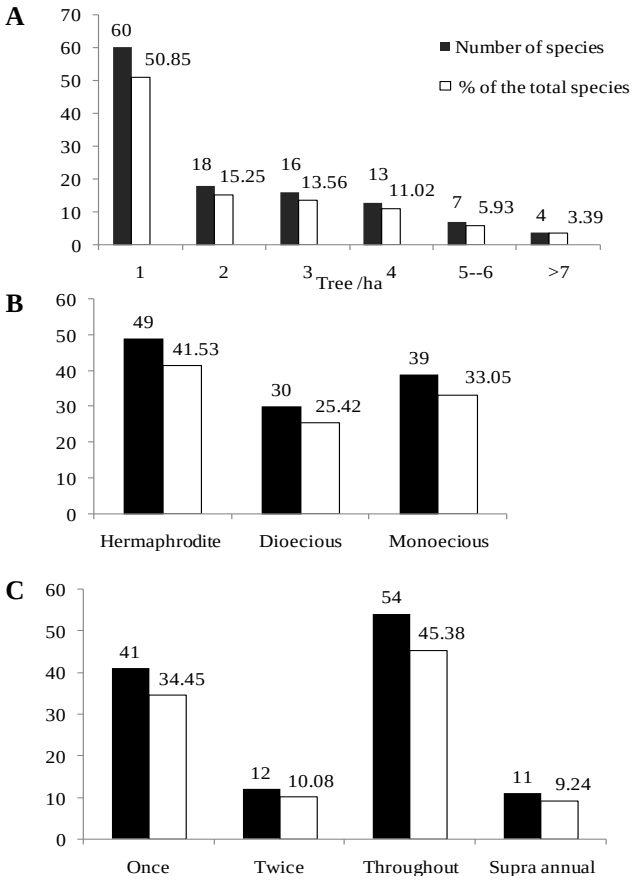

Figure 2. The structure of Taba Penanjung (Bengkulu, Indonesia) lowland rainforest according to: A. Tree density, B. Floral sexuality, C. Flower phenology of its species

Taba Penanjung lowland rainforest has diverse tree species based on flowering phenology. According to flowering phenology, the majority of species of Taba Penanjung rain forest perform either throughout or once flowering phenology (Figure 2.C). Flowering phenology shows the incidence of reproductive efforts of the species, which reflects and will determine the probability of the reproductive success. The throughout flowering species has relatively higher probability to ensure seed production and tree regeneration in the future than the supra annual category, simply because the former produces more frequent flowers and fruit than the later, which only produces flower and fruit once for every two to five years. Flowering phenology is an important factor in tree regeneration, because the length, timing of flowering and fruiting coupled with seasons will determine seed production, seedling mortality, establishment, and growth (Augspurger 1981). Furthermore, the role of environments becomes a pivot point in tree regeneration because the flowering phenology shows a strong correlation with climate, rainfall, and humidity, drought and temperature (Kushwaha et al. 2011; Sulistyawati et al. 2012). Species with throughout flowering contributed to 45.38% of the total species (54 species), while those with once flowering a year accounted to 34.45% (41 species). The number of

species with twice a year and supra annual flowering patterns was not as many as both throughout and once flowering phenologies. Both patterns respectively accounted to 10.08% (13 species) and 9.14% (11 species). The number of species with supra annual flowering in the site was much lower than that of Central Kalimantan (Brearley et al. 2007), and of Lambir hill of Sarawak (Sakai et al. 1999). The supra annual flowering species at those two last sites respectively account to 75%, and 54%. Both forests are dominated by species of Dipterocarpaceae (Brearley et al. 2007; Sakai et al. 1999), which are well known to perform mass flowering or supra annual flowering. Meanwhile, Taba Penanjung rainforest is dominated by species of Euphorbiaceae and Moraceae, which are recorded to have throughout flowering patterns (Whitmore 1983).

Most of species fallen into medium risk category, contributing to 38.14% of the total species. Species with this medium risk indicate that they do not face an immediate risk which may further threaten their regeneration. Other categories namely very high, low, and high risks had almost similar values, ranging from 19.49%, 18.64%, to 17.80% (Figure 3). Both very high and high-risk categories altogether accounted to 37.29%, indicating that more than one-third of the species will face more serious threat to their tree regenerations in the near future.

Twenty three species (Figure 3) from five families were included in very high-risk category, consisting of species from Euphorbiaceae, Myristicaceae, Lauraceae, Flaucourticaeae, Moraceae, and Rubiaceae. Each of these families respectively contributed to 52.17% (12 species), 21.74% (5 species), 13.04% (3 species), and 4.3% (1 species). Of these families, all species of Myristicaeae, namely *Knema globularia*, *Knema glauca*, *Horsfieldia polyspherula*, *Horsfieldia costulata*, and *Gymnacranthera forbesii*, were included in this category. The very highrisk category was dominated by dioecious species (22 of 23 species). The only monoecious included in this category was *Artocarpus kemando* (Moraceae). *Artocarpus kemando* was characterized by a single tree per species per ha, large seed category, and supra annual flowering. The combination of these biological characters makes this species have very highrisk. A note of this species is that the incident of supra annual flowering is relatively longer that of the other supra annual flowering species. It has been recorded to not produce flowers within 7 years (Sosef et al. 1998). The very high risk category consisted not only of species with a single tree; in fact, 7 of them had more than one tree per ha, namely *Aporosa accuminatisima* (2), *Actinodaphne peduncularis (*2 trees), *Drypetes longifolia* (2 trees), *Hydnocarpus curtisi* (3 trees), *Horsfieldia costulata* (2 trees), and *Knema globularia* (3 trees).

All of these species have similar characters such as dioecious and extra large seeds, with various flowering phenology. Among these species, *Aporosa accuminatisima* and *Actinodaphne peduncularis* are recorded to have ecological disadvantages, where the former has been reported for its low regeneration, and the later has been known for its restricted distribution (Sosef et al. 1998). It appears that the extra large seed category, and the species density of more than one tree per ha will put the dioecious species into very high risk regardless of their flowering phenologies. The extra large-size seed is generally recalcitrant, which has very fast germination, low capability of dormancy, and high sensitivity to drought (Marcos-Filho 2005). Water content within seeds of this type determines germination success, and varies according to species and habitat quality. For example, the seed of *Shorea roxburghii*, which has habitat with low rainfall, is tolerant to low water content and still be able to germinate when the water content reaches as low as 35%. Meanwhile, the seeds of other species such as *S. almon*, and *S. robusta* can not germinate when the water content is less than 40%. Recalcitrant seeds generally are not able to germinate if the water content reaches as low as 20%-30% (Davies and Ashton 1999). This is the reason why the species with large seeds prefer to grow and become common in the moist condition under canopy trees, but hardly survive in open canopy, or a dry, warm, and disturbed habitat.

Davies and Ashton (1999) raise the issues on the disadvantages for large-seed species. A large seed tends to have lower fecundity and can hardly thrive at a forest gap habitat. On the other hand, a large seed has the advantages of having more energy reserved in its cotyledon. In a good-quality environment, the large seed germinates and grows rapidly, and has high seedling survivorship due to the large energy stored in cotyledon (Arunachalam et al. 2003). On the contrary, the small-size seed size is generally more tolerant to decreasing water contents (Chin et al. 1989). Small-size seeds are categorized as orthodox which generally tolerate drought, and are well known to have long dormancy. Therefore, species with small seeds are able to wait until suitable environments become available for their germination. The presence of gap generating more light intensity, drier and less moist conditions triggers small seeds to germinate and dominate the open habitat (Marcos-Filho 2005). Furthermore, a small seed has many ecological advantages of having wider dispersal, being able to select suitable microclimates, and having high fecundity (Davies and Ashton 1999).

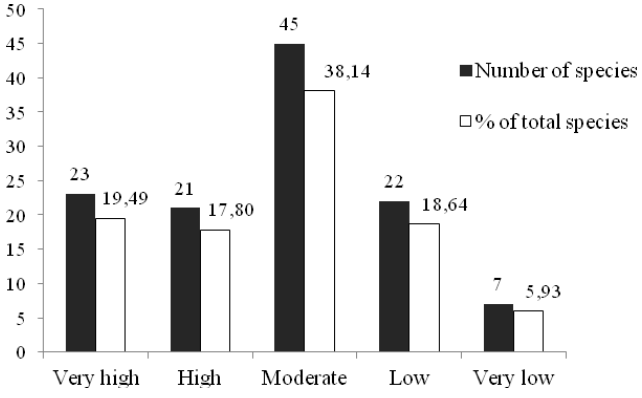

Figure 3. The potential risk of regeneration failure of trees of Taba Penanjung (Bengkulu, Indonesia) lowland rainforest

The majority of the very high-risk category had throughout flowering (12 species), followed by once flowering phenology type (6), while supra annual flowering only consisted of 3 species. This flowering phenology variation shows that the phenology does not determine the very high-risk category. Furthermore, the very high risk comprised 8 and 7 species respectively characterized by extra large and small seed categories. Similar to the phenology, seed size does not determine the very high-risk category either. Therefore, in general, if a species is dioecious and rare, then the species is likely to belong to very high category regardless of seed size and flowering phenology. Dioecious species generally fall into very high-risk category, because of the complexity of reproduction biology. To carry out reproduction efforts, they require flowering synchronization and the presence of pollinators. Dioecious species also show more limited reproduction capacity than those having other flower sexuality types, simply because they have only half of their mature trees contributing to seed production. Their flowering and fruiting successes are also influenced by both the distance between male and female trees and the pollinator movement from male to female trees (Renner 2014). During pollination process, pollinators travel a certain distance which further adds up to the uncertainty of fruit production. The farther the distance between mature male and female trees, the more uncertain pollination process to occur. It was estimated that the closest distance between the same tree species could reach up to 131 m, and the distance between female and male trees could be even farther (Abebbe, 2008). The difficulty of the regeneration of dioecious species is even greater due to the fact that the microclimates beneath male mature trees play a determining role in regeneration. It has been known that seedling and sapling recruitments tend to be greater under the male trees than the female trees (Arai and Kamitani 2005).

A rare species is expected to face the regeneration problem due to its difficulty to maintain its population density. A rare or single-tree per ha species is more likely to experience failed regeneration simply because it statistically has a lower chance to regenerate than those of more than one tree per ha. Forest structure dominated by species having very few individual trees appears a common ecological attribute of species-rich Southeast Asia rainforest (Susatya 2010) and Nigerian rain forest (Adekunle et al. 2013). From the forest tree regeneration perspective, this attribute has been worsened by the fact that even hermaphrodite species tend to be not self-compatible (Bawa 1979; Bawa et al. 1989). However, in this research, a species with a single tree alone does not necessarily determine whether the species belongs to either very high or high-risk categories. In fact, 55% of the total of rare species are classified into either medium risk (27 species) or low risk (5 species) category. Only rare species with either dioecy or monoecy are most likely to belong to either very high or high-risk category. Rare species with high and very high-risk categories accounted up to 12 species and 16 species, respectively.

Moreover, rare hermaphrodite species will fall into medium risk category regardless of seed size and flowering phenology. However, rare hermaphrodite species with both small seed category and throughout phenology such as *Bhesa paniculata* (Celastraceae), *Astronia macrophylla* (Melastomaceae), *Neolamarckia cadamba* (Rubiaceae), *Micromelum minutum* (Rutaceae), and *Rinorea anguifera* (Violaceae) were included in low-risk category. These combined criteria make these five species have better reproductive success as well as better seedling survivorship than the monoecious and dioecious species. This pattern shows that being dioecious or monoecious is more influential in determining very high and high risks than being rare species. As long as a rare species does not belong to dioecious and monoecious categories, it will not be included in either very high or high-risk category.

The high-risk category was composed of 21 species of 10 families (Figure 3), of which family of Meliaceae contributed most with 7 species, while the other nine families only contributed from one to four species. High-risk category comprised various species with all types of the flower sexuality. Monoecious species contributed most with 13 species (61.90%), followed by dioecious species (6 species). Meanwhile, hermaphrodite species only accounted to 2 species. Interestingly, of the 48 hermaphrodite species generally belonging to either medium or very low risk, two were included in high-risk category, namely *Shorea ovalis* and *Palaquium hexandrum*, both of which are characterized by extra large seed and supra annual flowering phenology. The combination of extra large seed and less frequent incidence of flowering and fruiting makes those two species classified into high-risk category. In addition to these biological aspects, an external factor in the form of timber harvesting becomes an imminent threat to these two species. The population of *Shorea ovalis*, the member of commercial light Red Meranti group, is also dwindling due to logging. Like other species of *Palaquium*, *Palaquium hexandrum* faces a reproductive problem, because its flowers hardly reach maturity due to insect predation. If they pass through fruit development, then their fruit suffer high predation by bats, birds, and squirrels (Soerianegara and Lemmens 1994). Furthermore, a special attention has to be made for a rare species of *Diospyros sumatrana* which has been classified into high-risk category. The species appears to face fruit development problem, because it needs long period of time to reach fruit ripening (Lemmens et al. 1995). Such a long period could result in being more vulnerable to fruit predation, which could further lead to lower its regeneration capability.

Not all of the monoecious species fall into a single category. Most of the monoecious species fallen into medium risk (18 species), subsequently followed by high risk (13 species), low risk (6 species), and very high and very lows risk categories which respectively consisted of only 1 species. Monoecious species with one to two trees per ha, large and extra large seed categories, and once flowering pattern will fall into high-risk category. Meanwhile, similar monoecious species with very small seed and throughout phenology (12 species) will belong to

medium risks category. Interestingly, monoecious species with 3-4 trees but with once and supra annual flower will also belong to medium risk category regardless of seed size. It appears that whether a monoecious species will fall into a certain category is not solely defined by its rareness, but also by the seed size and flowering phenology.

Species belonging to medium risk category should not face immediate threats for their regeneration. However, timber harvesting will potentially jeopardize their future. Among the medium risk category (45 species), 26.27% (12 species) are either included into major or minor commercial timbers (Soerianegara and Lemmens 1994; Lemmens et al. 1995). This indicates that these species are likely to become a target for logging in the near future. In addition to a timber harvesting factor, their ecological attributes could potentially increase the risks of several medium risk species. For example, the tree population of *Alstonia angustiloba* has been locally depleted due to logging (Soerianegara and Lemmens 1994), while *Baccaurea racemosa* has been recorded as uncommon species at the lower strata of the Southeast Asia rain forest (Sosef et al. 1998). Furthermore, *Endospernum diadenum* faces a high predation of its seeds and is known to have low seed viability (Soerianegara and Lemmens 1994). Three species of *Polyalthia, P. hookeriana, P. michaelii*, and *P. rumphii* are noted to have scattered distribution, and their seedlings are sensitive to drought (Sosef et al. 1998).

The hermaphrodite species were included in various categories from medium to very low risk. Rare hermaphrodite species are likely to fall into medium risk, if they have medium to extra large seeds, regardless of their flowering phenologies. However, rare hermaphrodite species with small to very small seed categories and throughout flowering pattern will fall into low risks. Furthermore, hermaphrodite species with more than one tree are most likely to belong to low and very low-risk categories. Hermaphrodite species with more than 4 trees will come up into two different categories depending on their seed sizes. Those with small and medium sizes will end up to very low-risk category, while those with large and extra large seeds will fall into low category. The former consists of *Shorea platyclados, Barringtonia lanceolata,* and *Syzygium rostrata*, while the later are *Geunsia hexandra*, *Dillenia excelsa*, *Strombosia javanica*, *Neonauclea gigantea, Microcos laurifolia, Euonymus javanicus,* and *Cratoxylum sumatrana.*

Of the 118 tree species, only 21 species (17.79%) are listed in 2017's IUCN red list. The other species are listed as not assessed species, meaning the conservation statutes of these species have not been evaluated according to IUCN's criteria. The tree species listed at IUCN consists of 2, 1, 3, and 15 tree species respectively categorized into endangered (E), Vulnerable (V), near threatened (NT), and least concerned (LC). Furthermore, of the 23 species with very high-risk category, only three tree species, namely *Litsea spathacea, Knema glauca, K. globularia* have been included into least concerned. Of the 21 species with high-risk category, seven are categorized into four different IUCN's conservation status. Two species, namely *Aglaia speciosa* and *Sterculia oblongata,* are classified as endangered, while *Sterculia parvifolia* is included as vulnerable. Furthermore, three species of Meliaceae, namely *Aglaia odoratissima, A. oligophylla,* and *A. rubiginosa,* are grouped into near threatened. Species of *K. glauca, K. globularia, Litsea spathacea, Aglaia tomentosa, Sterculia parviflora, Archidendron ellipticum, Magnolia sumatrana, Microcos laurifolia, Alstonia angustiloba, Bhesa paniculata, Euonymus javanicus, Payena maingayi, P. lanceolata, Polyalthia hookeriana,* and *Prunus arborea,* are categorized as the least concerned species. Comparing the IUCN RedList and the results of the potential risk analysis resulted in interesting outcomes. Among the 15 species listed as least concerned by IUCN, nine are classified into medium to low risk categories, which is almost similar to least concerned category, while the other six species, namely *K. glauca, K. globularia, Aglaia tomentosa, Archidendron ellipticum, Litsea spathacea*, and *Sterculia obolongata* are either included in high risk or very high-risk category. The first two were very high-risk species characterized by dioecious species with supra annual flowering phenology, while the rest were high-risk monoecious species with large seed category. *S. oblongata,* vulnerable species by IUCN, was classified into high risk. Both seem to be comparable status, where both indicate that in the near future, the species will face difficulty to maintain its population density in order to avoid local extinction. Interestingly, *Shorea platiclados* that has long been classified as endangered species (Ashton 1998), did fall into low risk. Low-risk category of this species indicates that it relatively does not face immediate threat on its tree regeneration locally, and is considered to be able to ensure its future regeneration. The number of tree per ha (4 trees) became the main reason for the species to be classified as low-risk category. Moreover, among the 97 species whose conservation statuses have not been evaluated by IUCN, 22 and 13 were respectively included in very high and high potential risk categories.

The conservation statuses of most of the very high and high-risk tree species have not been evaluated according to IUCN's criteria. IUCN is aware that there is a need for more detailed evaluation for conservation status at local level because differences between global and local threats are very important for determining the status. It further indicates that a species which has been globally categorized into endangered could be the least concerned category due to steady population at a local level. On the other hand, species with least concern status can turn into endangered category due to its small and locally dwindling population (IUCN 2012). The results of this research make more detailed ecological information concerning the potential risk of the failure of tree regeneration available, which is not always provided by IUCN red list documents. The results are very important to serve as both substitutes and guidance at local level for conservation purposes in the absence of conservation status of IUCN of the tree species.

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Table S1. Tree species of Taba Penanjung Lowland Rainforest and their potential risks of the regeneration failure

Species	Family	Rarity		Floral sexuality		Seed size		Flowering phenology		Total score	PRR
		Tree/ha	Score	Type	Score	Type	Score	Type	Score		
Actinodaphne peduncularis L	Lauraceae	2	0.238	Dio	0.581	Exl	0.3260	Onc	0.281	34.1826	VH
Aglaia affinis Merr	Meliaceae	2	0.238	Mon	0.309	Med	0.1770	Onc	0.281	25.6374	H
Aglaia faveolata Pannell	Meliaceae	1	0.286	Mon	0.309	Med	0.1770	Onc	0.281	27.8982	H
Aglaia odoratissima Blume	Meliaceae	1	0.286	Mon	0.309	Lrg	0.2960	Onc	0.281	29.1834	H
Aglaia oligophylla Miq	Meliaceae	3	0.19	Mon	0.309	Exl	0.3260	Onc	0.281	24.9858	M
Aglaia rubiginosa (Hiern.) Pannell1	Meliaceae	1	0.286	Mon	0.309	Lrg	0.2960	Onc	0.281	29.1834	H
Aglaia speciosa Blume	Meliaceae	2	0.238	Mon	0.309	Lrg	0.2960	Onc	0.281	26.9226	H
Aglaia tomentosa Teijm ex Binn	Meliaceae	1	0.286	Mon	0.309	Lrg	0.2960	Onc	0.281	29.1834	H
Alstonia angustiloba Miq.	Apocynaceae	1	0.286	Hmp	0.11	Vsm	0.087	San	0.412	24.0132	M
Antidesma brachybotrys Airy Shaw	Euphorbiaceae	1	0.286	Dio	0.581	Sml	0.114	Thr	0.116	31.4313	VH
Antidesma griffithii Hoof. F	Euphorbiaceae	1	0.286	Dio	0.581	Lrg	0.2960	Thr	0.116	33.3969	VH
Antidesma leucocladon Hook.f	Euphorbiaceae	1	0.286	Dio	0.581	Lrg	0.2960	Thr	0.116	33.3969	VH
Antidesma montanum Blume	Euphorbiaceae	2	0.238	Dio	0.581	Vsm	0.087	Thr	0.116	28.8789	H
Antidesma velutinosum Blume	Euphorbiaceae	3	0.19	Dio	0.581	Sml	0.114	Thr	0.116	26.9097	H
Aporosa accuminatisima Merr	Euphorbiaceae	2	0.238	Dio	0.581	Sml	0.114	Onc	0.281	31.8930	VH
*Archidendron ellipticum (*Blume) Nielsen	Leguminosae	1	0.286	Mon	0.309	Exl	0.3260	Thr	0.116	26.7849	H
Artocarpus anisophyllus Miq.	Moraceae	4	0.143	Mon	0.309	Lrg	0.2960	San	0.412	24.6096	M
Artocarpus elasticus Reinw. ex Blume	Moraceae	4	0.143	Mon	0.309	Exl	0.3260	Onc	0.281	22.7721	M
Artocarpus kemando Miq	Moraceae	1	0.286	Mon	0.309	Lrg	0.2960	San	0.412	31.3449	VH
Astronia macrophylla Blume	Melastomaceae	1	0.286	Hmp	0.11	Sml	0.114	Thr	0.116	19.4208	L
Baccaurea bracteata Mull. Arg	Euphorbiaceae	1	0.286	Dio	0.581	Med	0.1770	Thr	0.116	32.1117	VH
Baccaurea edulis Merr	Euphorbiaceae	1	0.286	Dio	0.581	Exl	0.3260	Onc	0.281	36.4434	VH
Baccaurea racemosa (Reinw. ex Blume) Mull. Arg.	Euphorbiaceae	6	0.095	Dio	0.581	Lrg	0.2960	Thr	0.116	24.4008	M
*Baccaurea sumatrana (*Miq.) Mull. Arg.	Euphorbiaceae	3	0.19	Dio	0.581	Med	0.1770	Thr	0.116	27.5901	H
Barringtonia lanceolata (Ridl.) Payen	Lecythidaceae	4	0.143	Hmp	0.11	Exl	0.3260	Thr	0.116	14.9751	L
Bhesa paniculata Arn	Celastraceae	1	0.286	Hmp	0.11	Sml	0.114	Thr	0.116	19.4208	L
Bridelia insulana Hance	Euphorbiaceae	3	0.19	Mon	0.309	Sml	0.114	Thr	0.116	19.9737	M
Campnosperma auriculatum (Blume) Hook.f	Anarcadiaceae	1	0.286	Mon	0.309	Med	0.1770	Thr	0.116	25.1757	M
Casearia capitellata Blume	Flacourtiaceae	1	0.286	Hmp	0.11	Exl	0.3260	Thr	0.116	21.7104	M
Casearia clarkei King *var. kunstleri* (King) Ridl.	Flacourtiaceae	1	0.286	Hmp	0.11	Exl	0.3260	Thr	0.116	21.7104	M
Castanopsis inermis (Lindl.) Benth. ex Hook. F	Fagaceae	4	0.143	Mon	0.309	Lrg	0.2960	Onc	0.281	22.4481	M
Chionanthus pluriflorus (Knob) Kew	Oleaceae	2	0.238	Hmp	0.11	Lrg	0.2960	Thr	0.116	19.1256	L
Chionanthus spicata Blume	Oleaceae	1	0.286	Hmp	0.11	Lrg	0.2960	Thr	0.116	21.3864	M
Commersonia bartramia (L) Merr.	Sterculiaceae	2	0.238	Mon	0.309	Sml	0.114	Thr	0.116	22.2345	M
Cratoxylum sumatrana (Jack.) Blume	Hypericaceae	6	0.095	Hmp.	0.11	Sml	0.114	Onc	0.281	13.1472	VL
Croton argyratus Blume	Euphorbiaceae	15	0.048	Mon	0.309	Med	0.1770	Thr	0.116	13.9659	VL
Dacryodes rugosa (Blume) H. J. Lam	Burseraceae	3	0.19	Dio	0.581	Exl	0.3260	Thr	0.116	29.1993	H
Dillenia excelsa (Jack.) Gilg	Dilleniaceae	6	0.095	Hmp	0.11	Sml	0.114	Onc	0.281	13.1472	VL

Diospyros sumatrana Miq	Ebenaceae	1	0.286	Mon	0.309	Sml	0.114	Onc	0.281	27.2178	H
Drypetes longifolia (Blume) Pax ex. K. Hoffm	Euphorbiaceae	2	0.238	Dio	0.581	Exl	0.3260	Onc	0.281	34.1826	VH
Durio zibethinus L	Bombacaceae	1	0.286	Hmp	0.11	Exl	0.3260	Twi	0.191	22.9479	M
Dysoxylum arborescens (Blume) Miq	Meliaceae	2	0.238	Mon	0.309	Exl	0.3260	Onc	0.281	27.2466	H
Dysoxylum densiflorum (Blume) Miq.	Meliaceae	4	0.143	Mon	0.309	Sml	0.114	Onc	0.281	20.4825	M
Dysoxylum excelsum Blume	Meliaceae	4	0.143	Mon	0.309	Exl	0.3260	Onc	0.281	22.7721	M
Elaeocarpus nitidus Jack	Elaeocarpaceae	1	0.286	Hmp	0.11	Med	0.1770	Onc	0.281	22.8237	M
Elateriospernum tapos Blume	Euphorbiaceae	31	0.048	Mon	0.309	Exl	0.3260	Thr	0.116	15.5751	L
Endospermum diadenum (Miq.) Airy Shaw	Euphorbiaceae	12	0.048	Dio	0.581	Vsm	0.087	Thr	0.116	19.9299	M
Erismanthus obliquus Wall ex. Mull. Arg.	Euphorbiaceae	1	0.286	Mon	0.309	Sml	0.114	Thr	0.116	24.4953	M
Euonymus javanicus Blume	Celastraceae	3	0.19	Hmp.	0.11	Lrg	0.2960	Onc	0.281	19.5873	L
Ficus benjamina L	Moraceae	1	0.286	Mon	0.309	Vsm	0.087	Thr	0.116	24.2037	M
Ficus depressa Blume	Moraceae	1	0.286	Mon	0.309	Vsm	0.087	Thr	0.116	24.2037	M
Ficus fistulosa Reinw. ex. Blume	Moraceae	2	0.238	Mon	0.309	Vsm	0.087	Thr	0.116	21.9429	M
Ficus fulva Reinw. ex. Blume	Moraceae	2	0.238	Mon	0.309	Vsm	0.087	Thr	0.116	21.9429	M
Ficus heteropleura Blume	Moraceae	3	0.19	Mon	0.309	Vsm	0.087	Thr	0.116	19.6821	L
Ficus lepicarpa Blume	Moraceae	2	0.238	Mon	0.309	Vsm	0.087	Thr	0.116	21.9429	M
Ficus ribes Reinw. ex Blume	Moraceae	3	0.19	Mon	0.309	Vsm	0.087	Thr	0.116	19.6821	L
Ficus sundaica Blume	Moraceae	1	0.286	Mon	0.309	Vsm	0.087	Thr	0.116	24.2037	M
Ficus variegata Blume	Moraceae	4	0.143	Mon	0.309	Vsm	0.087	Thr	0.116	17.4684	L
Flacourtia rukam Zoll. et. Moritzi	Flaucourtiaceae	3	0.19	Hmp	0.11	Sml	0.114	Onc	0.281	17.6217	L
Geunsia hexandra (Teijsm. et. Binn) Koord	Verbenaceae	4	0.143	Hmp	0.11	Sml	0.114	Thr	0.116	12.6855	VL
Gironniera subaequalis Planch	Ulmaceae	5	0.095	Mon	0.309	Sml	0.114	Thr	0.116	15.4992	L
Gordonia maingayi Dyer	Theaceae	1	0.286	Hmp	0.11	Exl	0.3260	Twi	0.191	22.9479	M
Gordonia multinervis King	Theaceae	3	0.19	Hmp	0.11	Exl	0.3260	Twi	0.191	18.4263	L
Gymnacranthera forbesii (King) Ward	Myristicaceae	1	0.286	Dio	0.581	Exl	0.3260	Twi	0.191	34.9584	VH
Horsfieldia costulata Warb	Myristicaceae	2	0.238	Dio	0.581	Exl	0.3260	Thr	0.116	31.4601	VH
Horsfieldia polyspherula (Hook. F) J. Sinclair	Myristicaceae	1	0.286	Dio	0.581	Lrg	0.2960	Thr	0.116	33.3969	VH
Hydnocarpus curtisii King	Flacourtiaceae	3	0.19	Dio	0.581	Exl	0.3260	Onc	0.281	31.9218	VH
Knema glauca (Blume) Petermann	Myristicaceae	4	0.143	Dio	0.581	Exl	0.3260	San	0.412	31.8696	VH
Knema globularia (Lam.) Warb.	Myristicaceae	3	0.19	Dio	0.581	Exl	0.3260	San	0.412	34.0833	VH
Lansium domesticum Corra	Sapindaceae	1	0.286	Mon	0.309	Exl	0.3260	Onc	0.281	29.5074	H
Litsea cubeba (Laur.) Pers	Lauraceae	4	0.143	Dio	0.581	Lrg	0.2960	Onc	0.281	29.3841	H
Litsea sessilis Boerl.	Lauraceae	1	0.286	Dio	0.581	Med	0.1770	Onc	0.281	34.8342	VH
Litsea spathacea Gamble	Lauraceae	1	0.286	Dio	0.581	Med	0.1770	Onc	0.281	34.8342	VH
Macaranga hosei King ex Hook	Euphorbiaceae	1	0.286	Dio	0.581	Vsm	0.087	Thr	0.116	31.1397	VH
Macaranga hulletii King ex Hook. F.	Euphorbiaceae	1	0.286	Dio	0.581	Sml	0.114	Thr	0.116	31.4313	VH
Macaranga triloba (Blume) Mull. Arg.	Euphorbiaceae	1	0.286	Dio	0.581	Sml	0.114	Thr	0.116	31.4313	VH
Magnolia uvariifolia Dandy ex Noot 2	Magnoliaceae	1	0.286	Hmp	0.11	Med	0.1770	Onc	0.281	22.8237	M
Mallatus leptophyllus Pax et C.K. Hoffm.	Euphorbiaceae	2	0.238	Hmp	0.11	Vsm	0.087	Onc	0.281	19.5909	M
Mallotus auriculatus Merr	Euphorbiaceae	3	0.19	Dio	0.581	Sml	0.114	Thr	0.116	26.9097	H
Mallotus montanus (Mull. Arg) Airy Shaw	Euphorbiaceae	1	0.286	Dio	0.581	Sml	0.114	Thr	0.116	31.4313	VH
*Mallotus peltatus (*Geisel.) Mull. Arg.	Euphorbiaceae	1	0.286	Dio	0.581	Sml	0.114	Thr	0.116	31.4313	VH
Microcos laurifolia (Hook et Mast) Burret	Tiliaceae	14	0.048	Hmp	0.11	Med	0.1770	Thr	0.116	8.8914	VL
Micromelum minutum (G. Forst.) Wright and Arn.	Rutaceae	1	0.286	Hmp	0.11	Vsm	0.087	Thr	0.116	19.1292	L

Naphelium lappaceum L	Sapindaceae	1	0.286	Hmp	0.11	Exl	0.3260	Onc	0.281	24.4329	M
Neolamarckia cadamba (Roxb) Basser	Rubiaceae	1	0.286	Hmp	0.11	Vsm	0.087	Thr	0.116	19.1292	L
Neonauclea excelsa Merr	Rubiaceae	1	0.286	Hmp	0.11	Med	0.1770	Thr	0.116	20.1012	M
Neonauclea gigantea Merr	Rubiaceae	6	0.095	Hmp	0.11	Med	0.1770	Thr	0.116	11.1051	VL
Ochanostachys amentacea Mast	Olacaceae	2	0.238	Mon	0.309	Lrg	0.2960	Thr	0.116	24.2001	M
Palaquium hexandrum (Griff.) Baill	Sapotaceae	1	0.286	Hmp	0.11	Exl	0.3260	San.	0.412	26.5944	H
Payena lanceolata Ridl.	Sapotaceae	1	0.286	Hmp	0.11	Lrg	0.2960	Thr	0.116	21.3864	M
Payena maingayi Clarke	Sapotaceae	1	0.286	Hmp	0.11	Lrg	0.2960	Thr	0.116	21.3864	M
Pittosporum ferrugineum W.T. Aiton	Pittosporaceae	3	0.19	Hmp	0.11	Vsm	0.087	Thr	0.116	14.6076	L
Polyalthia hookeriana King	Annonaceae	1	0.286	Hmp	0.11	Lrg	0.2960	Twi	0.191	22.6239	M
Polyalthia michaelii C.T. White	Annonaceae	1	0.286	Hmp	0.11	Med	0.1770	Twi	0.191	21.3387	M
Polyalthia rumphii (Blume) Merr.	Annonaceae	1	0.286	Hmp	0.11	Med	0.1770	Twi	0.191	21.3387	M
Pometia pinnata J.R. Forst et G. Frost	Sapindaceae	1	0.286	Mon	0.309	Exl	0.3260	Onc	0.281	29.5074	H
Prainea limpato (Miq.) Beumee	Moraceae	4	0.143	Mon	0.309	Vsm	0.087	Onc	0.281	20.1909	M
Prunus arborea (Blume) Kalkman	Rosaceae	1	0.286	Hmp	0.11	Sml	0.114	Onc	0.281	22.1433	M
Prunus lamponga (Miq.) Kalkman	Rosaceae	3	0.19	Hmp	0.11	Sml	0.114	Onc	0.281	17.6217	L
Quercus argentata Korth	Fagaceae	7	0.048	Mon	0.309	Exl	0.3260	Onc	0.281	18.2976	L
Rhodamnia cinerea Jack	Myrtaceae	1	0.286	Hmp	0.11	Med	0.1770	Thr	0.116	20.1012	M
Rinorea anguifera (Lour.) Kuntze	Violaceae	1	0.286	Hmp	0.11	Vsm	0.087	Thr	0.116	19.1292	L
Shorea ovalis (Korth.) Blume	Dipterocarpaceae	1	0.286	Hmp	0.11	Exl	0.3260	San	0.412	26.5944	H
Shorea parvifolia Dyer	Dipterocarpaceae	3	0.19	Hmp	0.11	Med	0.1770	San	0.412	20.4636	M
Shorea platyclados Slooten ex. Fox	Dipterocarpaceae	4	0.143	Hmp	0.11	Lrg	0.2960	San	0.412	19.5351	L
Sterculia oblongata R. Br.	Sterculiaceae	1	0.286	Mon	0.309	Sml	0.114	San	0.412	29.3793	H
Sterculia parviflora Roxb. ex. G. Don	Sterculiaceae	1	0.286	Mon	0.309	Lrg	0.2960	Onc	0.281	29.1834	H
Strombosia javanica Blume	Olacaceae	6	0.095	Hmp	0.11	Med	0.1770	Onc	0.281	13.8276	VL
Symplocos crassipes C. B. Clarke	Symplocaceae	2	0.238	Hmp	0.11	Sml	0.114	Twi	0.191	18.3975	L
Syzygium flosculiferum (M. R. Hensd.) Sreek	Myrtaceae	2	0.238	Hmp	0.11	Lrg	0.2960	Twi	0.191	20.3631	M
Syzygium kunstleri (King). Bahadur et R.C. Gour	Myrtaceae	1	0.286	Hmp	0.11	Lrg	0.2960	Twi	0.191	22.6239	M
Syzygium lineatum (DC) Merr. ex L. M. Terry	Myrtaceae	1	0.286	Hmp	0.11	Lrg	0.2960	Onc	0.281	24.1089	M
Syzygium politum (King). I.M. Turner	Myrtaceae	1	0.286	Hmp	0.11	Sml	0.114	Twi	0.191	20.6583	M
Syzygium rostrata Blume	Myrtaceae	4	0.143	Hmp	0.11	Lrg	0.2960	Twi	0.191	15.8886	L
Urophyllum macrophyllum Korth	Rubiaceae	1	0.286	Dio	0.581	Sml	0.114	Thr	0.116	31.4313	VH
Vitex vestita Wall. ex Schauer	Verbenaceae	1	0.286	Hmp	0.11	Sml	0.114	Onc	0.281	22.1433	M
Xylopia caudata Maingay ex. Hook. F. et Thomson	Annonaceae	2	0.238	Hmp	0.11	Lrg	0.2960	Onc	0.281	21.8481	M
Xylopia elliptica Hook.f and Thomson	Annonaceae	1	0.286	Hmp	0.11	Lrg	0.2960	Onc	0.281	24.1089	M

Note: Floral sexuality code; Dioecious (Dio), Monoecious (Mon), Hermaphrodite (Hmp). Seed size code; Extra large (Exl), Large (Lrg), Medium (Med), Small (Sml), Very small (Vsm). Flowering phenology code; Once (Onc), Twice (Twi), Throughout (Thr), Supra annual (San). PRR refers to the potential risk of regeneration failure. PRR code: Very high risk (VH), High risk (H), Medium risk (M),Low risk (L), and Very low (VL).

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Short Communication: Variation in vocal cord morphometric characters among dangdut type and the slow type Gaga Chicken

ABINAWANTO[1,♥], ALFI SOPHIAN[1], PIPIH SUNINGSI EFFENDI[2], TITIN SISWANTINING[3]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia. Jl. Lingkar UI, E Building UI Campus, Depok 16242, West Java, Indonesia. Tel.: +62-21-7270163, Fax.: +62-21-78829010, ♥email: abinawanto.ms@sci.ui.ac.id, abinawanto@gmail.com

[2]Laboratory of Genetics, Faculty of Husbandry, Institut Pertanian Bogor. Jl. Raya Dramaga, Bogor 16680, West Java, Indonesia

[3]Department of Mathematics, Faculty of Mathematics and Natural Sciences, Universitas Indonesia. Depok 16242, West Java, Indonesia

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Abstract. *Abinawanto, Sophian A, Effendi PS, Siswantining T. 2018. Short Communication: Variation in vocal cord morphometric characters among dangdut type and the slow type Gaga Chicken. Biodiversitas 19: 1902-1905.* Gaga chicken is one of the ornamental chicken originating from Sidendreng and Rapang (South Sulawesi). Gaga chicken has a unique crowing sound, like people laugh. Gaga's chicken which has a long and fast crowing sound known as the dangdut type, while those with short and slow crowing sound are known as slow type. Study was conducted in Pinrang (South Sulawesi), where one of the pure strains are located. Samples were collected from Kanie, Bullo, Macege, Rappang, and Sidenreng. The purpose of study was to determine the characteristics of the vocal cord morphometric among the dangdut type and the slow type of Gaga's chicken. All of the morphometric data were recorded and analyzed by Mean Test, using SPSS (version 22). The results showed that there was no significant difference ($\alpha = 0.010$) among the dangdut type and the slow type based on syrinx morphometric. Meanwhile, according to the trachea muscle morphometric the results showed that the trachea muscle of dangdut type was longer than the slow type ($\alpha < 0.010$). In addition, either the right or the left trachea muscle of dangdut type was longer than either the right or the left trachea muscle of the slow type ($\alpha < 0.010$).

Keywords: Gaga chicken, morphometric, vocal cord, dangdut type, slow type

INTRODUCTION

Gaga chicken comes from an area in South Sulawesi named Sidrap (Prawira 2014). At a place in Sidrap called Baranti, chicken laughed or better known to the people by the name of manu "Gaga", has been maintained from generation to generation (Andrianto et al. 2015). Because of its melodious voice, gaga chickens is often made a contest (Robin et al. 2015). Gaga chicken that often wins the contest has a price reaching hundreds of millions of million rupiahs (IDR) so that this chicken has the potential to be developed as a chicken with a good economic value (Prawira 2014; Andrianto et al. 2015; Robin et al. 2015).

Based on the type of crowing sound, gaga chicken is classified into two types, i.e., the slow type and dangdut type (Junaedi 2012). The grouping of type of crowing sound of gaga chicken into dangdut and slow types was based on agreement on the chickens laughing (KOMPAK) (Abinawanto and Efendi 2017). When viewed from the color of the feathers, gaga chicken can be categorized into several types, namely: korro (black base color with golden yellow back), ceppaga (white color scattered on the chest to chicken stomach), lappung (red dominated color yellowish on his body) and bakka (white dominance on all parts of his body) (Roiz 2011; Bahmid 2015).

Chicken diversity can be identified based on several analysis, one of which is morphometric analysis (Abinawanto and Efendi 2017). Morphometric is an identification technique carried out by observing the physical characters (Rusfidra 2004). Campbell and Lack (1985) stated that morphometric consists of two large components, namely the size and the shape. According to Ishii et al. (1996), body size and shape can be used to distinguish a series within a population. The quantitative nature of morphometrics also plays an important role in mapping the productive traits in the utilization of a species; these quantitative properties are influenced by genetic factors (genetic), environmental and genetic and environmental interactions (Campbell and Lasley 1985). According to Miller et al. (2007), study of chicken sound variation can be done by morphometric analysis on vocal cord organ. In a study by Eliyani et al. (2015) revealed that the shape and size of producing organs between roosters and hens were different. The sound in chickens is produced by the organs at the end of the tracheal tract, syrinx (Setijanto 1998). Sound variations in the singer's cock are affected by vocal cord organ including trachea, trachea and syrinx muscles (McLelland 1990; Setijanto (1998). Mutations in sound-producing organs including trachea, tracheal and syrinx muscles cause a certain variation in species Chicken Ketawa (Prawira 2014).

The use of morphometric method to measure chicken kinship has so far been done on Bangkok chicken, Katai chicken (Sitanggang et al. 2016), chicken (Kurnia 2011; Mariandayani et al. 2013; Sitanggang et al. 2016), Chicken Sentul, Chicken Kedu (Kurnia 2011) and Chicken Broiler (Mariandayani et al. 2013) by using body weight analysis,

femur length, tibia length, shank length, shank circumference, third finger length, wing length, maxilla length, jengger, long bone neck, chest length and chest width.

Our previous studied shown the relationship between some characters of body morphometrics characteristics and biodiversities of gaga chicken, particularly slow type and dangdut type. However, the role of vocal cord morphometrics on song expression of gaga chicken, such as slow type and dangdut type has not been studied, yet. Therefore, this research was conducted.

MATERIALS AND METHODS

Study area

The study was conducted in January 2018-July 2018 in Pinrang District, Province of South Sulawesi, Indonesia in chicken farms in 5 Subdistricts (Malimpung, Tiroan, Mattiro Bulu, Lamrisang, and Sawito/Wattang Sawito). Geographically, Pinrang is located at 3º19'13 "to 4º10'30" South Latitude and 119º26'30 "to 119º47'20" East Longitude. The area is located at an altitude of 0-2600 meters above sea level. The research area is approximately ± 1,961.77 km^2, consisting of three regional dimensions covering lowland, sea, and highland. Pinrang district, administratively, consists of 12 sub-districts, 40 urban-village, and 67 villages covering 96 neighborhoods and 181 hamlets. The total subdistricts in coastal areas have 1,457.19 km^2 or 74.27% of the total area of Pinrang District with a total coastline length of ± 101 km (Center for Statistical Bureau of Pinrang District 2018).

Procedures

Twenty Gaga chicken (10 dangdut type and 10 slow type) was collected from several farms in Pinrang, South Sulawesi, Indonesia. Pinrang consists of the village of Malimpung, Tiroan, Mattiro Bulu, Lamrisang, and Sawito (Wattang Sawito) (Figure 1). The morphometric characters measured were the trachea, right tracheal muscle, left tracheal muscle and syrinx (Figure 2). Morphometric characters were measured using a sliding range.

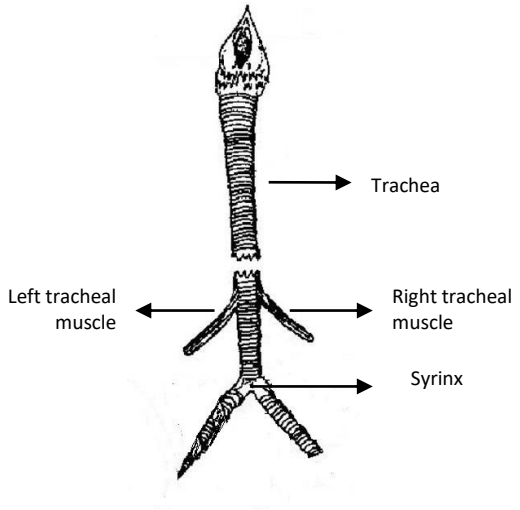

Figure 2. Vocal cord organ of gaga chicken

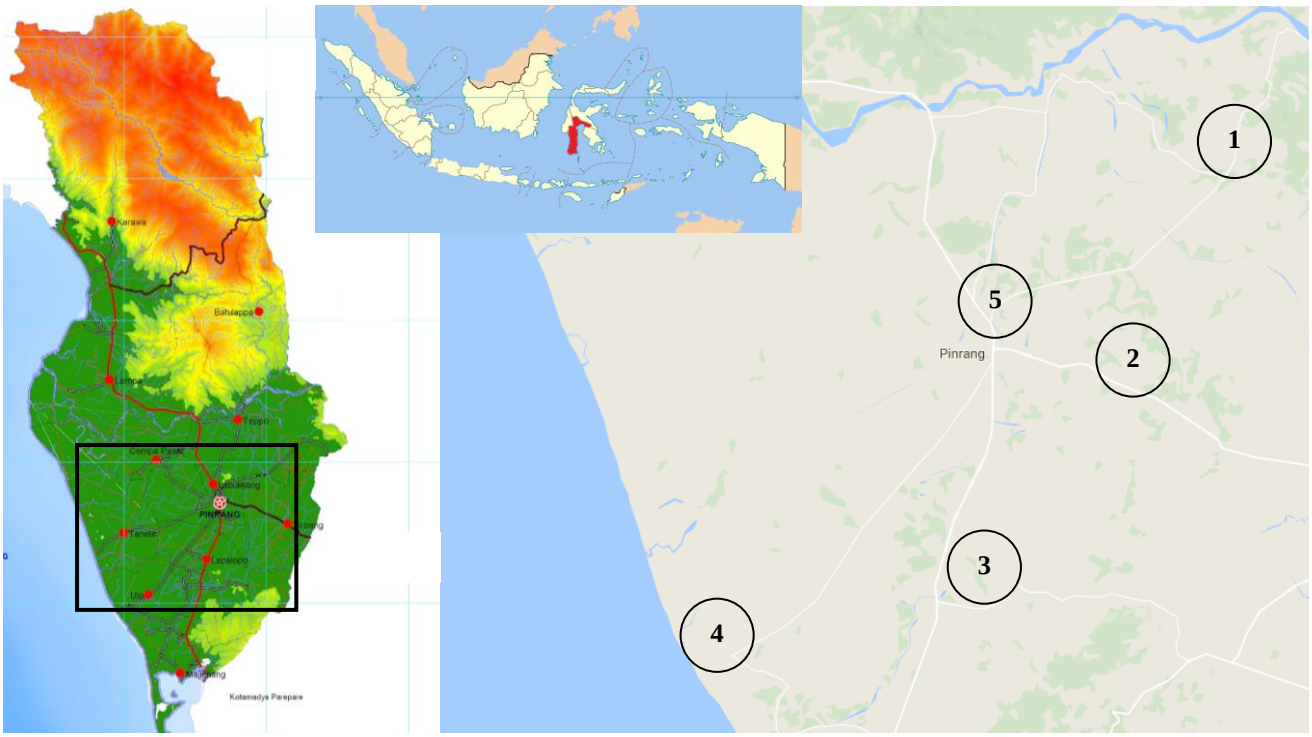

Figure 1. Sampling site in five subdistricts from Pinrang, South Sulawesi, Indonesia. A. Malimpung, B. Tiroan, C. Mattiro Bulu, D. Lamrisang, and E. Sawito (Wattang Sawito)

Data analysis

Discriminant analysis was used to determine the significant differences between groups based on the observed morphometric characters. The results were further analysis and descriptively discussed. The variables used consisted of dependent variable (gaga chicken type) and independent variable (length of trachea, right tracheal muscle length, left tracheal muscle length and syrinx length).

RESULTS AND DISCUSSION

Mean of morphometric gaga chicken vocal cord

The average length of dangdut type chicken trachea was 104.04 mm while that of the slow type chicken was 89.55 mm. The average length of the right tracheal muscle of the dangdut type was 13.52 mm and that of slow type gaga chicken was 11.00 mm. Dangdut type had an average length of left tracheal of 11.94 mm while the slow type gaga chicken had a mean left trachea muscle of 10.34 mm. The average length of syrinx of dangdut type was 14.73 mm while that of the slow type was 13.58 mm (Table 1 and Figure 3). Test of Mean differences between dangdut and slow types in each independent is presented in Table 2.

The significance value (α) specified was 0.01. Based on Table of Tests of Equality of Group Means, the value of Sig. for syrinx variable was 0.016> 0.010, suggesting that, the length of syrinx cannot explain the different types of gaga chickens. Syrinx, in some birds there are muscles attached directly to the syrinx to regulate the syrinx voltage. The amount of syrinx muscle affects the sound produced (McLelland 1990; Setijanto 1998). However, according to McLelland (1990), internal muscle syrinx in chickens does not exist. This is in line with Sudjana (2017), who which stated that Syrinx is part of respiratory tract which capable of producing sounds. However, the high low sound variations in chickens are not determined by syrinx. Syrinx is a ballot box in poultry. The sound of the chicken is generated from the air pressure on the sound valve and is modified by muscle tension (McLelland 1990). Syrinx consists of ossified cartilage, membranes, and muscles (Myers 1917). Chicken Syrinx is located behind the heart organ (Onuk et al. 2010). According to Eliyani et al. (2015), syrinx morphometric can be used to differentiate variations in the frequency of resonant sound between chickens and females.

As for tracheal variables, the sig values of right tracheal muscle and left tracheal muscle, were less than 0.010, meaning that these variables can explain the different types of chickens. This finding is in accordance with McLelland (1990) and Setijanto (1998), who stated that the sound setting in chickens is affected by the trachea and the tracheal muscles, the sternotrachealis musculus. The sternotracheal muscle consists of a pair of membranes that are attached to the craniolateral of sternum process and extend to the trachea to the cranial part of the syrinx (McLelland 1990). Both tracheal muscles appear smaller in the hens than in the rooster (Myers 1917).

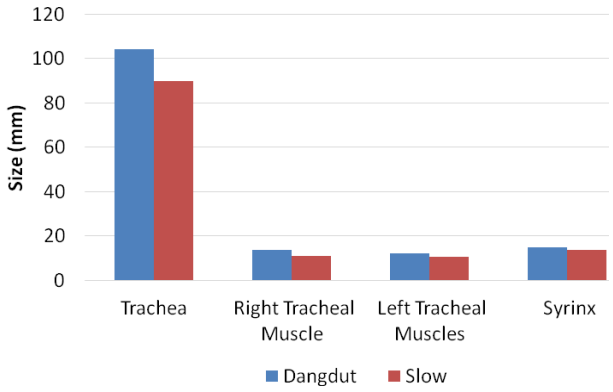

Figure 3. The average size of the vocal cords organ

Table 1. Means of morphometric data of chicken vocal cord

Gaga chicken type	Vocal cord			
	Trachea (mm)	Right tracheal muscle (mm)	Left tracheal muscle (mm)	Syrinx (mm)
Dangdut	104.04	13.52	11.94	14.73
Slow	89.55	11.00	10.34	13.58

Table 2. Test of mean difference between groups of dangdut and slow gaga chicken

Vocal cord	Wilks' Lambda	F	df1	df2	Sig
Trachea	0.321	38.110	1	18	0.000
Right tracheal muscle	0.505	17.624	1	18	0.001
Left tracheal muscle	0.644	9.929	1	18	0.006
Syrinx	0.717	7.116	1	18	0.016

The cocking sound in chickens occurs when the air in the lungs passes through the tympanic membrane of the internal form and the external tympanic membrane associated with the lateral bronchial wall. Sound variation is generally caused by differences in the poultry ballot contained in the trachea and the lower trachea, lying between the branched trachea and the two bronchi (Tanudimadja 1974). The vocalizations depend on proper syrinx and airflow configurations. Musculus tracheolateralis, musculus sternotrachealis, syrinx structures, clavicular air sacs, and ventilator muscles will work together to form a sound system (Gaunt and Gaunt 1977).

In conclusion, the result of morphometric analysis of the vocal cords can be used as one of the methods to reveal the relationship between gaga chicken dangdut and slow type. Variables that can be used to distinguish the type of gaga chicken, i.e., the dangdut and the slow type, were the length of trachea, right tracheal muscle length, and the length of the left tracheal muscle.

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Leaf anatomical characters of four epiphytic orchids of Sempu Island, East Java, Indonesia: The importance in identification and ecological adaptation

RIDESTI RINDYASTUTI, SITI NURFADILAH, APRIYONO RAHADIANTORO, LIA HAPSARI, ILHAM KURNIA ABYWIJAYA

Purwodadi Botanic Gardens, Indonesian Institute of Sciences. Jl. Raya Surabaya-Malang, Km. 65, Purwodadi, Pasuruan 67163, East Java, Indonesia
Tel./fax.: +62 343-615033. Email: ride17@gmail.com

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Abstract. *Rindyastuti R, Nurfadilah S, Rahadiantoro A, Hapsari L, Abywijaya IK. 2018. Leaf anatomical characters of four epiphytic orchids of Sempu Island, East Java, Indonesia: The importance in identification and ecological adaptation. Biodiversitas 19: 1906-1918.* Leaf anatomy features are important characters to support species identification and classification, and they are related to ecological adaptation of species. The aims of the present study were: (i) to investigate leaf anatomical characters of four epiphytic orchids of Sempu Island (*Ascochilus emarginatus*, *Dendrobium subulatum*, *Thrixspermum subulatum*, and *Thrixspermum acuminatissimum*) in relation to the significance in species identification and ecological adaptation in coastal habitats of Sempu Island, (ii) to compare the adaptive ability of the four species in coastal habitats based on adaptive anatomical characters. The procedure of leaf anatomical studies as follows: orchid leaves were fixed in ethanol 70% and sliced into thin pieces with a microtome, and stained with 1% Safranin. The leaf anatomical organization of orchids (stomata, epidermis, mesophyll, vascular bundles, and other characters such as hypodermis, fibre bundles, raphide bundles, and spiral thickenings) was observed under light microscope. The results showed the comparable data of leaf anatomical characters among the orchids. There was distinct variation in the anatomical characters of the orchids including stomata anomocytic, tetracytic, and cyclocytic; the presence or absence of hypodermis, spiral thickenings, fibre bundles, raphide bundles, and bundle sheaths; homogenous and heterogenous mesophyll; and variation in vascular bundle arrangement. Detailed leaf anatomical characters can be used to distinguish a species from others, which are important to support species identification. The similarity of anatomical characters among these orchids were they possessed relatively thick cuticle and other specific anatomical characters as a structural adaptation to coastal habitat with high irradiation to reduce leaf transpiration. *D. subulatum* can be considered as the most adaptive orchid species to coastal habitats based on adaptive anatomical characters as it possessed the largest number of adaptive anatomical characters. The implication of this study is the importance of leaf anatomical features to support species identification and to increase understanding of orchid biology and ecology which are important in orchid conservation.

Keywords: Anatomy, ecology, environment, Orchidaceae, small island

INTRODUCTION

Orchidaceae is one of the most successful plant families in evolution and speciation, resulting in approximately 25.000 species across the world with highly various morphological and anatomical characters (Dressler 1993). Anatomical characters of orchids have been widely studied in a wide range of species within tribes, subtribes, and genera levels, such as tribe *Calypsoeae* (Stern and Carlsward 2008); subtribes *Laeliinae* (Stern and Carlsward 2009); *Aeridinae*, *Angraecinae* and *Aerangidinae* (Carlsward et al. 2006); *Stanhopeinae* (Stern and Whitten 1999), *Orchidinae* (Stern 1997); *Habenariinae* (Stern 1997), *Oncidiinae* (Stern and Carlsward 2006); and genera *Caladenia* (Pridgeon 1993), *Dendrobium* sections *Aporum* and *Rhizobium* (Carlsward et al. 1997), *Ophrys*, *Orchis* and *Dactylorhiza* (Aybeke et al. 2010).

Anatomical features are important characters to support identification and classification in Orchidaceae (Pridgeon 1982; Aybeke et al. 2010, Fan et al. 2014). Pridgeon (1982) and Pridgeon and Norris (1979) showed diagnostic characters to distinguish genera within subtribe *Pleurothallidinae* (*Acostaea, Barbosella, Brachinionidium, Cryptophoranthus, Dracula, Dresslerella, Dryadella, Lepanthes, Masdevallia,* and other genera within the subtribe) using anatomical characters of leaf such as cuticle, epidermis, hypodermis, spiral thickenings, and vascular bundle number. Fan et al. (2014) reported that *Holcoglossum* could be distinguished from the related genera, such as *Ascocentrum, Luisia, Papilionanthe, Rhynchostylis,* and *Vanda* based on leaf cuticular wax characteristics. Furthermore, Aybeke et al. (2010) demonstrated the use of anatomical characters to distinguish species within genera of *Ophrys,* such as *O. speculum, O. fusca, O. lutea, O. sphegodes, O. cornuta, O. umbilicata, O. bucephala, O. apifera* and *Orchis*, such as *O. coriophora*, *O. tridentata, O. militaris, O. papilionacea, O. mascula,* and *O. palustris*. The species had particular comparable anatomical characters that can be used to distinguish it from others using anatomical characters, such as thickness of epidermal walls and shape of chlorenchyma cells.

Anatomical characters have been shown to have the relationship with the ecological adaptation of orchids. Moreira et al. (2013) showed the leaves of *Epidendrum secundum* growing in the luminous area had a relatively thick cuticle, indicating adaptation to the environment with intense solar radiation, with cuticle functions as a barrier to reduce transpiration because of the high intensity of sunlight. Fan et al. (2014) demonstrated other anatomical characters showing structural adaptation in other orchids. They reported that *Holcoglossum* had structural adaptations to strong winds and ample rains in subalpine region of the Hengduans Mountains by having laterocytic and polarcytic stomata in their leaf epidermal layer.

Previous studies on orchids anatomy in Indonesia have been conducted including anatomical characters of roots of orchids of Sempu Island (Nurfadilah et al. 2016), leaf anatomy of nine species of *Bulbophyllum* (Orchidaceae) (Betty 2011), comparative leaf and root anatomy of two species of *Dendrobium* (Metusala et al. 2017). The present study of orchid leaf anatomy provides additional data to support species identification and to increase understanding of orchid biology and ecology, concerning structural adaptation of orchids in coastal habitats in Sempu Island. The clear species identification, biology and ecology data of orchids are required in the management of orchid conservation. Furthermore, understanding of the adaptive ability of orchids to coastal habitats based on adaptive anatomical characters also supports the assessment of the susceptibility of orchid species to the environmental alteration, which is important for species conservation priority.

Various orchids can be found in Sempu island, especially on coastal areas exposed to irradiation. Approximately 15 orchid species have been recorded in Sempu island, that consisted of mostly epiphytic orchids (14 species) and one terrestrial orchid (Rindyastuti et al. 2018). The most common orchids that could be found in Sempu island included *Ascochilus emarginatus* (Blume) Schuit., *Dendrobium subulatum* (Blume) Lindl., and *Thrixspermum subulatum* (Blume) Rchb.f. (Figure 1). The present study aimed (i) to investigate the anatomical characters of these orchids of Sempu Island (*A. emarginatus*, *D. subulatum*, *T. subulatum*)., as well as *Thrixspermum acuminatissimum* (Blume) Rchb.f. (ii)) to compare the adaptive ability of the four orchids to coastal habitats based on adaptive anatomical characters.

Figure 1. Four orchids species which grow in coastal habitats of Sempu Island. A. *Ascochilus emarginatus* (Blume) Schuit., B. *Dendrobium subulatum* (Blume) Lindl., C. *Thrixspermum subulatum* (Blume) Rchb.f., D. *Thrixspermum acuminatissimum* (Blume) Rchb.f. Courtesy: Figure 1.A. Siti Nurfadilah, Figure 1.B. Nina Dwi Yulia, Figure 1.C. Apriyono Rahadiantoro, Figure 1.D. Comber (1990)

MATERIALS AND METHODS

Study area

Sempu Island is a small island located off the south coast of East Java, Indonesia (Figure 1). The island is a nature reserve under the Ministry of Forestry with an area of approximately 877 ha. The island altitude ranges between 0 and 102 m asl. The epiphytic orchids were collected in the coastal habitat in Waru-Waru and Air Tawar, Sempu Island with temperatures around 27-29 ºC, high humidity 90-94 %, and sunlight intensity around 52-163 lux. The epiphytic orchids grew on some host trees (phorophytes), such as *Terminalia catappa* L., *Streblus asper* Lour., *Sophora tomentosa* L. in the coastal habitats that were exposed to high irradiation.

Anatomical sample preparations

The leaves were fixed in ethanol 70%, and sliced into thin pieces with a microtome, and stained with 1 % Safranin (Stern and Judd, 2000). The leaf anatomical organization of orchids was observed under a light microscope (Olympus CX21) with three replication of slices of leaf for each orchid species. The leaf anatomical features of all orchids were characterized, including stomata, epidermis, mesophyll, vascular bundles, and other characters (hypodermis, fibre bundles, raphide bundles, and spiral thickenings). The size of stomata, epidermis, and cuticle thickness was measured using micrometer.

Assessment of the adaptive ability of orchid species to coastal habitats

Adaptive anatomical characters, such as smaller stomata, larger epidermis, the presence of hypodermis, many layers of mesophyll, the presence of fibre bundles, spiral thickenings and bundle sheaths were used to assess the adaptive ability of orchid species to coastal habitats (Aybeke et al. 2010; Fahn 1982; Guan et al. 2011; Roth 1984; Reginato et al. 2009; Hsiao 1973; Metusala et al. 2017; Vincent 2000; Richter et al. 2011; Placet et al. 2014; Leroux et al. 2010; Lack and Evans 2001). The data of adaptive anatomical characters were obtained by comparing the size of stomata and epidermis, the presence of hypodermis, many layers of mesophyll, the presence of fibre bundles, spiral thickenings and bundle sheaths among four orchid species. Assessment of the most adaptive orchid species was established through the largest number of adaptive anatomical characters it had.

Data analyses

Data of stomata size, cuticle thickness, and epidermis size among four orchid species were analyzed using Analysis of Variance (ANOVA) and Tukey post-test in confidence level of 95% using MINITAB 15.0. Comparison of the adaptive ability of orchid species to coastal habitats was based on the presence and absence of adaptive anatomical characters, such as smaller stomata, larger epidermis, the presence of hypodermis, more layers of mesophyll, the presence of fibre bundles, spiral thickenings, and bundle sheaths.

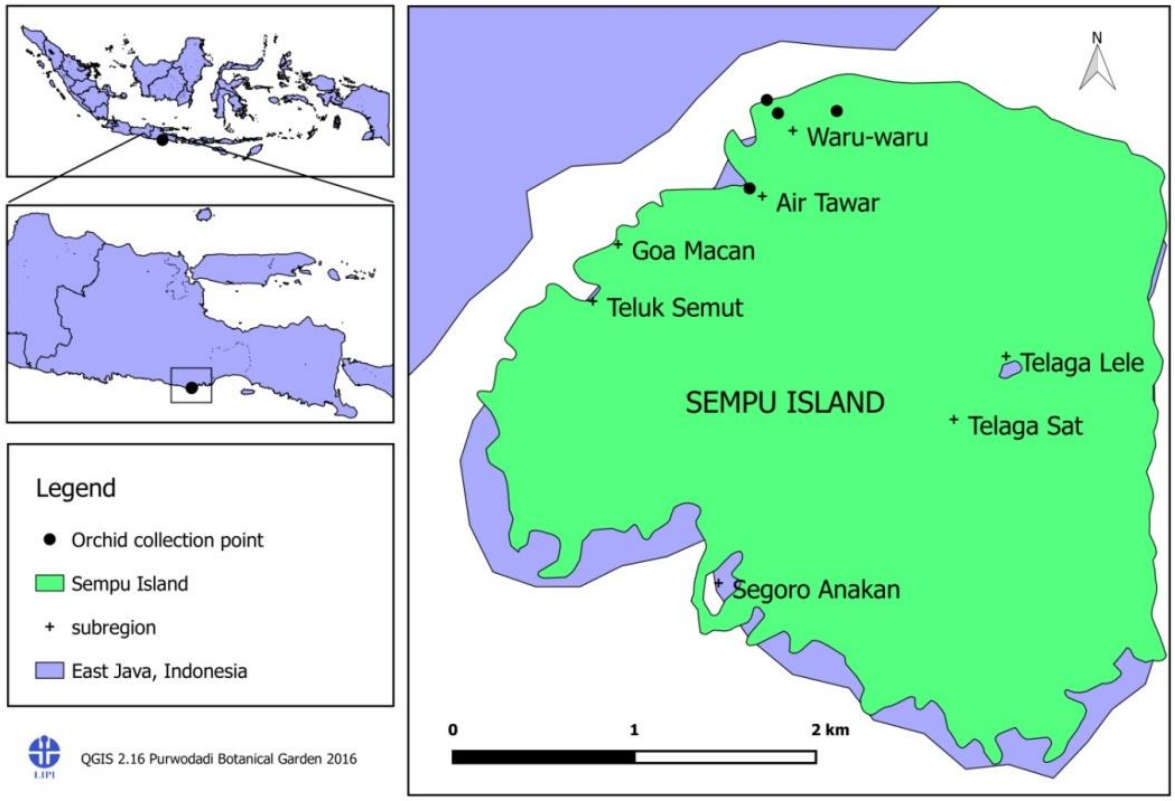

Figure 2. The study site of epiphytic orchid species in Waru-Waru and Air Tawar within Sempu Island, East Java, Indonesia

RESULTS AND DISCUSSION

The results of this study showed leaf anatomical features of orchids of Sempu Island and comparable data among the orchids to support species identification and to understand their structural adaptation to coastal habitat in Sempu Island.

Leaf anatomical features

The leaf anatomical structures showed organization of components forming leaves consisted of stomata, cuticle, epidermis, mesophyll, and vascular bundles. There were specific characters, such as hypodermis, spiral thickenings, fibre bundles, and raphide bundles in epidermal cells in particular orchid species (Table 1).

Ascochilus emarginatus

Leaf transverse section of *Ascochilus emarginatus* showed the leaf anatomical structures including stomata with anomocytic and tetracytic configuration. Cuticle was striate, relatively thick ranging from 6.3-11 µm. The epidermis was uniseriate, composed of elongated-shaped cells. Hypodermis was absent or not clear. Mesophyll was homogenous, consisted of 10-14 layers and consisting of thin-walled and rounded-shaped parenchymatous cells. Parenchymatous cells contained chlorophylls (chlorenchyms). Spiral thickenings were present in parenchymatous cells of mesophyll. Vascular bundles consisted of xylem and phloem arranged collaterally. There were 9-10 arches of vascular bundles arranged in a single row embedded in parenchymatous cells of mesophyll. There were no sclerenchyma cells associated with vascular bundles. Thin-walled bundle sheath surrounded the vascular bundle (Table 1; Figure 2).

Dendrobium subulatum

Dendrobium subulatum anatomical characters showed that stomata configuration was cyclocytic. Cuticle was striate, relatively thick ranging from 2.9-5.4 µm. The epidermis was a single layer composed of elongated-shaped cells. There was a specific anatomical character of *D. subulatum*, fibre bundles subtended epidermal layer, forming hypodermal layers (Figure 3). Mesophyll was 19-22 layers, heterogeneous, consisted of elongated-shaped parenchymatous cells in the outer mesophyll and polygonal-shaped parenchymatous cells in the inner mesophyll. Chlorophylls were present in the parenchymatous cells of mesophyll. Vascular bundles were arranged radially within parenchymatous areas of mesophyll, consisted of 10-12 arches of vascular bundles. Sclerenchymatic cells were associated with phloem. Bundle sheath was thin-walled surrounding vascular bundles (Table 1; Figure 3).

Thrixspermum subulatum

Thrixspermum subulatum had anomocytic and tetracytic stomata configuration, smooth and thick cuticle layer ranging from 9.4-11.8 µm. The epidermis was uniseriate composed of polygonal-shaped cells. Hypodermis was absent. Mesophyll was 9-12 layers; homogenous consisted of polygonal-shaped parenchymatous cells. There were chlorophylls in parenchymatous cells of mesophyll. Spiral thickenings were present in parenchymatous mesophyll. Vascular bundles consisted of xylem and phloem arranged collaterally. No sclerenchyma cells were associated with vascular bundles. Bundle sheath was indistinct. There were 11-13 arches of vascular bundles arranged in a single row embedded within parenchymatous cells of mesophyll (Table 1; Figure 4).

Table 1. Leaf anatomical characters of four epiphytic orchids in Sempu Island

Anatomical characters	*Ascochilus emarginatus*	*Dendrobium subulatum*	*Thrixspermum Subulatum*	*Thrixspermum acuminatissimum*
Leaf shape	blade-like	cylindric	blade-like	blade-like
Leaf thickness	512.4-584.4 µm	1225-1773 µm	523.4-585.5 µm	681.6-923 µm
Stomata				
Stomata configuration	anomocytic, tetracytic	cyclocytic	anomocytic, tetracytic	tetracytic
Cuticle				
Thickness (µm)	6.3-11	2.9-5.4	9.4-11.8	5.7-14.9
Cuticle Pattern	striate	striate	smooth	smooth
Epidermis				
Number of epidermis cell layer	uniseriate	uniseriate	uniseriate	uniseriate
Epidermis cell shape	elongated	elongated	polygonal	elongated
Hypodermis	absent or indistinct	single layer, thin-walled	absent or indistinct	absent or indistinct
Mesophyll				
Number of mesophyll layer	10-14	19-22	9-12	14-17
Mesophyll cell shape	rounded	elongated, polygonal	polygonal	polygonal
Mesophyll cell thickening	no	no	no	no
Mesophyll homogenous	yes	no	yes	yes
Vascular bundles				
Bundle sheath	thin-walled	thin-walled	indistinct	thin-walled
Arch number	9-10	11-12	10-11	10
Vascular bundle arrangement	single row	radial	single row	single row
Specific characters				
Fibre bundles	absent	present	absent	absent
Raphide bundles in epidermis	absent	absent	absent	present
Spiral thickening	absent	absent	present	present

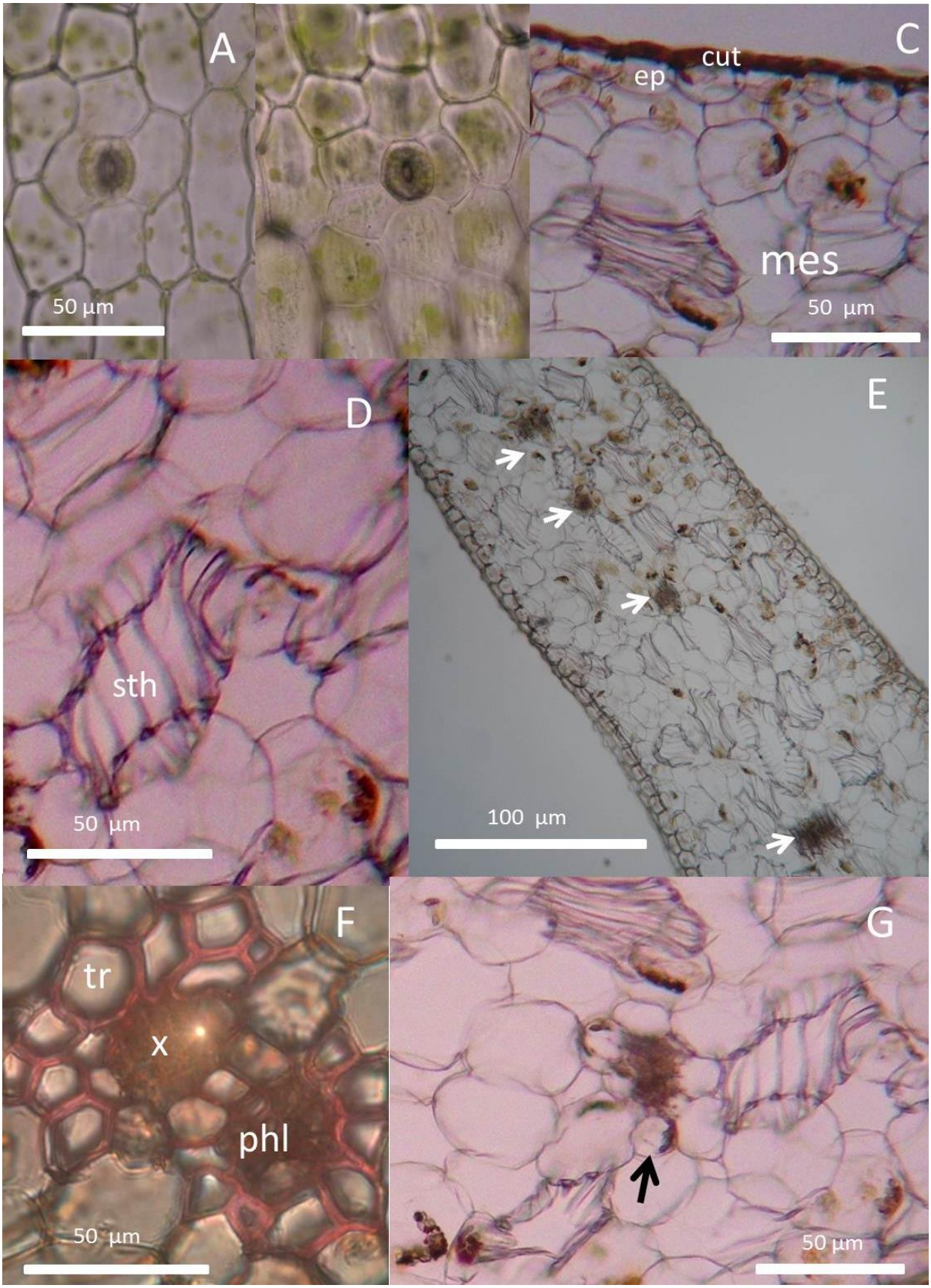

Figure 2. Transverse section of *Ascochilus emarginatus* leaf. A. Stomata, tetracytic; B. Stomata anomocytic; C. Cuticle (cut), epidermis (ep), mesophyll (mes); D. Spiral thickening in parenchymatous cell (Sth) within mesophyll (mes); E. Homogenous mesophyll and vascular bundles arranged in a single row, vascular bundle (arrow); F. Xylem (x), phloem (phl), tracheid (tr); G. Thin-walled cells of bundle sheath surrounding the vascular bundle (black arrow).

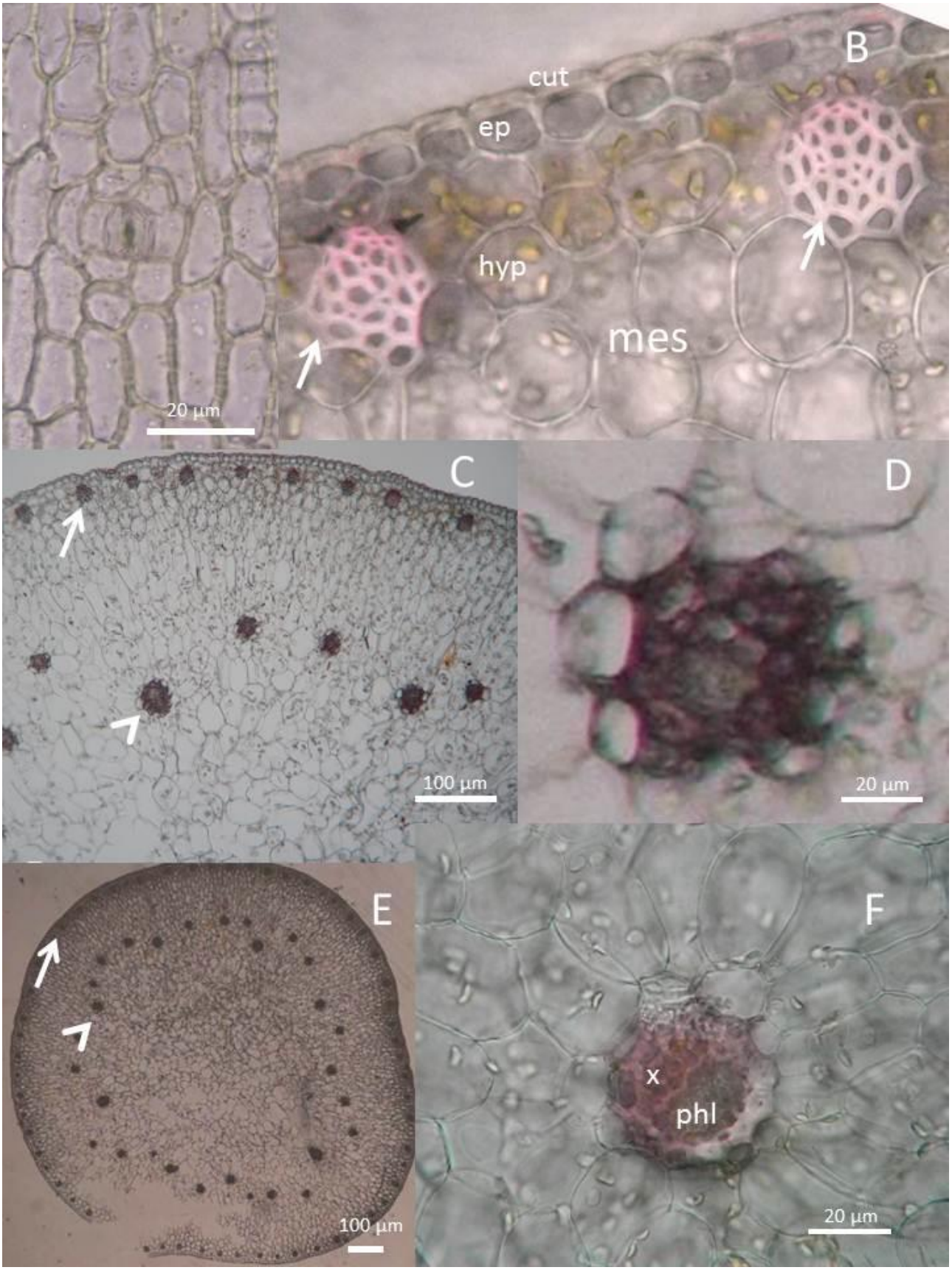

Figure 3. Transverse section of *Dendrobium subulatum* leaf. A. Cyclocytic Stomata; B. Cuticle (cut), sclerenchym (arrow), epidermis (ep), hypodermis (hyp), fibre bundle (arrow), mesophyll (mes); C. Heterogenous mesophyll, fibre bundle (arrow), vascular bundle (arrowhead); D. Thin-walled bundle sheath surrounding vascular bundle; E. Vascular bundles arranged radially, vascular bundle (arrowhead), fibre bundle (arrow); F. Xylem (x), phloem (phl), sclerenchyma associated with phloem (under phloem).

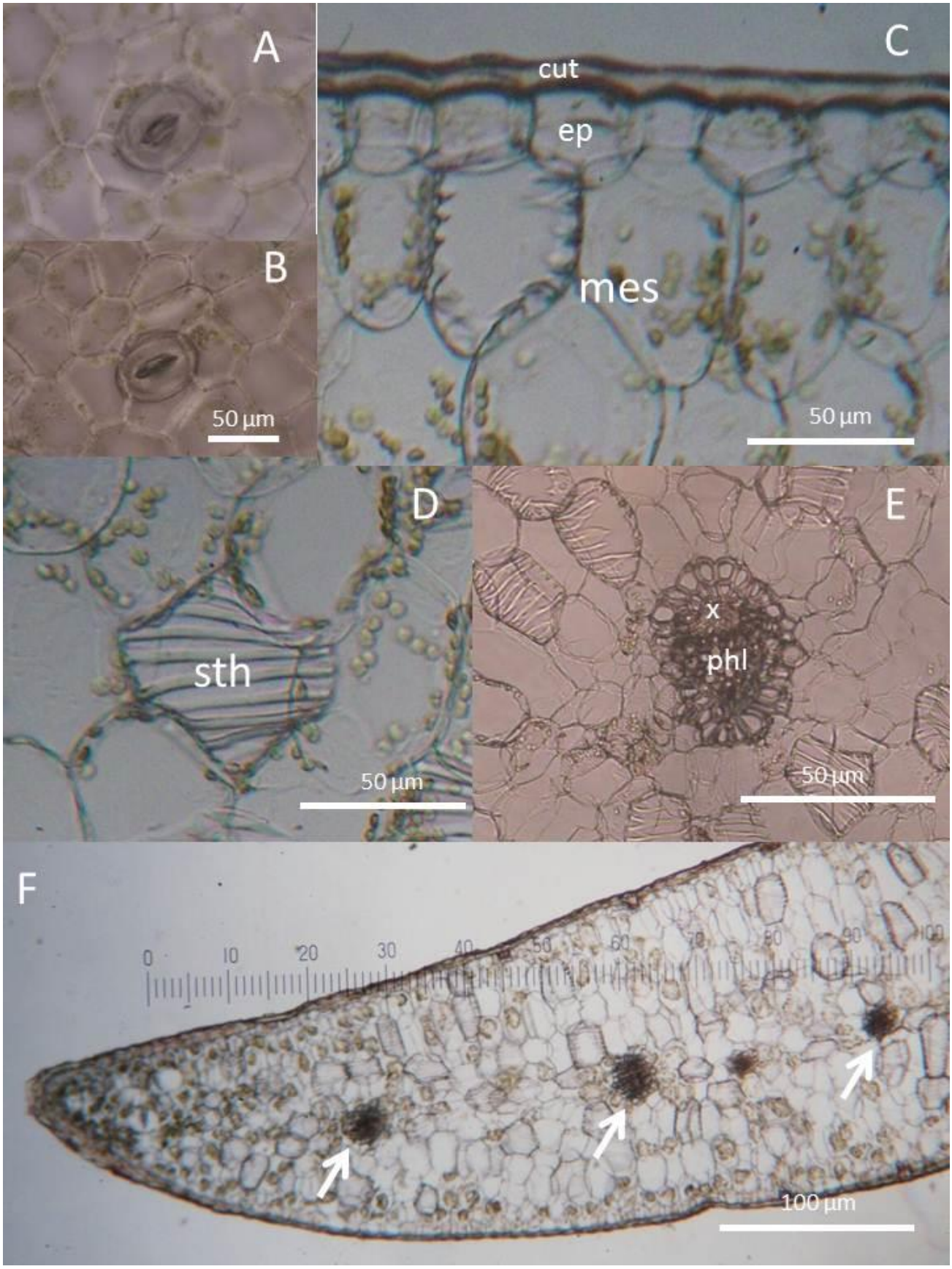

Figure 4. *Thrixspermum subulatum* leaf transverse section. A. Tetracytic stomata; B. Anomocytic stomata; C. Cuticle (cut), epidermis (ep), mesophyll (mes); D. Spiral thickening in parenchymatous cell (Sth); E. Xylem (x), phloem (phl), indistinct. bundle sheath; F. Homogenous mesophyll and vascular bundles arranged in a single row, vascular bundle (arrow)

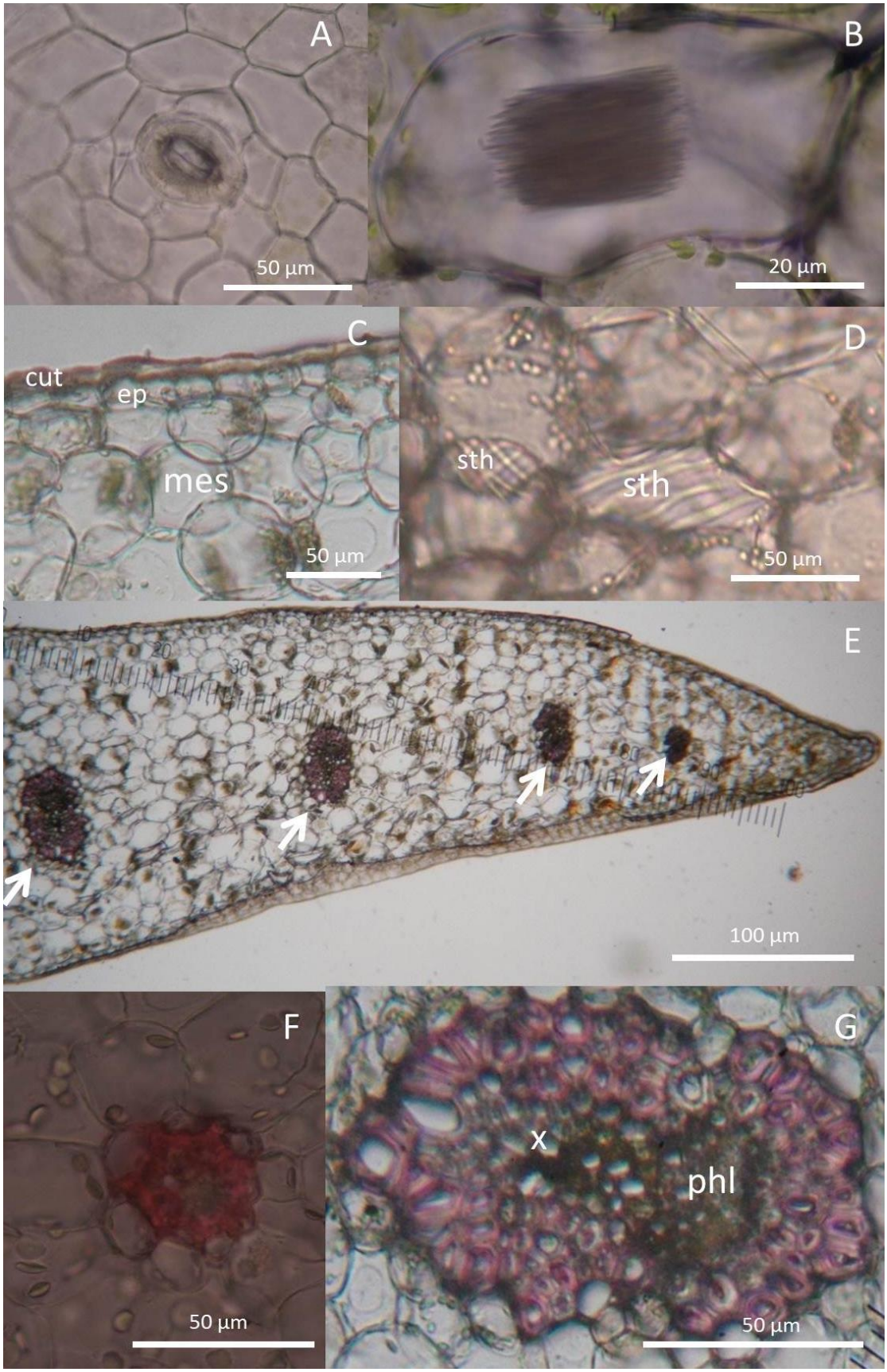

Figure 5. Leaf transverse section of *Thrixspermum acuminatissimum*. A. Stomata, anomocytic; B. Raphide bundles in epidermis cell; C. Cuticle (cut), Epidermis (ep), mesophyll (mes); D. Spiral thickening (Sth); E. Mesophyll homogenous, vascular bundles arranged in a single row, vascular bundle (arrow); F. Thin-walled bundle sheath surrounding vascular bundle; G. Xylem (x), phloem (phl), sclerenchyma cells associated with xylem and phloem.

Table 2. Means of stomata size, cuticle thickness and epidermis size of orchids Sempu Island

Anatomical characters	*Ascochilus emarginatus*	*Dendrobium subulatum*	*Thrixspermum subulatum*	*Thrixspermum acuminatissimum*
Stomata				
Stomata width (mean±SE)	36.83 ±3.35^{b}	36.50 ±0.72^{b}	55.82 ±2.05^{a}	59.70 ±0.47^{a}
Stomata length (mean±SE)	44.10 ±2.1^{b}	37.40 ±2.07^{b}	75.92 ±2.63^{a}	79.65 ±1.02^{a}
Cuticle				
Cuticle thickness (mean±SE)	8.82 ±0.96^{a}	3.880 ± 0.45^{b}	10.00 ± 0.32^{a}	9.99 ± 0.84^{a}
Epidermis				
Epidermis width (mean±SE)	11.73 ±1.18^{ab}	8.98 ±1.635^{b}	9.71 ±0.26^{b}	21.38 ±4.74^{a}
Epidermis length (mean±SE)	25.54 ±2.73^{a}	13.02 ±1.59^{b}	31.74 ±1.34^{a}	31.26 ±1.94^{a}

Note: Size was performed in µm; Different letters indicate significant differences among orchid species at $p < 0.05$ based on ANOVA and Tukey test

Thrixspermum acuminatissimum

Thrixspermum acuminatissimum had anomocytic stomata configuration and cuticle with 5.7-14.9 µm thick. Epidermis had one layer composed of elongated-shaped cells. Raphide bundles were present in epidermis cells. Hypodermis was absent. Mesophyll was homogenous, 14-17 layers consisting of polygonal shaped-parenchymatous cells. Chlorophylls and spiral thickenings were present in mesophyll parenchymatous cells. Vascular bundles consisted of xylem and phloem arranged collaterally. There were 8-11 vascular bundles arches arranged in a single row embedded in the mesophyll. Sclerenchyma cells associated with xylem and phloem. Thin-walled bundle sheath is surrounding vascular bundle (Table 1; Figure 5).

Anatomical features of the four epiphytic orchid species of Sempu Island

The leaf anatomical characters of four orchids of Sempu Island was revealed. There were similarities and differences in their anatomical features which showed variations in ecological adaptation among orchid species (Table 1 and Table 2).

Stomata

Stomata are small pores on leaves surfaces and have role of facilitating the gases movement in and out of leaves and, thus, the gas exchange in plants as a whole. Stomata have significant importance in the plant physiology, evolution, and ecology (Hetherington and Woodward 2003). Stomata configuration of plants varied, such as anomocytic, tetracytic, cyclocytic, and diacytic. The stomata configuration of four epiphytic orchids studied were various, anomocytic and tetracytic in *Ascochilus emarginatus* and *Thrixspermum subulatum*, (Figure 2 and Figure 4), cyclocytic in *Dendrobium subulatum* (Figure 3), and anomocytic in *Thrixspermum acuminatissimum* (Figure 5).

The stomata size (width and length) of the orchids was also significantly different among species (Table 2). According to Guan et al. (2011), larger stomata are slower to close and have greater potential for hydraulic dysfunction under drought condition. Small stomata is more tolerant to drought than the larger stomata. In the present study, *A. emarginatus* and *D. subulatum* had smaller stomata size than two other species (*T. subulatum T. acuminatissimum*). The smaller stomata of *Ascochilus emarginatus* and *D. subulatum* can support the species to be more adaptive to coastal habitat with warmer temperature and high irradiation.

Cuticle

The cuticle is the outermost layer of orchid leaves, deposited on the surface of epidermal cells. Plant cuticle plays an important role in the interaction of plants with the environment, such as to reduce absorbed solar radiation and temperature by reflecting the sunlight and to reduce transpiration (Domínguez et al. 2011; Fahn 1982; Rosso 1966). Cuticle contains polysaccharides, flavonoids and cutin matrix. These characters provide structural and chemical modifications for surface wetting, ranging from super hydrophilic to superhydrophobic to adapt to the environment (Koch and Barthlott 2009). Furthermore, according to Pridgeon (1993), cuticular patterns varies among species but are a consistent feature within each of them.

Orchidaceae has a large variation in cuticle thickness. In the present study, the orchids of Sempu Island had relatively thick cuticle (> 2.5 or 3 µm) (Carlsward et al. 2006), with various cuticle thickness, 8.82 µm in *A emarginatus;* 3.88 in *D. subulatum*; 10.005 in *T. subulatum*, and 9.99 in *T. acuminatissimum* (Table 2). The thick cuticle is an ecological adaptation to reduce transpiration (Moreira et al. 2013) and dry condition (Guan et al. 2011). The thick leaf cuticle of the orchids is significantly different among species based on the variance analysis. The cuticle of *D. subulatum* is thinner than that of three other species (*A. emarginatus, T. subulatum* and *T. acuminatissimum*). The thicker cuticle of these three species can also support for more adaptive capability of these species in coastal habitats. There are some types of cuticle including smooth, striate, papillate, or pitted, verrucose along the contours of epidermal cells. In the present study, two types of cuticle were recorded on the orchid leaf of Sempu Island, striate and smooth. *Ascochilus emarginatus* and *D. subulatum* had striate cuticle, while *T. subulatum* and *T. acuminatissimum* had smooth cuticle.

Epidermis

The epidermis is the outer cell layer of a plant leaf that functions as a barrier interconnecting and separating leaves from the environment, and is important in the response of plants to external stimulus. Ecophysiologically, important functions of leaf epidermis either in the direct interaction with environmental factors (Dietz and Hartung 1996). According to Darling (1989), the epidermis is important to protect mesophyll and chlorenchyma from high solar radiation and reduce the heat load on leaves. The thicker the epidermal cells, the more radiation and heat can be reflected and reduced. Moreover, large epidermal cells in many species of orchids serve as water storage (Guan et al. 2011). Based on the variance analysis, the width and length of the epidermal cells significantly different among species. *T. acuminatissimum* has the largest epidermal cells among the species in this study, while *D. subulatum* has the smallest epidermal cells. The larger epidermal cells of *T. acuminatissimum* can support the species to be more adaptive to the warmer environment compared to other species. There are various shapes of leaf epidermis of orchids such as polygonal, isodiametric, rectangular, and elongated (Aybeke et al. 2010). The epidermis of orchids of Sempu Island (*A. emarginatus, D. subulatum, T. subulatum,* and *T. acuminatissimum*) in the present study had various shape of epidermal cells. *A. emarginatus, D. subulatum,* and *T. acuminatissimum* had elongated epidermal cells, while *T. subulatum* had polygonal epidermal cells (Table 1; Figure 2-5).

Hypodermis

The hypodermis is a structure beneath the epidermis. This structure can be present or absent in orchid species (Pridgeon 1982; Stern 1997). Together with the epidermis, it has a role to protect the underlying layers, parenchymatous mesophyll, from too high sun radiation especially UV A and UV B and reduce the heat load in the leaves (Roth 1984; Darling 1989). Another role of hypodermis is for water storage (Roth 1984; Reginato et al. 2009).

The hypodermis is an important anatomical character. The development of hypodermis is useful in distinguishing monocotyledon genera and species. For example, within *Pleurothallidinae*, such as *Acostaeae, Barbosella, Dryadella, Lepanthes, Masdevallia* and *Pleurothallis* had hypodermis, while other genera within the same section such as *Brachionidium* and *Dracula* did not exhibit to posses hypodermis (Pridgeon 1982).

In the present study, hypodermis was only present in *D. subulatum* with a single layer hypodermic cell, while it was absent or not clear in other species (*A. emarginatus, T. subulatum,* and *T. acuminatissimum*). Hypodermis in *D. subulatum* is a single layer and composed of thin-walled hypodermic cell. The thin-walled hypodermic cells usually have a role in water storage (Carlsward et al. 2006; Stern and Carlsward 2008). Hypodermal layers in orchids also serve as additional structures on leave thickness and succulence (Metusala et al. 2017). The presence of hypodermis in *D. subulatum* in the present study is one of the important structures to store water and as a structural adaptation in coastal habitat in Sempu Island, with relatively high irradiation to reduce water loss because of leaf transpiration. Some other orchids of Sempu Island (*A. emarginatus, T. subulatum,* and *T. acuminatissimum*) that did not possess hypodermis, had another additional structure for adaptation in relatively high illumination in coastal habitat in Sempu Island (spiral thickening; discussed below).

Mesophyll

The mesophyll is an important structure in leaves containing a vital component for photosynthesis (chlorophyll) to assimilate nutrients. The number of mesophyll layers in the present study varied; *A. emarginatus* (10-14 layers), *D. subulatum* (19-22 layers), *T. subulatum* (9-12 layers), and *T. acuminatissimum* (14-17 layers). The thicker mesophyll layers also support the leaves succulence. The succulence level of leaves related to the parenchymal capacity of mesophylls to provide water supply for photosynthetic process and leaves cells turgor especially in the arid environment (Hsiao 1973; Lack and Evans 2001; Metusala et al. 2017). In the present study, *D. subulatum* had more mesophyll layers than the other three orchid species indicating that *D. subulatum* was more succulent compared to the other three orchid species and had more capability to store water, which is important to survive in the habitats with high irradiation.

The mesophyll parenchymatic cells are known to be homogenous for some orchids and heterogeneous for some others (Carlsward et al. 2006; Stern and Whitten 1999). In the present study, there was variation in the homogeneity and heterogeneity of mesophyll of orchids of Sempu Island. *A. emarginatus, T. subulatum,* and *T. acuminatissimum* possessed homogenous mesophyll, while *D. subulatum* had heterogenous mesophyll. Homogeneity and heterogeneity of mesophyll of other orchids were also reported to be varied.

Vascular bundles

The vascular bundle is a transport system containing xylem and phloem that are important in the water and nutrients transport (Lack and Evans 2001; Fahn 1982). The vascular bundles arrangement in the mesophyll of orchids of Sempu Island varied. *A.emarginatus, T. subulatum,* and *T. acuminatissimum* had vascular bundles aligned in one row in the center of mesophyll, while the vascular bundles of *D. subulatum* arranged radially in the mesophyll (Figure 2-5).

Other orchids were also reported to have variation in the alignment of vascular bundles in the mesophyll. Vascular bundle arrangement in a single row was reported in *Campylocentrum micranthum* (Carlsward et al. 2006); in *Habenaria cornuta, H. holothrix, H. monorrhiza, H. occidentalis, H. odontopetala, H. snowdenii, H. vaginatum, Stenoglottis fimbriata, S. longifolia,* and *S. woodii* (Stern 1997); in *Calypso bulbosa, Govenia tingens, Tipularia discolor* (Stern and Carlsward 2008). Another vascular bundle arrangement, symmetric arrangement, was reported in *Dendrobium anceps* (Carlsward et al. 1997).

The presence or absence of sclerenchyma associated with vascular bundles (xylem and phloem) can be one of the anatomical characters in the differentiation between species (Stern and Carlsward 2009). In the present study, sclerenchyma cells associated with xylem and phloem were recorded in *Thrixspermum acuminatissimum*, associated with phloem only in *D. subulatum;* while *A. emarginatus* and *T. subulatum* did not exhibit to possess sclerenchyma associated with xylem or phloem. Other studies also showed the presence and absence of sclerenchyma associated with vascular bundles (xylem and or phloem) in some other orchid species.

Other orchids which were reported to possess schlerenchyma associated with vascular bundles were *Octomeria* sp. (Pridgeon 1982), *Campylocentrum micranthum* (Carlsward et al. 2006), *Epidendrum secundum* (Moreira et al. 2013), *Dendrobium leonis, D. anceps, D. brevimentum, D. aloifolium, D. distichum, D. indivisum, D. mannii, D. rosellum*, and *D. nathaniele* (Carlsward et al. 1997); *Arpophyllum giganteum* (Stern and Carlsward 2009), while other orchids which reported to not exhibit schlerenchyma cells associated with vascular bundles were *Barbosella cucullata* (Pridgeon 1982) and *Calypso* (Stern and Carlsward 2008).

Bundle sheath consisted of cells surrounding the vascular bundle. Bundle sheath is an anatomical structure of leaf which indicates that leaf contains two photosynthetic enzymes both Rubisco and PEP carboxylase to catalyze the CO_2 fixation in photosynthetic reactions. This feature is important to adapt to the higher temperature through the photosynthetic effectiveness in maintaining plant productivity (Lack and Evans 2001). Of four orchids of Sempu Island, three orchids (*A. emarginatus, D. subulatum*, and *T. acuminatissimum*) had thin walled bundle sheath; while the bundle sheath in *T. subulatum* was indistinct.

Other studies also reported variation in the bundle sheath features of other orchid species. Distinct and thin-walled bundles sheath were reported in *Ophrys iricolor, O. heldreichii, O. bucephala, Orchis coriophora, O. fragrans, O. punctulata, O. purpurea, O. morio subsp. morio, O. papilionacea var. papilionacea, O. laxiflora*, and *Ophrys tenthredinifera* (Aybeke et al. 2010), *Cynorkis fastigiata* (Stern 1997); *Platanthera flava* (Stern 1997); *Dendrobium leonis, D. anceps, D. brevimentum, D. aloifolium, D. distichum, D. indivisum, D. mannii, D. rosellum*, and *D. nathaniele* (Carlsward et al. 1997). Bundle sheath of other orchids was reported indistict, such as in *Ancistrorhynchus clandestinus, A. refractus, Rhipidoglossum curvatum, R. kamerunense* and *R. obanense* (Carlsward et al. 2006).

Specific characters

Fibre bundles

Fibre is elongated cells, typically sclerenchyma cells, with thick-walled cells composed of cellulose and lignin. The sclerenchymatic fibres organized, glued together by a pectin interface form fibre bundles. The thick-walled sclerenchymatic cells in fibre or fibre bundles act as mechanical protection and a barrier to reduce water loss (Vincent 2000; Richter et al. 2011; Placet et al. 2014).

In the present study, fibre bundles were only present in *D. subulatum* (Figure 3), forming hypodermal layers under epidermis, while they were absent in *A. emarginatus, T. subulatum*, and *T. acuminatissimum*. The presence of fibre bundles can be a diagnostic character in species identification and differentiation. Other studies also reported the presence and absence of fibre bundles in some other orchid species, such as the presence of fibre bundles in *Ionopsis utricularioides, Helcia sanguinolenta, Aspasia lunata, Oncidium boothianum* (Stern and Carlsward 2006), *Gongora portentosa, G. truncata*, and *Cirrhaea dependens* (Stern and Whitten 1999), *Epidendrum secundum* (Moreira et al. 2013), *Cattleya skinneri, C. forbesii, Laelia anceps, Arpophyllum giganteum* (Stern and Carlsward 2009); while fibre bundles were absent in some others, such as in *Campylocentrum micranthum* (Carlsward et al. 2006), *Brachtia andina, Erycina echinata, Lemboglossum maculatum, Odontoglossum cordatum* (Stern and Carlsward 2006), *Caladenia* (Pridgeon 1993), *Epidendrum anceps, Prosthechea boothiana*, and *P. radiata* (Stern and Carlsward 2009).

Raphide bundles

Raphides are bundles of narrow, elongated needle-shaped crystals containing calcium oxalate (Prychid and Rudall 1999). The raphide function is as a storage form for calcium or oxalate, regulation levels of calcium oxalate, mechanical support, and osmotic regulation (Franceschi and Horner 1980; Paiva and Machado 2005).

Raphide bundles in parenchymatous cells are common in Orchidaceae and are of little or no systematic value (Carlsward et al. 1997). However, raphide bundles in epidermal cells are of taxonomic value (Carlquist 1961; Tomlinson 1961,1969). In this study, raphide bundles can be used in species identification as they were only present in leaf epidermal cells in *T. acuminatissimum*, while they were absent in other orchids (*A. emarginatus, D. subulatum*, and *T. subulatum*). Pridgeon (1982) also showed similar results of useful characters of raphide bundles in epidermal cells in the systematic and identification within subtribe Pleurothallidinae.

Spiral thickening

Spiral thickening is spiral-shaped cell wall thickening in parenchymatic cells that function as mechanical stabilization of parenchymatic cells, prevention from desiccation, and for water storage (Leroux et al. 2010). Spiral thickenings were present in parenchymatic mesophyll of *A. emarginatus, T. subulatum*, and *T. acuminatissimum*, while they were absent in *D. subulatum* (Table 1, Figure 2, Figure 4, Figure 5). The spiral thickenings presence in *A. emarginatus, T. subulatum*, and *T. acuminatissimum* is a structural adaptation to high illumination in coastal forests of Sempu Island to reduce water loss because of transpiration. Although *D. subulatum* did not exhibit to posses spiral thickenings in the mesophyll, it had fibre bundles that have the same function as spiral thickenings (discussed above). In the previous study, spiral thickenings were also found in the cortical roots of *A. emarginatus* (Nurfadilah et al. 2016).

The presence or absence of spiral thickenings in orchids of Sempu Island in the present study can be used in species differentiation. *D. subulatum* is characterized by spiral thickenings absence, while they were present in other orchids of Sempu Island. Pridgeon (1982) also reported that spiral thickening is one of useful diagnostic anatomical characters in orchids systematic and identification.

Assessment of the adaptive ability of four orchid species to coastal habitats

To assess the adaptive ability of orchid species to coastal habitats for the classification of the most to the less adaptive species was based on adaptive anatomical characters. The adaptive anatomical characters used included: (i) smaller stomata; smaller stomatas are faster to close under the dry condition that leads to high capacity to reduce transpiration rates (Fahn 1982; Guan et al. 2011); (ii) thicker cuticle; the thicker cuticle the more ability to reflect sunlight and to reduce water loss (Dominguez et al. 2011; Fahn 1982; Rosso 1966); (iii) larger epidermal cell; the larger epidermal cell; the more water can be stored in epidermal cells which is important for water storage in dry condition (Guan et al. 2011); (iv) thicker leaf, this character is related to the succulence level for the adaptation in dry condition (Hsiao 1973; Lack and Evans 2001; Metusala et al. 2017); (v) presence of hypodermis; presence of hypodermis is important for water reservation to increase adaptive ability in warmer environment (Roth 1984; Reginato et al. 2009); (vi) thicker layer of mesophyll; thicker layer also supports the leaves succulence which is important to provide water supply for photosynthetic process and leaves cells turgor especially in arid environment (Hsiao 1973; Lack and Evans 2001; Metusala et al. 2017); (vii) presence of fibre bundles; fibre bundles composed of cellulose, pectin and lignin which is important to prevent from water loss (Vincent, 2000; Richter et al. 2011; Placet et al. 2014); (viii) presence of bundle sheath; bundle sheath containing PEP carboxylase to catalyze the CO_2 fixation in photosynthetic reactions under higher temperature condition for the efficacy of photosynthetic in dry area (Lack and Evans 2001).

Orchids of Sempu Island had various adaptive anatomical characters to ecologically adapt to coastal habitats with high irradiation that can induce high transpiration (Table 3). *A. emarginatus* had three adaptive anatomical characters including smaller stomata, the presence of bundle sheath surrounding vascular bundles, and spiral thickenings in the mesophyll to reduce water loss from leaves. *D. subulatum* had seven adaptive anatomical characters including smaller stomata, thicker cuticle, thicker leaf, the presence of hypodermis, thicker layer of mesophyll, the presence of fibre bundles and bundle sheath to reduce leaf transpiration. *T. subulatum* had two adaptive anatomical characters including thicker cuticle and the presence of spiral thickenings in mesophyll to inhibit water loss. *T. acuminatissimum* had four adaptive anatomical characters including thicker cuticle, larger epidermal cells, the presence of bundle sheath surrounding vascular bundles and spiral thickenings in mesophyll to prevent from the high rate of transpiration.

Table 3. Adaptive anatomical characters of four epiphytic orchids of Sempu Island, East Java, Indonesia

Adaptive anatomical characters	***Ascochilus emarginatus***	***Dendrobium subulatum***	***Thrixspermum subulatum***	***Thrixspermum acuminatissimum***
Smaller stomata	+	+	-	-
Thicker cuticle	-	+	+	+
Larger epidermal cell	-	-	-	+
Thicker leaf	-	+	-	-
Presence of hypodermis	-	+	-	-
Thicker layer of mesophyll	-	+	-	-
Presence of fibre bundles	-	+	-	-
Presence of bundle sheath	+	+	-	+
Presence of spiral thickenings	+	-	+	+

Note: + indicates the presence of the character, - indicates the absence of the character

Dendrobium subulatum had more adaptive anatomical characters (7 adaptive anatomical characters) compared to other orchid species *T. acuminatissimum* (4 adaptive anatomical characters), *A. emarginatus* (3 adaptive anatomical characters), and *T. subulatum* (2 adaptive anatomical characters) (Table 3). This result indicates that *D. subulatum* had more adaptive ability to coastal habitats compared to other orchid species in Sempu Island. Other studies also showed that other orchids species have adaptive anatomical characters, such as *Epidendrum secundum* growing in luminous area had relatively thick cuticle (Moreira et al. (2013); *Holcoglossum* growing in subalpine region of mountainous area possessed laterocytic and polarcytic stomata in their leaf epidermal layer showing structural adaptation to strong winds and ample rains (Fan et al. 2014).

Implication for conservation

This study implied the importance of anatomical characters in species identification. According to Aybeke et al. (2010), detailed anatomical characters are useful to support species identification. Taxonomic knowledge and species identification are required in the conservation. Selecting target species for conservation, identifying endangered species, inventory for biodiversity assessments, and monitoring, all depend on taxonomy and species identification (Schuiteman and de Vogel 2003).

Furthermore, orchids anatomical characters of Sempu Island explain ecological adaptation to the habitat with high irradiation in the coastal habitats. Relatively thick cuticle, smaller stomata, larger epidermis, the presence of hypodermis, many layers of mesophyll, the presence of fibre bundles, spiral thickenings and bundle sheaths, in these orchids are anatomical structures that have key roles to adapt to coastal habitats with high intensity of sunlight.

Of four epiphytic orchids, *D. subulatum* can be considered as the most adaptive species to coastal habitat in

Sempu Island based on the larger number of anatomical adaptive characters it possessed. As epiphytic orchids need higher humidity for growth, four orchid species in Sempu island are facing greater threats of global climate change especially temperature rising. A more adaptive orchid species will be less threatened by extinction. On the other hand, *A. emarginatus*, *T. subulatum*, and *T. acuminatissimum* were potentially influenced by even slight environmental alteration. Therefore, these orchids species need more attention in conservation than the more adaptive species, for their future survival.

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Spatial distribution of echinoderms in littoral area of Ambon Island, Eastern Indonesia

ANA SETYASTUTI[1,♥], WAHYU PURBIANTORO[2], HADIYANTO[1]

[1]Research Center for Oceanography, Indonesian Institute of Sciences. Jl. Pasir Putih Raya No.1, North Jakarta 14430, Jakarta, Indonesia. Tel.: +62-21-64713850, ♥email: ana.setyastuti@gmail.com

[2]Research Center for Deep Sea, Indonesian Institute of Sciences. Jl. Pasir Putih Raya No.1, North Jakarta 14430, Jakarta, Indonesia

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Abstract. *Setyastuti A, Purbiantoro W, Hadiyanto. 2018. Spatial distribution of echinoderms in littoral area of Ambon Island, Eastern Indonesia. Biodiversitas 19: 1919-1925.* Echinoderms samples were collected in Ambon Island as part of the marine resource inventories designed to study the biodiversity of Maluku Archipelago. The purpose of this works was to investigate the abundance and genus richness of echinoderms within the five sampling sites using single transect orthogonal to the coast consisted of 10-quadrat plot of 5x5m, the distance between plots was 10m at each site. Furthermore, this study also aimed to understand the relation of substrate type and community composition within those five sites. The substrate was classified into four types (sea-grass, macro-algae, sand, rocks and/or dead coral) noted by presence/absence mark at each plot. The differences in total abundance and genus richness among those sites were analyzed using an Analysis of Deviance following a Generalized Linear Models (GLMs) fitted by Poisson and log link. However, in term of genus composition, the data were visualized using a Principal Coordination Analysis (PCoA) plots calculated from Bray-Curtis dissimilarity based on the log (x+1) transformed abundance data. A total of 910 individuals of echinoderms belonging to 19 genera was successfully recorded. Total abundance of echinoderms was different significantly among sites ($p<0.05$). The most abundant echinoderms were collected from Tanjung Tiram, approximately 68.71 individuals/quadrat, which was almost 1.4 times than those in Liang and even 4 times than those in Suli. The genus composition between sites was significantly different ($p<0.05$). The composition of substrate types among sites was not significantly different ($p>0.05$), however the composition of substrates correlated significantly with the composition of echinoderms genus ($p<0.05$, rho=0.36). In conclusion, more complex the substrate variation in an area will affect the diversity and abundance of Echinoderms community therein.

Keywords: Ambon-Maluku, Echinodermata, littoral area, spatial distribution

INTRODUCTION

The assessment of biodiversity in the marine realms are important for understanding ecological patterns, ecosystem functioning, and conservation management, thus magnetize many ecologist, societies, conservationist and decisions maker. Other importance of biodiversity inventories are to detects, monitor, measures and estimates the fluctuation of diversity list and the implications of its changes to the ecosystem function (Iken et al. 2010; Wheeler et al. 2012).

Echinoderms are the crucial part of marine biodiversity because of its ecological role (Birkeland 1988; Iken et al. 2010; Lampe 2013). For example Holothuroidea is positive as a major bioturbator because they can increase the productivity of benthic microalgae and seagrass system and through their excretory physiology they also can increase local nutrients (Uthicke and Klumpp 1998; Uthicke 2001; Wolkenhauer et al. 2010; Jamieson et al. 2011; Costa et al. 2014; Wolfe and Byrne 2017) and potential to help buffer the effects of ocean acidification (Schneider et al. 2011; Schneider et al. 2013; Wolfe and Byrne 2017). Echinoidea as a herbivore has an important role in controlling the algal cover on hard substrates, and as a bioturbator (or their foraging behavior) they may play a key role as a limiting factor of reef growth (Bak 1994; Mokady et al. 1996; Carreiro-Silva and McClanahan 2001; Fjukmoen 2006). Some species of Asteroidea are a corallivore, their grazing activity can strongly influence the coral reef ecosystem structure and diversity (Yamaguchi 1986; Lane 1996; Wakeford et al. 2008). Ophiuroidea as a dominant taxon in Arctic region may account for a large portion of remineralization (Piepenburg et al. 1995; Ambrose et al. 2001).

Estimation number of echinoderms diversity over the world which already described is roughly 7.291 species, consist of Asteroidea is 1922 species, Echinoidea is 999 species, Ophiuroidea is 2.064 species, Crinoidea and Holothuroidea are 623 and 1.683 species, respectively (Appeltans et al. 2012). Data of Indonesian echinoderms diversity seem to scatter in specific location and most are about Holothuroidea. Supono et al. (2014) listed echinoderms species in Lembeh Strait, North Sulawesi, total species observed were 76 species; Setyastuti (2014) listed 23 species of echinoderms diversity in Nusa Laut Island, Central Maluku; Uneputty et al. (2017) observed 17 species of echinoderms of Ambon, Maluku. Other publications are about Holothuroidea, Massin (1996) enlisted 27 species from Ambon, Maluku; Massin (1999) enlisted 56 species from Spermonde, South Sulawesi; Setyastuti (2009) enlisted 9 species from West Seram, Maluku; Lane and Limbong (2013) successfully reviewed 28 species from Bunaken National Marine Park, North

Sulawesi. Those differences in number of taxa at each publication seem to correlate with different sampling methods they used. However, it is difficult to quantify because complete species inventories require extraordinary efforts (Longino et al. 2002; Shen et al. 2003) and there are undiscovered species in almost every taxonomic survey or species inventory (Shen et al. 2003).

Echinoderms samples were collected in Ambon Island (Figure 1) as part of the marine resources' inventories designed to study the biodiversity of Maluku Archipelago. The purpose of this works were to investigate the abundance and genus richness of echinoderms within the five sampling sites; two sites at outer of Ambon Island (Liang and Suli), three sites at Ambon Bay (Lateri, Halong, and Tanjung Tiram) using sampling technique of quantitative biodiversity assessment that we tried to expand from previous methodologies. Furthermore, this study also aimed to understand the relation of substrate type and community composition within those five sites.

MATERIALS AND METHODS

Study area

Liang and Suli beach are located in the outer side of Ambon Island, Maluku, Indonesia, but specific location of both sites are different. Liang beach is interrupted most by oceanic flow from Seram Sea. Suli, however, is located inside the Baguala Bay thus making the area more sheltered than Liang. As a fringing reef area, both sites are characterized by a very vast area of intertidal whereas mangrove, seagrass, and coral reef ecosystem is positioned in adjacent. By visual observation, it can be considered that seagrass density at Liang is lower than Suli. Substrates at Liang are mostly sandy bottom with a few boulders/rubble found. Otherwise, at Suli, the substrates composition are more complex than Liang, sandy bottom of which most areas are mixed with rubble and died corals. Seagrass species found at Liang are dominated by *Thalassia hemprichii,* and *Cymodocea rotundata*, whereas at Suli are *T. hemprichii* and *Enhalus acoroides*.

Halong, Lateri, and Tanjung Tiram are located in the inner of Ambon Bay thus making the current less heavy than Liang/Suli. Halong and Lateri have similar habitat characteristics such as spotty seagrass stands and sandy-muddy substrate. Seagrass species that found at Halong are *E. acoroides* and *T. hemprichii,* while at Lateri is only *E. acoroides.* The intertidal area is approximately less than 100 m perpendicular to the slope. Furthermore, only a few boulders are found at these sites. On the contrary, Tanjung Tiram has a very vast intertidal area as Liang and Suli. Seagrass meadow, sandy, rubble, and boulders bottom are dominance characteristic at the site. *E. acoroides* is the most dominant seagrass species herein.

Procedures

Sampling technique

The fieldwork took place four days during two weeks period in late March and early April 2014 at five sites, two sites at outer of Ambon Island (Liang and Suli beach), three sites at inner Ambon Bay (Lateri, Halong, and Tanjung Tiram beach) (Figure 1). Investigation on echinoderm diversity and abundance used single transect orthogonal to the coast at each site. Each transect consisted of 10-quadrat plot of 5x5m, the distance between plots was 10m. Sampling was conducted during the low tide to get the better view while inventories the echinoderms benthic community. Each individual found within the plot was noted and identified up to genus level using taxonomy references (Rowe 1969; Clark and Rowe 1971; Massin 1996; Vandenspiegel et al. 1998; Massin 1999; Albuquerque et al. 2001; Massin et al. 2002; Purwati and Lane 2002; Fujita and Marsh 2004; Samyn et al. 2006; O'louglin and Rowe 2006; Clark and Jewett 2010; Clark and Jewett 2011; Kim et al. 2013; O'Loughlin and Birbiesca-Contreras 2015). Furthermore, substrates that classified into four types (sea-grass, macro-algae, sand, rocks, and/or dead coral) at each plot were also noted by presence/absence mark.

Figure 1. Study sites in Ambon Island, Maluku, Indonesia. 1. Liang, 2. Suli, 3. Halong, 4. Lateri, 5. Tanjung Tiram

Data analysis

Since the transect result at Lateri and Halong sites were nil, data analyses included differences in total abundance, genus richness, and genus composition of echinoderms was applied only for three sites, i.e. Liang, Suli, and Tanjung Tiram beach. The composition of substrate types among those sites was also analyzed based on the binomial data, i.e. absent (0) and present (1).

The differences in total abundance and genus richness among those sites were analyzed using an Analysis of Deviance following a Generalized Linear Models (GLMs) fitted by Poisson and log link. A GLMs was used because the data were count data with a lot of zero (Crawley 2015). Pairwise tests were also done to analyses which sites that differ significantly in total abundance and genus richness ($p<0.05$).

In term of genus composition, the data were visualized using a Principal Coordination Analysis (PCoA) plots calculated from Bray-Curtis dissimilarity based on the log (x+1) transformed abundance data. The differences in genus composition among study sites were analyzed using a Per-mutational Multivariate Analysis of Variance (PERMANOVA) calculated from Bray-Curtis dissimilarity based on the log (x+1) transformed abundance data with 999 permutations. A log (x+1) transformation was used because the data variance was higher than those mean (Bakus 2007). Pairwise tests were also conducted to analyses which sites that differ significantly in genus composition ($p<0.05$). The genus contributing to those differences was determined based on the genus vector that had significant correlations with both axes of PCoA plots (i.e. PCO1 and PCO2) ($p<0.05$) and the value of rho >0.6 according to Spearman correlation tests.

Similarly, the composition of substrate types among study sites was also visualized and analyzed using a PCoA and a PERMANOVA, respectively, but those were calculated from Bray-Curtis dissimilarity based on the binomial data. The correlation between genus composition and substrate composition was then analyzed using a Mantel Test for Dissimilarity Matrices.

Data analyses and visualizations were performed using R software (*https: //www.r-project.org*) using various packages. The package of vegan was used to perform PCoA, PERMANOVA, and Mantel Test (Oksanen et al. 2016), while the package of ggplot2 was used to visualize the data (Wickham 2009).

RESULTS AND DISCUSSIONS

Results

A total of 910 individuals of echinoderms belonging to 19 genera were observed in this study. Table 1 present the list of genus obtained at each site except Halong and Lateri which null result. The total abundance of echinoderms was different significantly among sites ($p<0.05$). The most abundant echinoderms that were collected from Tanjung Tiram approximately 68.71 individuals/quadrat, which was almost 1.4 times than those in Liang and even 4 times than those in Suli (Figure 2.A). However, the genus richness was not significantly different among sites ($p>0.05$), around 3-5 genera/quadrat (Figure 2.B).

In term of genus composition, the PCoA plots represented 39.37% of total variations, i.e. PCO1 (23.37%) and PCO2 (16.00%) (Figure 2.C). PERMANOVA and Pairwise tests showed that the genus composition between sites was significantly different ($p<0.05$). Genera contributed to those differences were *Bohadschia*, *Ophiocoma*, and *Synapta*, while *Bohadschia* was only found in Tanjung Tiram. *Ophiocoma* was absent from this site but abundant in Liang. *Synapta* was abundant in both Tanjung Tiram and Liang, but few in Suli (Figure 2.E).

Compared to the genus composition, the PCoA plots represented more variations in the composition of substrate types, about 91.99% of total variations, i.e., PCO1 (91.52%) and PCO2 (0.47%). The composition of substrate types among sites was not significantly different ($p>0.05$) (Figure 2.D). However, the composition of substrates correlated significantly with the composition of echinoderm genera ($p<0.05$, rho= 0.36). The presence of seagrass, rock, and dead corals increased the diversity and abundance of echinoderms in the Ambon Bay.

Table 1. Genus distribution at each site in Ambon Island, Maluku, Indonesia

No.	Genus	Liang	Suli	Tanjung Tiram
Class Holothuroidea				
1	*Actinopyga*	-	-	√
2	*Bohadschia*	-	-	√
3	*Holothuria*	√	√	√
4	*Stichopus*	-	-	√
5	*Synapta*	√	√	√
6	*Opheodesoma*	√	-	√
				-
Class Asteroidea				
7	*Culcita*	√	-	-
8	*Linckia*	√	-	-
9	*Protoreaster*	-	√	-
10	*Archaster*	-	-	√
				-
Class Ophiuroidea				
11	*Ophiocoma*	√	√	-
12	*Ophiarachnella*	√	-	-
13	*Macrophiotrix*	√	-	-
14	*Ophiolepsis*	√	-	-
				-
Class Echinoidea				
15	*Diadema*	√	√	√
16	*Echinometra*	√	√	-
17	*Echinotrix*	-	-	√
18	*Mespilia**	-	-	√
19	*Tripneustes**	-	-	√
Total genus		11	6	11

Note: √: present, -: absent; *free handpicking collection/not in the transect plot

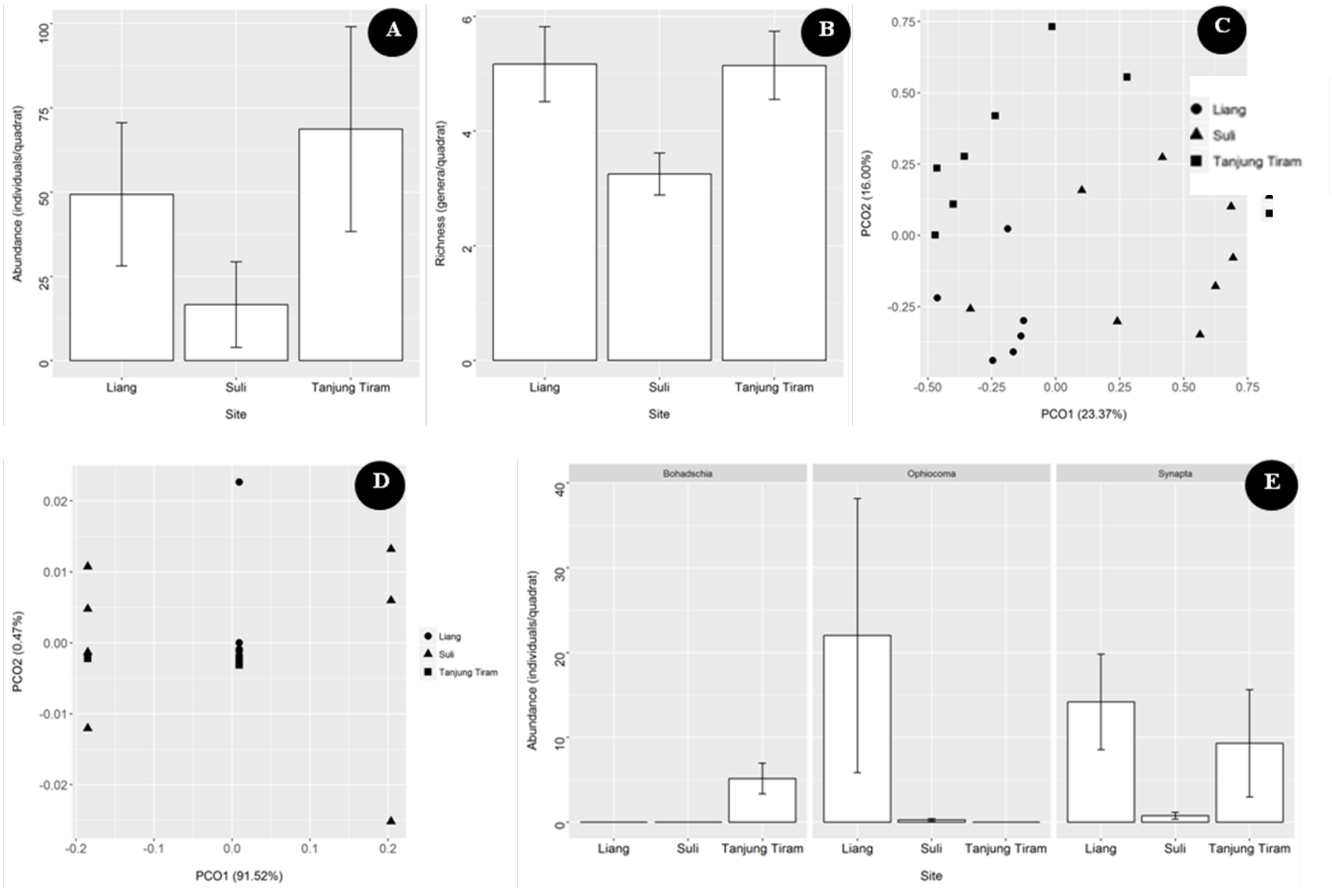

Figure 2. A. Total abundance of echinoderms among study sites; B. Genus richness of echinoderms among study sites; C. The ordination of genus composition of echinoderms among study sites; D. The ordination of the composition of substrate types among study sites; E. The distribution of genus contributed to differences in genus composition among study sites

Total abundance of echinoderms was different significantly among sites ($p<0.05$). The most abundant echinoderms were collected from Tanjung Tiram approximately 68.71 individuals/quadrat, which was almost 1.4 times than those in Liang and even 4 times than those in Suli (Figure 2.A). However, the genus richness was not significantly different among sites ($p>0.05$), around 3-5 genera/quadrat (Figure 2.B).

Discussion

Tracing on publications of echinoderms diversity and density in the intertidal zone or shoreline specifically showed that the methodology used for the sampling were varied. Several publications used standardized protocols of the Census of Marine Life NaGISA program (Natural Geography in Shore Areas, www.coml.nagisa.org), of which transect perpendicular to the coast by using 5-10 plot of 0.0625 m^2 (Chenelot et al. 2007; Iken et al. 2010; LIacuna et al. 2016). While other publications used line transect/belt transect of 40 x 40 m /10 x 10 m/50 x 2 m along the beachside or perpendicular to the coast (Massin and Doumen 1986; Darsono et al. 1998; Hasan 2009; Selano et al. 2014; Uneputty et al. 2017). The others used quadrat transect of 0.5 x 0.5 m/1 x 1 m perpendicular to the coast (Yusron 2003, 2003b; Dobo 2009). Each methodology has its own justification. However, the pluses and minuses of each methodology are inevitable. This study tried to expand the methods, considering the middle efforts compare to previous different methods by using the transect plot of 25 m^2 perpendicular to the coast.

Out of five sites location, two sites (Halong and Lateri) were excluded from the statistical analyses because of no echinoderms found during the survey. Several causes to explain it could be the habitat condition does not support anymore for the sustainable living of echinoderms. Based on visual observation, the sedimentation occurred in those two areas making the bottom substratum covered most by mud. Moreover, liquid anthropogenic waste from the village surrounding is also recorded very high. At those two areas, we could not find any boulders/rubbles/corrals, and only a few *E. acoroides* stands noted, as ecologically common known either boulders or seagrass stands worthwhile beneficially as shelter area and nutrient trapping respectively (Hereu et al. 2004; Entrambasaguas et al. 2008). The composite of those factors is possibly making the sites not suitable anymore for echinoderms to live, and this condition shows the declining of habitat quality compared to many previous publications that still

recorded several echinoderms species on those areas (PPLD-LIPI 2014). However, this kind of habitat degradation may also lead to declining of food supply and the capability of recruitment that affected the patchiness of marine organism (Chenelot et al. 2007).

During the study, we only observed four classes out of five extant classes of Echinodermata, i.e., Holothuroidea, Echinoidea, Asteroidea, and Ophiuroidea. The absence of class Crinoidea is no clear reason. However, from the point of view of habitat preference, it could be a consequence of its habitat restrictions related to food resource and attachment to the stratum that affected it anatomy adaptation (Ausich 1980; Entrambasaguas et al. 2008). Since crinoid itself is an animal group that eats plankton from seawater and nocturnal, it makes them prefer to live in the area with persistent currents such as in the floor of deep sea or coral reef ecosystem (Meyer 1976; Ausich 1980; Kirkendale and Messing 2003). However, several publications noted that crinoid species in Indonesia or the world usually recorded not in the exposed water/shoreline (Clark and Rowe 1971; Meyer 1976; Ausich 1980; Messing 2007).

Genus richness was not significantly different among sites ($p<0.05$) (Figure 2B). Tanjung Tiram and Liang showed the same number of genus diversity even their composition was totally different (Figure 2.C, 2.E). Tanjung tiram and Liang possessed 11 genera each, of which four genera were the same (*Opheodesoma, Synapta, Holothuria,* and *Diadema*). Other genera such as *Bohadschia*, *Actinopyga, Echinothrix, Archaster, Stichopus, Mespilia,* and *Tripnesuates* were exclusively only on Tanjung Tiram. However, statistical analysis showed that genus contributed to those genus composition differences were *Bohadschia*, *Ophiocoma*, and *Synapta* (Figure 2.E). While *Bohadschia* was only found in Tanjung Tiram, *Ophiocoma* was absent from this site but abundant in Liang. *Synapta* was abundant in both Tanjung Tiram and Liang but few in Suli. These findings showed that several genera might have their specific microhabitat preference because different genus responds differently to environmental drivers that contribute to some of the disparate patterns (Iken et al. 2010). However, no single environmental variable was the sole driver. The more complex the habitat in the sites the more favorable places for more organism to live in, since it will provide more places for recruitment, food supply, and shelter from the predator. These findings also supported by the correlation analysis of genus richness and substrates composition at each sites using PcoA that resulted in the composition of substrates correlated significantly ($p<0.05$, rho=0.36) with the composition of echinoderm genus which means that the presence of seagrass, rock, and dead corals increased the diversity and abundance of echinoderms in the Ambon Bay.

Our data showed that total abundance among three observed sites was different significantly ($p<0.05$), of which Tanjung Tiram was the most abundant and then Liang and Suli afterward (Figure 2.A). In Tanjung Tiram was successfully observed 481 inds. echinoderms, meanwhile in Liang and Suli noted about 296 and 133 inds., respectively. Out of those, the highest individual number in Tanjung Tiram was genus *Diadema* (306 inds.). Thoroughly investigation showed that most of this genus individually captured in the area near the slope where the substrates were mostly rubble, coral/dead coral, and algae. This finding supports the information about specific microhabitat of *Diadema* as a herbivore of algal turf that is usually covering the hard substratum (Bak 1994; Aziz 1996; Fjukmoen 2006). It also strengthens the result of (Bechtel et al. 2006) that the presence of *Diadema* is instrumental in initiating the transition from algal to the coral-dominated reef since our result noted that most of its aggregation individuals were discovered on the transition area of seagrass bed and coral reef ecosystem.

Some notes that we can conclude from our research are: (i) patchily distribution of echinoderms as a megabenthic community is commonly known. Our present results generated the same things, that was in our two sites (Lateri and Halong) did not contain any echinoderms. However, the causes of this patchiness can be numerous, including the physical environment (such as the habitat changing that obviously seen in those two sites because of environmental damage caused by water pollution, sedimentation, and liquid household waste). This kind of condition may lead to habitat degradation that could interrupt the food supply, recruitment process, and shelter area. Another reason might be related to the cryptic behavior of certain species. (ii) Abundance and species richness of echinoderm are correlated significantly with substrate composition. More diverse the substrate composition, more abundance the individual and more varies the diversity of echinoderms will be recorded.

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The impacts of traditional homegarden conversion into the commercial one: A case study in Sukapura Village of the Upstream Citarum Watershed, West Java, Indonesia

JULIATI PRIHATINI[1], JOHAN ISKANDAR[2], RUHYAT PARTASASMITA[2,♥], DEDEN NURJAMAN[1]

[1]The Government Institute of Home Affairs (IPDN). Jl. Jl. Raya Bandung-Sumedang Km 20, Jatinangor Sumedang 45363, West Java, Indonesia
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran. Jl. Raya Bandung-Sumedang Km 21, Jatinangor, Sumedang 45363, West Java, Indonesia. Tel./fax.: +62-22-7796412, ♥email: ruhyat.partasasmita@unpad.ac.id; rp2010rikkyo@gmail.com

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Abstract. *Prihatini J, Iskandar J, Partasasmita R, Nurjaman D. 2018. The impacts of traditional homegarden conversion into the commercial one: A case study in Sukapura Village of the Upstream Citarum Watershed, West Java, Indonesia. Biodiversitas 19: 1926-1940.* In the past, rural homegardens in West Java were planted with various annual and perennial crops. As a result, the vegetation structure of traditional homegardens in rural areas of West Java, Indonesia was very complex, similar to that of forest vegetation. Nowadays, however, due to rapid development of market economic system in rural areas, many traditional homegardens in West Java have been converted into the commercial ones. Consequently, the structure and functions of the homegardens have drastically changed. For example, the vegetation structure has become simpler and dominated by commercial crops, and the gardens serve mostly economic function instead of providing various ecological, socio-economic and cultural functions. The aim of this study was to elucidate: (i) the ecological history of traditional homegardens, (ii) the changes of structure and functions of the homegardens converted from the traditional into the commercial one, and (iii) the positive and negative impacts of conversion of the traditional homegardens into the commercial ones in the Village of Sukapura, the Subdistrict of Kertasari, the District of Bandung, Upstream Citarum Watershed, West Java. The combination of qualitative and quantitative methods were used, while some techniques, including observations, and in-depth interviews with competent informants were applied in this study. The results of study showed that initially the traditional homegardens in Kertasari Village had been predominantly cropped with various annual and perennial crops. However, due to market economic development, the homegardens have been drastically changed. For example, the commercial vegetable crops, including Welsh onion (*Allium fistulosum* L), carrot (*Daucus carota* L) and cabbage (*Brassica oleracea* var *capitata*) have been predominantly cultivated in the commercial homegardens. Consequently, the household income of the village people who own the commercial homegardens increased, however, some ecological and socio-cultural functions of the commercial homegardens drastically decreased. In addition, some negative impacts of the commercialization of the homegardens have occurred. We suggest that to develop the sustainable village homegardens for the future, the diversity of plants must be maintained to provide ecological function or ecosystem services and the economic production must be improved to increase the income of the rural people.

Keywords: Changes of homegarden, commercial homegarden, traditional homegarden, Upper Citarum Watershed

INTRODUCTION

Homegarden is one of the traditional agroforestry systems which may be defined as "a piece of land with a definite boundary surrounding a home, cultivated with a diverse combination of perennial and annual plant species, having a multilayered vertical structure, and it is often used as a place for raising livestock, and managed mainly by household members for subsistence production." (Karyono 1990; Iskandar and Iskandar 2011; Iskandar et al. 2018).

According to environmental history, the rural homegarden of West Java has evolutionally devolved from forest ecosystem and culturally developed into the homagarden (*pekarangan*), the perennial mixed garden (*kebun campuran* or *talun*), the garden (*kebun*), and the rice field (*sawah)* (Iskandar and Iskandar 2011). The homegarden as one of traditional agroforestry systems has both subsistence and commercial functions. The subsistence production functions have been recognized as providing the household needs, including starchy or carbohydrate foods, spices, vegetables, ornaments, medicines, handicraft, and traditional materials for rituals, while the commercial production functions is providing cash income from the trade of production surpluses, including fruits (Iskandar and Iskandar 2016a; Iskandar 2017).

Initially the rural homegardens in West Java had been managed using the traditional ecological knowledge (TEK) and had been strongly embedded in local culture (cf. Toledo 2002; Iskandar 2010). In addition, it had been managed mainly for subsistence and not for commercial function (cf. Warton 1970). In the past, the homegardens were planted with high diversity of annual and perennial plants. Since homegarden is a man-made ecosystem, various plants planted in the homegardens have been determined by ecological factors, including altitude, water availability, soil condition, and climate, and by socioeconomic-cultural factors, including land size, education level, income, distance from market, and market development (Iskandar and Iskandar 2016a). The size of a

homegarden varies between less than 100 m^2 and more than 200 m^2 (Arifin 2013). There is a positive correlation between the size of a homegarden and the diversity and the number of individual plants in the homegarden (Karyono 1990; Iskandar and Iskandar 2016a). The results of inventory of the Indonesian homegarden plants of the framework of the consortium of genetic resources of conducted by the Agricultural Technology Research Centers in 2013, showed that the food, horticultural, spice and medicinal plants in the homegardens contributed of 17, 57, and 26%, respectively. The genetic resources of food crops in the homegardens which have been planted for a long time were considered as the ones adapted to the local environment and can be used for plant breeder programs (cf. Surat and Yaman 2017). As a result, the homegardens have played an important role in conserving genetic sources and in supporting food security referred to in the Act No. 18 of 2012 (cf. Saliem 2011). According to the reports of case studies in East Kalimantan and Bengkulu Provinces, the utilization of homegardens cultivated with high diversity of plants can support food self-sufficiency of the traditional people and village communities (Afrilia and Rizal 2015; Wiryono et al. 2016). However, unlike the village homegardens, the urban homegardens are usually small in size and have low diversity of plants, except for ornamental plants which are relatively high (Iskandar and Iskandar 2016a).

In the past, the homegardens got low external inputs, including seeds, inorganic fertilizers, and pesticides. However, since the homegardens have high diversity of plants, they have high stability, equitability, and resilience (cf. Soemarwoto and Conwey 1992; Kehlenbeck and Maass 2004; Arifin 2013; Iskandar and Iskandar 2016a).

The people in Sukapura Village initially owned traditional homegardens. However, in the last several decades, a lot of traditional homegardens in Sukapura Village have been converted into the modern ones, including by intensification of monoculture vegetable crops, due to many factors, particularly intensive market economic penetration. Consequently, several positive and negative impacts on ecological, socio-economic and cultural aspects have been inevitable. Some studies on changes of the homegarden were undertaken by some scholars, including Hadikusmah (2003), Kubota et al. (2003), Prihartini (2004) that were focused on vegetation structures and economic aspects. However, the study on changes of the homegardens in ecological and socio-economic-cultural aspects as in integrated systems has rarely been undertaken.

This paper elucidates: (i) the ecological history of traditional homegardens, (ii) the changes of structure and functions of the homegardens converted from the traditional into the commercial one, and (iii) the positive and negative impacts of conversion of the traditional homegardens into the commercial ones in the Village of Sukapura, Upstream Citarum Watershed, the Subdistrict of Kertasari, the District of Bandung, the Province of West Java, Indonesia conducted in 2004 (Prihatini 2004) and 2018.

MATERIALS AND METHODS

Study area

This research was conducted in 2004 in Sukapura Village, upper Citarum watershed, Kartasari Sub-district, Bandung District, West Java, Indonesia (Figure 1), and the results were used as baseline data (Prihartini 2004), while the updated data were collected in the same location in April 2018.

Data collection

This study used a combination of quantitative and qualitative methods. The quantitative methods were applied to record species of plants in both the traditional and commercial homegardens. Total samples of 40 homegardens, consisting of 20 traditional homegardens and 20 commercial homegardens, were selected. Each unit of homegarden was considered as a plot. The species of every plant and number of individuals of each species in every plot were recorded.

The qualitative data were applied to collect social-economic aspects, including ecological history of land use types, particularly the homegarden ecosystems, homegarden functions, changes of farming practices of the homegardens. Some techniques including observation and interview were applied to collect primary data in the field. Observations were conducted to observe general local environmental conditions, such as that of settlement and homegarden, and homegarden vegetation. In-depth interviews with competent informants or local experts who were purposively selected were conducted (cf. Martin 1995; Iskandar 2012). The informants consisted of formal village leaders, hamlet leaders, informal/religion leaders, old farmers, vegetable farmers, village vegetable traders, village market traders, and village middlemen.

Data analyses

The structure and floristic composition of homegardens were analyzed using some indexes, including Summed Dominance Ratio (SDR), Index of Similarity, and Index of Diversity. The qualitative data of social-economic aspects of the homegardens were analyzed by cross-checking to get valid data collected by observations.

Summed Dominance Ratio (SDR)

SDR index was used to analyze the plant species dominance and frequency of both the traditional and commercial homegardens. SDR was calculated using the formula below (Numata 1974; Iskandar and Iskandar 2016a):

$$SDR = (FR + DR) /2$$

Where;
SDR : Summed dominance ratio;
F : Absolute frequency;
FR : Relative frequency;
Di : Absolute dominance of species -i;
DR : Relative dominance

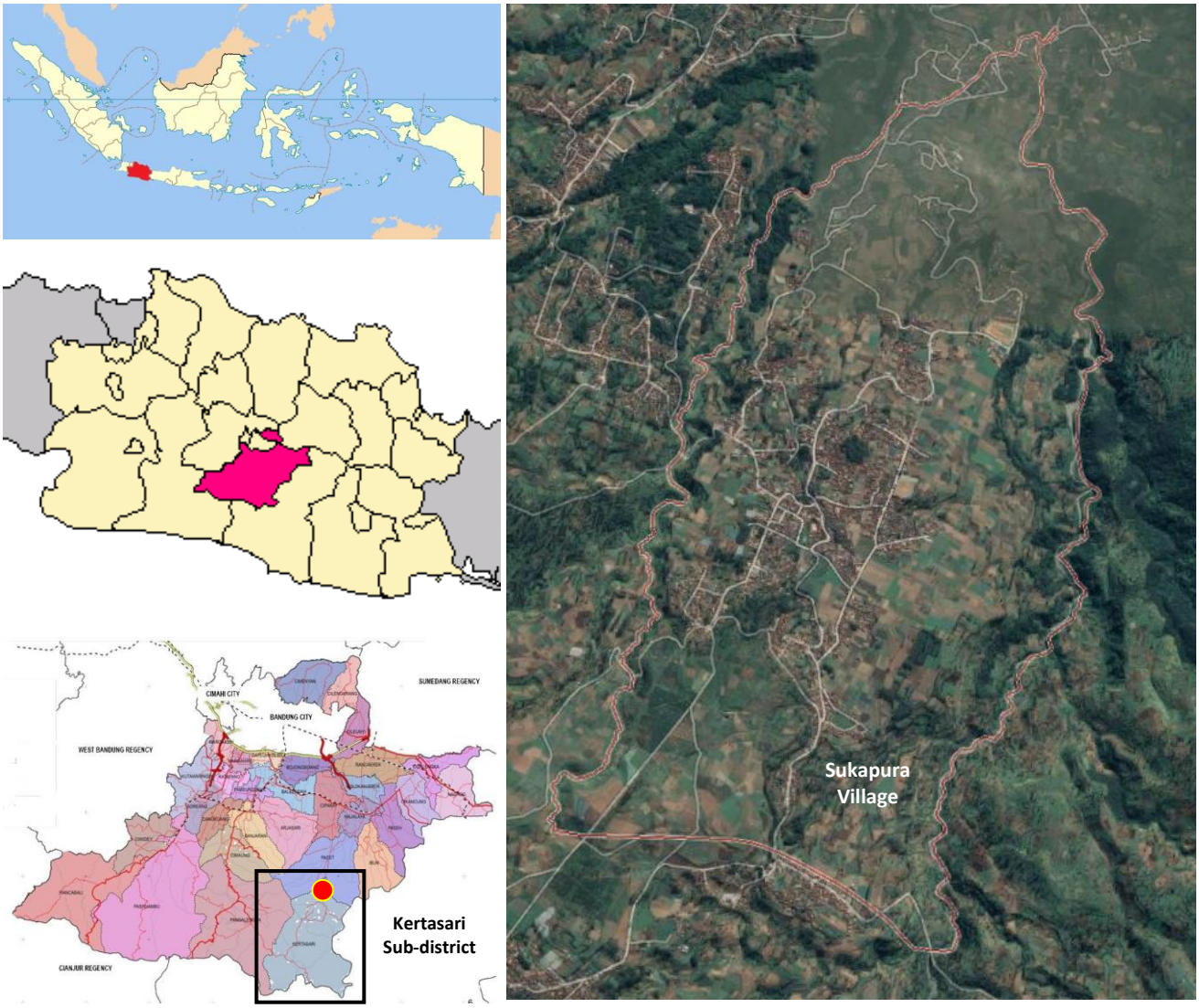

Figure 1. Map of location of study area in Sukapura Village (●), Kertasari Sub-district, Bandung District, West Java, Indonesia

These parameters were computed as follows:

$$F = \frac{Number\ of\ home\ gardens\ in\ which\ a\ particular\ species\ occurs}{Total\ homegarden\ samples} X100\%$$

$$FR = \frac{Frequency\ of\ species\ -\ i}{Sum\ frequency\ of\ all\ species}\ x\ 100\ \%$$

$$Di = \frac{Individual\ number\ of\ species\ -\ i}{ndividual\ number\ of\ all\ species} X100\%$$

$$DR = \frac{Dominance\ of\ species\ -\ i}{Dominance\ of\ all\ species}$$

The plant species which are found in many samples and have many individuals have a high-value index of SDR.

Diversity index

Diversity index is based on the relationship between the total number of individuals of plant present and the number of individuals per species of plant of the homegarden samples. In other words, diversity index integrates species richness and evenness into a single value. A measure diversity is useful when investigating the interactions of physical and biotic factors in an ecosystem, including human factors, particularly in the homegarden ecosystem (cf. Williams 1987; Magurran 1988; Iskandar and Kotanegara 1995).

The formula of diversity index of Shannon-Wiener is:

$$H' = -\sum_{i-1}^{n}\left[\frac{n_i}{N}\ ln\ \frac{n_i}{N}\right]$$

Where:

H' : The diversity index of Shannon-Wiener

ni : Total number of individuals of the i-th species in the samples

N : The total number of individuals of all species in samples

The diversity index can be used in analyzing the quality of communities, particularly in natural ecosystems, including forest ecosystem. The community, including homegarden, that has high diversity index has a good quality (Iskandar and Iskandar 2016a).

Similarity index

To compare the floristic communities of homegarden plants in different times, namely in 2004 and 2018, similarity index of Sørensen was used (cf. Mueller-Dombois and Ellenberg 1974; Iskandar and Iskandar 2016a):

$$ISs = \frac{2\,C}{A + B} \times 100\ \%$$

Where:

ISs : Index of similarity of Sørensen

A : Total number of plant species recorded in 2004

B : Total number of plant species recorded in 2018

C : Number of plant species common in both 2004 and 2008

IDS : Dissimilarity index is 100 %-ISS

High similarity index means that the homegardens in 2004 and 2018 have similar species composition.

Analyses of social data

The qualitative data of social aspects were analyzed by cross-checking, summarizing, synthesizing, and narrating (Newing et al. 2011). Cross-checking was carried out to check the validity of information based on the information obtained from different techniques, namely observation and in-depth interviews, and information from different informants. Moreover, the data were summarized, synthesized and made into systematic descriptions with descriptive and evaluative analyses.

RESULTS AND DISCUSSION

Study site

Administratively, Sukapura is one of the villages of Kertasari Sub-district, Bandung District of West Java, Indonesia (Figure 1). Sukapura Village has located about 52 km from Bandung city, the capital city of West Java, and has distance of approximately 39 km from Soreang, the capital of Bandung District.

Sukapura is bordered by other neighboring villages. To the north, it is bordered by Resmitingal Village of Kertasari, to the south by Cibeureum Village of Kertasari Sub-district, to the east by Cihawuk Village and Forest area of Kertasari Sub-district, and to the west by Girimulya Village of Pacet Sub-district (Sukapura 2016).

The agricultural land use types of Sukapura are homegarden (*pekarangan*), vegetable garden (*kebun sayur*), mixed-perennial crop garden (*kebon tatangkalan* or *talun*), bamboo talun (*kebon awi*), and rice field (*sawah*). Almost all households in Sukapura Village have homegardens. They obtained the homegardens by various means, mainly heritage, buying, and heritage and buying.

Sukapura Village is categorized as a village of highland located at an altitude of 1,300 m. The daily air temperature is between 20 and 24 degrees Celsius and the average rainfall is between 600-700 mm/month. Its high altitude makes Sukapura Village appropriate for vegetable farming. In recent changes of development, the commercial vegetable crops, including the Welsh onion (*Allium fistulosom* L), carrot (*Daucus carota* L) and cabbage (*Brassica oleracea* var *capitata*) were not only planted in the vegetable gardens but also in homegardens. As a result, Sukapura Village has been known as one of vegetable center areas of Bandung District, West Java.

According to the village statistical data, the total area of Sukapura Village is 596.7 Ha. The population of Sukapura in 2016 was 8,636, consisting of 4,415 males and 4,221 females with a total of 2,844 households (Sukapura 2016).

The main occupations of people are farmers (547 persons) and farmer labors (1,230 persons).In addition, various off-farm occupations, such as merchants of village stalls, peddlers, and carpenters are also found (Table 1).

Ecological history and changes of the homegarden

According to ecological history, in the past, the upper Citarum watershed of West Java was predominantly forest. Like other upland areas of West Java, the forest of the upper Citarum watershed was traditionally used by local villagers for practicing the swidden cultivation (*ngahuma*) (cf. Iskandar and Iskandar 2011; Iskandar et al. 2017). The forest of the upper Citarum drastically changed due to the introduction of cultivation system (*cultuur stelsel* or *tanam paksa)* in Java between 1830 and 1870. The forests were predominantly planted with quinine/*kina* (*Cinchona pahudiana* Howard) and tea (*Camellia sinensis* (L.) Kuntze). In 1870, the cultivation system was abolished and the land was taken by private commercial plantation.

Table 1. Composition of people occupations in Sukapura Village, Kertasari Sub-district, Bandung District, West Java, Indonesia

People occupations	Number of people (persons)
Free detailer	2120
Labor farmer	1230
Farmer	547
Merchant of village stall	199
Civil servant	59
Micro/Middle craftsmen	42
Livestock farmer	30
Peddler	28
Carpenter	26
Soil digger	10
Retired civil servant	10
Trained village healer	9
Mechanic	8
Businessmen	6
Servant of Army/Police of Republic of Indonesia	6
Army/Police of Republic of Indonesia	4
Barber	3
Midwife/Nurse	3
Total	4,340

Note: Sukapura (2016)

Then, after the Indonesian Independence, the quinine and tea plantation were managed by Perkebunan Nusantara (PTPN) VIII based on '*Hak Guna Usaha'* (*HGU*-Plantation concession permit) and the permit expired at the end of 1997 (Kurniawan et al. 2011). Afterward, since the beginning of the Reform Order, some abandoned the quinine, and the plantation areas were illegally cultivated with commercial vegetable crops by farmers. At the same time with forest conversion to plantation, some rural people continuously practiced swidden farming. Furthermore, they established the settlement by converting the secondary forest to a farmhouse and developing into semi-permanent houses in a cluster called *catihan* and new hamlet (*babakan*) and more permanent hamlet (*kampung* or *ampian*). Then the forest areas decreased and the population increased, so the shifting cultivation was formally prohibited by the government. As a result, the traditional swidden farming was gradually changed to several agroecosystem types, including homegarden (*pekarangan*), wet rice field (*sawah*), perennial mixed-garden (*kebun campuran* or *kebon tatangkalan*) and *bamboo talun* (*talun bambu*). However, with the introduction of commercial vegetable crops, some traditional agroforestry systems, including the perennial mixed-garden and bamboo talun have been gradually converted into the commercial vegetable garden. Indeed, the effect of intensive farming of commercial vegetable crops in the gardens has caused the conversion of the traditional homegarden into commercial one.

According to the informants, in the period between 1900s and 1980s the homegardens in Sukapura Village were predominantly managed by traditional system which provided very low or zero inputs from outside or markets. The homegardens were planted with a variety of annual crops, including corn (*Zea mays* L), cassava (*Manihot esculenta* Crantz), banana (*Musa x paradisiaca* L), tomato (*Solanum lycopersicum* L), ginger (*Zingiber officinale* Roscoe), sand ginger/*kencur* (*Kaempferia galanga* L), tumeric/*koneng* (*Curcuma domestica* Valeton), sweet potato/ *hui boled* (*Ipomoea batatas* L), peanut (*Arachis hypogaea* L), and lemongrass (*Cymbopogon citratus* (DC) Stapf). In addition, some perennial crops, including fruit plants, such as common guava (*Psidium guajava* L), soursop/*sirsak* (*Annona muricata* L), jackfruit (*Artocarpus heterophyllus* Lam) and mango (*Mangifera indica* (L) Pulp) were also planted in combination with annual crops in the homegardens. Most production of the homegardens was mainly used for home consumption instead of being sold to obtain cash income. In the 1980s some traditional homegardens drastically changed into the commercial ones. At that time, potato (*Solanum tuberosum* L), cabbage (*Brassica oleracea* var *capitata*) and carrot (*Daucus carota* L) were first introduced and planted in the traditional homegardens in Sukapura Village. The seeds of those plants were brought from Cisarua, Lembang. As a result, between 1990 and 2004, 65% of respondents of the villagers of Sukapura adopted the commercial vegetable crops and drastically changed the traditional homegardens into the commercial ones (Prihartini 2004).

Moreover, since 2000s a lot of people of Sukapura Village have planted Welsh onion (*Allium fistulosum* L) in their homegardens. As mentioned by Hadikusumah (2003), the homegardens in Sukapura had been drastically changed from the traditional into the commercial one as indicated by the cultivation of mostly commercial vegetable crops, particularly Welsh onion (*Allium fistulosum* L) (see Figure 2). The villagers have perceived that farming the vegetable crops instead of other crops in the homegardens can provide benefits because the vegetable crops have relatively shorter harvest age and the produce can be sold at a high price. Generally, the produce of traditional homegarden crops is mainly for daily household home consumption, while that of the commercial homegarden crops is predominantly sold to middlemen or village market (Hadikusumah 2003). The external inputs, including seeds, chemical fertilizers, and pesticides of the commercial homegardens are high, while the external inputs of the traditional homegardens are very low, even zero. In addition, the diversity of plant species in the commercial homegardens is very low because the vegetation is dominated by only commercial vegetable crops. Conversely, the diversity of plant species of traditional homegardens is high. For example, staple food, spice, vegetables, and ornamental plants have traditionally been planted in the traditional homegardens.

The traditional homegardens versus the commercial ones

Initially, the homegardens in the villages of upper Citarum watershed of West Java, including Sukapura Village were managed by the traditional ecological knowledge embedded in the local culture (cf. Toledo 2002; Iskandar 2012). In other words, the characteristics of homegardens in Sukapura village depend on local environment, local natural resources, local knowledge, and local institutions. The homegarden farming systems continued to develop in constant interaction with local culture and local ecology. As conditions for farming changed, e.g., because of the village's population growth and intensive penetration of market economy systems into the village ecosystems, including introduction of commercial crops, the homegardens of local people of Sukapura also changed. Some people had adopted the commercial homegardens, including adoption of commercial vegetable crops, use of external inputs, such as vegetable seeds, chemical fertilizer, and synthetic pesticides. In addition, most yielde of the commercial homegardens is sold to middlemen instead of being used for daily household consumptions. However, at the same time some people also still maintain the traditional homegardens, including application of internal inputs, such as various local annual and perennial crops, and organic fertilizers. In addition, most produce of the homegardens is used for fulfilling the household needs instead of being sold to middlemen (cf. Wharton 1970; Reinjntjes et al. 1992).

According to the respondents, from 1970s to 1990, some traditional homegardens in Sukapura were gradually changed into the commercial ones (Table 2). As a result,

the commercial homegardens have been predominantly planted with commercial vegetable crops as both monoculture and polyculture instead of planting of various annual and perennial plants, namely vegetable, spice, starchy or additional staple food, fruit, , and ornamental plants. However, some people still maintain the traditional homegardens for the following reasons, namely tradition (45,0 %) and concern with subsistence needs (55 %) (Table 3).

Plant species of the homegardens recorded in 2004 and 2018

The direct survey of plant diversity of both traditional and commercial homegardens in Sukapura Village in 2018 found 171 plant species belonging to 74 families. The total number of plant species of the homegardens increased from that recorded in 2004 survey by Prihatini (2004). In 2004, the total number of plant species of both traditional and commercial homegardens was 134, belonging to 63 families (Prihatini 2004). The complete list of plant species recorded in 2004 and 2018 are presented in Table 4.

It can be seen in Table 4 that some plant species, namely *handeuleum, wortel, jinteun, alamanda, taleus hias, gelombang cinta, salada bokor, kembang tai ayam, begonia* and *lobak* which are mainly vegetable and ornamental crops were recorded in 2018 but not in 2004. These results are similar to that of study undertaken by Kubota et al. (2003) regarding the changes of plant structure of the homegardens in Cibakung, Cianjur, and West Java. According to Kubota et al. (2003), the number of ornamental, vegetable, and fruit, spice plants was larger in the survey of 1999 than in 1980, and especially the number of ornamental plant species was more than twice of that in 1980. Similarly, study on changes of the plant structure of homegardens in Rancakalong, Sumedang for 10 years showed that the total number of ornamental plants increased, but the size of homegarden decreased due to population increase (Suryana et al. 2014). This fact indicates that the number of ornamental plant species increases because of socioeconomic changes of the farmers, including the increase of standard of living of the farmers in the village (Kubota et al. 2003). In other words, the increase of plant species of vegetables and ornament in Sukapura Village between the survey of 2018 and 2004 indicated that standard of living of the farmers of Sukapura has increased, because with the increasing the living standard, in general, the people become more interested in planting more ornamental plants (cf. Iskandar and Iskandar 2016a).

Index of similarity of the homegarden floristic composition

The species composition of homegardens in Sukapura Village in 2004 (Prihartini 2004) was highly similar with that in 2018, with a similarity index of 72.13%, higher than the similarity index between traditional and commercial homegardens in 2018, which was only 56.22%. The lower similarity index between the traditional and commercial homergadens is due to the introduction of commercial crops in the commercial homegardens.

Plant species diversity of the traditional and commercial homegardens in 2018

The study undertaken in 2018 found that the total plant species in the traditional homegardens in Sukapura Village was 156 belonging to 67 families, while that in the commercial homegardens was 61 from 47 families (Figure 3).

The commercial homegardens had lower number of plant species because they were predominantly planted with commercial vegetable plants only. Conversely, the traditional homegardens were planted with various crops, including spice, vegetable, ornamental, and fruit crops. Because the traditional homegardens have high diversity of plants, they provide some ecological and socioeconomic and cultural benefits, including conservation of local plant diversity, soil erosion protection, soil fertility maintenance, production of oxygen, production of subsistence economy and carbon sequestration, and serve as wildlife habitats, particularly for birds and insects (Soemarwoto 1989; Iskandar and Iskandar 216a). Conversely, because the commercial homegardens were dominated only by commercial vegetable plants, the economic function was very high, but the ecological functions, including soil erosion protection, soil fertility maintenance, and wildlife conservation were very low. In other words, because the traditional homegardens have a high diversity of plants, they play important roles for ecological functions and economic subsistence of village farmers, but their commercial economic function is low. Conversely, the commercial homegardens, due to their low diversity of plants; have low ecological function, but high commercial economic function (Soemarwoto 1989).

Vegetation structure of traditional and commercial homegardens

The life forms of plants of the homegardens in Sukapura Village can be divided into 5 categories mainly herb, bush, tree, liana, and succulent. In terms of life forms, the traditional and the commercial homegardens in Sukapura were dissimilar in that the traditional homegardens had a much higher number of species in all life forms than the commercial ones (Figure 4).

Table 2. Time period of changes of the traditional homegardens into the commercial one in Sukapura Village, Kertasari Sub-district, Bandung District, West Java, Indonesia (Prihatini 2004)

Time period	Number of households	Percentage of the total
Before 1970s	2	10
Between 1970s and 1979s	2	10
Between 1980s-1989	3	15
Between 1990s-2004s	13	65
Total	20	100

Table 3. The reasons of respondents for maintaining the traditional homegardens in Sukapura Village, Kertasari Sub-district, Bandung District, West Java, Indonesia (Prihatini 2004)

Reasons of the respondents	Number of households	Percentage of total
Tradition	9	45
Concern for subsistence needs	11	55
Total	20	100

Table 4. Comparison of species composition of homegardens of Sukapura Village, West Java, Indonesia recorded in 2004 and 2018

Family	Plant name		Year	
	Local name	Scientific name	2004*)	2018
Acanthaceae	Lolipop	*Pachystachys lutea* Nees	√	√
	Handeuleum	*Graptophyllum pictum* (L.) Griff.		√
	Suplir	*Adiantum venustum* D. Don	√	√
Amaranthaceae	Iresine	*Iresine herbstii* Hook.	√	√
	Jawer kotok	*Celosia cristata* L.	√	√
Amaryllidaceae	Bakung	*Hippeastrum reginae* (L.) Herb	√	√
	Bawang daun	*Allium fistulosum* L.	√	√
Anacardiaceae	Buah/Mangga	*Mangifera indica* L.	√	√
	Kedondong	*Spondias dulcis* Parkinson	√	√
Annonaceae	Sirsak	*Annona muricata* L.	√	√
	Sarikaya	*Annona squamosa* L.	√	√
Apiacea	Wortel	*Daucus carota* L.		√
	Saledri	*Apium graveolens L.*	√	√
	Adas	*Foeniculum vulgare* Mill.	√	√
	Jinteun	*T. roxburghianum* L.		√
Apocynaceae	Tapak dara	*Catharanthus roseus* (L.) G.Don	√	√
	Alamanda	*Allamanda cathartica* L.		√
Araceae	Taleus hias	*Caladium bicolor* (Aiton) Vent.		√
	Gelombang cinta	*Anthurium plowmanii* Croat		√
	Kuping gajah	*Anthurium andraeanum* Linden ex Andre	√	√
	Taleus	*Colocasia esculenta* (L.) Schott	√	√
	Srirejeki	*Aglaonema* sp.	√	
	Kasintu	*Dieffenbachia fournieri* N.E.Br.	√	
	Taleus	*Xanthosoma sagittifolium* (L.) Schott	√	√
Araliaceae	Daun kedondong	*Nothopanax fruticosum* (L.) Miq	√	√
	Waregu	*Rhapis humilis* Blume		√
	Kelapa	*Cocos nucifera* L.	√	√
	Palem beureum	*Cyrtostachys lakka* Burret	√	√
	Palem koneng	*Chrysalidocarpus lutescens* H.Wendl.	√	√
	Palem raja	*Roystonea* sp	√	√
	Buntut kala	*Euphorbia tithymaloides* L.	√	√
Asparagaceae	Hanjuang	*Cordylin fruticosa* (L.) A.Chev.	√	√
	Ganas sabrang	*Agave sisalana* Perrine	√	
Asteraceae	Salada bokor	*Lactuca sativa* L.		√
	Kembang tai hayam	*Tagetes erecta* L.		√
	Randa midang	*Cosmos caudatus* Kunth		√
	Krisan	*Chrysanthemum indicum* (Kovalevsk.)	√	√
	Dahlia	*Dahlia x hybrida* Huber	√	√
Balsaminaceae	Pacar air	*Impatiens balsamina* L.		√
Bambusaceae	Haur	*Bambusa vulgaris* Schrad.	√	
Basellaceae	Binahong	*Anredera cordifolia* (Ten.) Steenis		√
Begoniaceae	Begonia	*Begonia rex pan* (Putz.) Seem.	√	√
	Begonia	*Begonia maculata argentea* (Klotzsch) Voss		√
Brassicaceae	Sosin	*Brassica chinensis* L.	√	√
	Lobak	*Raphanus sativus* L.		√
	Kol	*Brassica oleracea* L		√
Bromeliaceae	Ganas	*Ananas comosus* (L.) Merr	√	
	Adam eva	*Rhoeo discolor* (L'Hér.) Hance		√
Cactaceae	Kaktus	*Opuntia ficus-indica* (L.) Mill.	√	√
	Wijayakusumah	*Epiphyllum anguliger* (Lem.) G.Don	√	√
	Buah naga	*Hylocereus undatus*(Haworth)		√
Cannaceae	Bunga Kana	*Canna indica* L.	√	√
	Ganyong	*Canna edulis* Ker Gawl.		√
	Gedang	*Carica papaya* L.	√	√
Caryophyllaceae	Anyelir	*Dianthus caryophyllus* L.	√	√
Compositae	Hebras	*Gerbera jamesonii* Bolus ex Hook.f.	√	√
	Krisan	*Chrysanthemum indicum* L.	√	√
Convolvulaceae	Boled	*Ipomea batatas* L.	√	√
Costaceae	Pacing	*Costus spicatus (*Jacq.) Sw.		√
Crassulaceae	Buntiris	*Kalanchoe pinnata* (Lam.) Pers.	√	√
Cucurbitaceae	Waluh gede	*Cucurbita pepo* L.	√	√
	Paria	*Momordica charantia* L.	√	√

	Waluh sieum	*Sechium edule (Jacq.)* Sw.	√	√
Dracaenaceae	Drasaena	*Dracaena* sp		√
Equisetaceae	Paku ekor kuda	*Equisetum hyemale* L		√
Ericaceae	Azalia	*Rhododendron ledifolium* G. Don	√	√
Euphorbiaceae	Puring	*Codiaeum variegatum* (L.) Rumph. ex A.Juss.	√	√
	Pakis giurang	*Euphorbia milii* Des Moul.	√	√
	Kastuba	*Euphorbia pulcherrima* Balf.f.		√
	Jarak pager	*Jatropha curcas* L.		√
	Sampeu	*Manihot esculenta* Crantz	√	√
	Dawolong	*Acalypha hispida* Burm.f.	√	
	Puring	*Coidaeum variegatum* (L.) Rumph. ex A.Juss.	√	
	Pakis giwang	*Euphorbia milii* Des Moul.	√	
	Sampeu	*Manihot esculenta* Crantz	√	√
	Buntut kala	*Euphorbia tithymaloides* L.	√	
Fabaceae	Hiris	*Cajanus cajan* (L.) Millsp.	√	√
	Kacang jepun	*Glycine max* (L.) Merr.		√
	Dadap	*Erythrina variegata* L.	√	√
	Albasiah	*Albizia chinensis* (Osbeck) Merr.		√
	Roay	*Dolichos* sp	√	√
	Buncis	*Phaseolus vulgaris* L.		√
	Kacang beureum	*Vigna angularis* (Willd.) Ohwi & H.Ohashi		√
	Kacang panjang	*V. unguiculata* L.		√
Ferbenaceae	Widara	*Duranta erecta* L.	√	√
Heliconiacea	Pisang hias	*Heliconia bihai* (L.) L.	√	
Hydrangeaceae	Borondong	*Hydrangea macrophylla* (Thunb.) Ser.	√	√
Iridaceae	Gladiul	*Gladiolus* sp.	√	
Lamiaceae	Pagoda	*Clerodendron paniculatum* L.	√	√
	Surawung	*Ocimum ×citriodorum* Lour.	√	√
	Kumis kucing	*Orthosiphon aristatus* (Blume) Miq.	√	√
	Lapender	*Lavandula angustifolia* Mill.		√
	Nona makan sirih	*Clerodendrum thomsoniae* Balf.f.		√
	Jati putih	*Gmelina arborea* Roxb.		√
	Seuseureuhan	*Clerodendron paniculatum* L.	√	
	Daun min	*Mentha cordifolia* Opiz ex Fresen		√
	Jawer Kotok	*Plectrannthus scutellarioides* (L.) R.Br.	√	√
	Cingcau	*Premna corymbosa* Rottler & Willd.	√	√
Lauraceae	Kayu manis	*Cinnamomum verum* J.Presl		√
	Alpuket	*Persea americana* Mill.	√	√
Laxmanniaceae	Hanjuang	*Cordyline banksii* Hook.f.	√	√
Leguminosae	Kacang suuk	*Arachis hypogaea* L.	√	√
	Buncis	*Phaseolus vulgaris* L.	√	√
	Kapri	*Pisum sativum* L	√	√
Lytheraceae	Dalima	*Punica granatum* L.	√	√
Malvaceae	Kembang wera	*Hibiscus rosa-sinensis* L.	√	√
	Daun edi	*Abelmoschus manihot* (L.) Medik.	√	√
	Duren	*Durio zibethinus* L.		√
Manisperceae	Batrawali	*Tinospora crispa* (L.) Hook. f. & Thomson	√	√
Maranthaceae	Kalatea batik	*Maranta lietzei* E.Morren	√	√
Maranthaceae	Sagu	*Maranta arundinacea* L	√	√
Meliaceae	Mahoni	*Swietenia macrophylla* King		√
Meliaceae	Suren	*Toona sureni* (Blume) Merr.	√	√
Menispermaceae	Cingcau	*Cylea barbata* Miers	√	√
Moraceae	Murbai	*Morus alba* L.	√	√
	Nangka	*Artocarpus heterophyllus* Lam.	√	√
	Karet kebo	*Ficus elastica* Roxb. ex Hornem.		√
	Sukun	*Artocarpus altilis*(Parkinson ex F.A.Zorn)		√
	Caringin	*Ficus benjamina* L	√	
Muntingiaceae	Kersen	*Muntingia calabura* L.		√
Musaceae	Cau	*Musa ×paradisiaca* L.	√	√
Myrtaceae	Kayu putih	*Melaleuca leucadendron* F.Muell.	√	√
	Pucuk merah	*Syzygium oleina* Merr.		√
	Jambu batu	*Psidium guajava* L.	√	√
	Jambu air	*Syzygium aqueum* (Burm.f.) Alston	√	√
	Cengkeh	*Syzygium aromaticum* (L.) Merr. & L.M.Perry	√	√
	Jambu kupa	*Vaccinium vitis* L.	√	√
	Jambu bol	*Syzygium malaccense* (L.) Merr. & L.M.Perry	√	√
	Jambu lokat	*Eriobotrya japonica* (Thunb.) Lindl.	√	√

Nyctaginaceae	Kembang kertas	*Bougenvillea spectabilis* Willd.	√	√
Orchidaceae	Anggrek Kala	*Arachnis hookeriana* (L) Rchb.f.	√	√
	Anggrek bulan	*Phalaenopsis amabilis* Blume.		√
	Anggrek japati	*Dendrobium crumenatum* SW.		√
Oxalidaceae	Calincing	*Averrhoa bilimbi* L.	√	√
Pandanaceae	Pandan	*Pandanus amaryllifolius* Roxb	√	√
Passifloraceae	Konyal	*Passiflora ligularis* Juss.	√	√
	Markisa	*Passiflora edulis* Sims	√	√
Phyllanthaceae	Katuk	*Sauropus androgynus* (L.) Merr.	√	√
	Cermai bogor	*Phyllanthus acidus* (L.) Skeels	√	√
Phytolaccaceae	Gegetihan	*Rivina humilis* L.		√
Pinaceae	Pinus	*Pinus merkusii* Jungh. & de Vriese	√	√
Piperaceae	Seureuh	*Piper betle* L.		√
Poaceae	Jagong	*Zea mays* L	√	√
	Sereh	*Cymbopogon citratus* (DC.) Stapf	√	√
	Tiwu	*Saccharum bengalense* Retz		√
Polypodiaceae	Paku tanduk rusa	*Platycerium superbum* de Jonch. & Hennipman		√
Portulacaceae	Gingseng jawa	*Talinum paniculatum* (Jacq.) Gaertn.	√	√
	Kriminil	*Portulaca amilis* Speg.	√	√
Rhamnaceae	Widara	*Ziziphus mauritiana* Lam.	√	√
Rosaceae	Eros	*Rosa hibrida* Wolley-Dod	√	√
	Arben	*Rubus rosaefolius* S.Vidal	√	√
	Stroberi	*Fragaria* × *ananassa* (Duchesne ex Weston)		√
	Jambu lokat	*Eriobotrya japonica* (Thunb.) Lindl.	√	√
Rubiaceae	Kaca piring	*Gardenia augusta* Merr.	√	√
	Soka	*Ixora javanica* (Blume) DC.	√	√
	Kopi	*Coffea arabica* L.	√	√
	Jabon	*Neolamarckia cadamba* (Roxb.) Bosser	√	√
Ruscaceae	Kasintu	*Sansevieria trifasciata* Prain	√	√
	Suji	*Dracaena angustifolia* (Medik.) Roxb.		√
	Kibeusi	*Dracaena* sp	√	√
Rutaceae	Jeruk	*Citrus aurantifolia* (Christm.) Swingle	√	√
	Jeruk lemon	*Citrus limon* (L.) Osbeck	√	
	Jeruk mangse	*Citrus* × *sinensis* L.	√	
	Jeruk papaya	*Citrus medica* L.		√
	Jeruk purut	*Citrus* × *hystrix* Pers.	√	√
	Kemuning	*Murraya paniculata* (L.) Jack		√
	Jeruk Bali	*Citrus grandis* (L.) Osbeck	√	√
Solanaceae	Leunca	*Solanum nigrum* L.	√	√
	Tomat	*Solanum lycopersicum* L.	√	√
	Cabe	*Capsicum annum* L.	√	√
	Cengek	*Capsicum frutescen* L.	√	√
	Terong kori	*Solanum betaceum* Cav	√	√
	Terung	*Solanum* sp.	√	√
	Terung roti	*Solanum melongena* L.		√
	Kentang	*Solanum tuberosum* L.		√
	Melati gunung	*Brunfelsia uniflora (*Pohl) D.Don		√
	Kecubung gunung	*Datura metel* L		√
Spindaceae	Lengkeng	*Dimocarpus longan* Lour.	√	√
Theaceae	Teh-	*Camellia sinensis* (L.) Kuntze	√	
Verbenaceae	Kinakal	*Duranta erecta* L.		√
	Ganas sabrang	*Agave sisalana* Perrine	√	
Xanthorrhoeaceae	Lidah buaya	*Aloe vera* (L.) Burm.f.	√	√
Zingiberaceae	Combrang	*Etlingera elatior (Jack)* R.M Smith	√	√
	Jahe	*Zingiber officinale* Roscoe	√	√
	Koneng	*Curcuma longa* L.	√	√
	Laja	*Alpinia galanga* (L.) Willd.	√	√
	Panglay	*Zingiber cassumunar* Valeton	√	√
	Temu lawak	*Curcuma xanthorrhiza* Roxb.	√	√

Note: *) Prihatini (2004)

Figure 2.A. The traditional homegarden in Sukapura Village, West Java, Indonesia is predominantly planted with various crops, including jackfruit (Artocarpus heterophllus), banana (*Musa x paradisiaca*), coffee (*Coffea arabica*), orange (*Citrus* sp), and laja (*Languas galanga*). B. The commercial homegarden in Sukapura Village is predominantly planted with a single species of Welsh onion (*Allium fistulosum*). C. The nursery of cabbage (*Brasica oleracea*) in the commercial homegarden in Sukapura Village. D. The carrot (*Daucus carota*) is planted in the commercial homegarden in Sukapua Village

Herb was the predominant life form recorded in the both the traditional and the commercial homegardens, i.e. 51 species in the traditional homegardens and 33 species in the commercial ones.

SDR (Summed Dominance Ratio) of plant species in the traditional and commercial homegardens

On the basis of SDR analysis, it can be seen that three species of plants which had high value of SDR in the traditional homegardens were Welsh onion (*Allium fistulosum* L), carrot (*Daucus carota* L), and carnation (*Dianthus caryophyllus* L) (Table 4), while in the commercial homegarden systems were Welsh onion (*Allium fistulosum* L), carrot (*Daucus carota* L) and cabbage (*Brassica oleracea* var *capitata*) (Table 5).

Table 6 shows that the vegetable crops had a high value of SDR in both the traditional and the commercial homegadens in Sukapura Village because the village is located in the mountainous upland of upper Citarum watershed of West Java which is appropriate for growing vegetables and the vegetables have high economic value (cf. Iskandar et al. 2017).

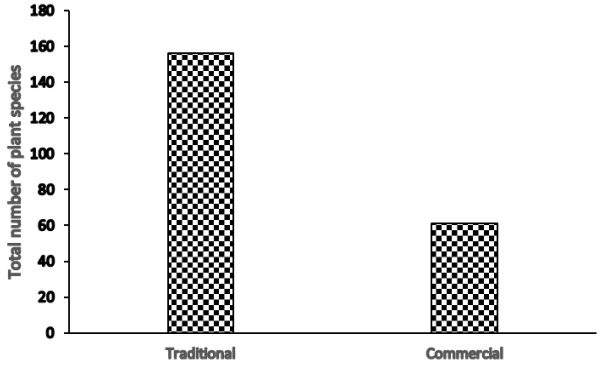

Figure 3. The number of plant species in the traditional and commercial homegardens of Sukapura Village, West Java, Indonesia

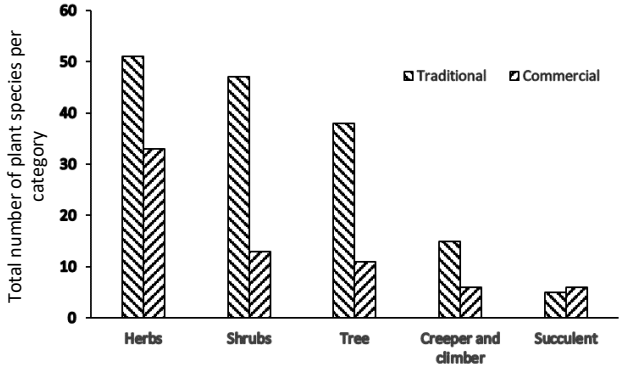

Figure 4. Comparison of total number plant species in the traditional homegarden and that in the commercial homegarden of Sukapura Village, West Java, Indonesia based on category of the living plant forms

The SDR values of plant species of both traditional and commercial homegardens in Sukapura Village in 2018 were generally similar to the results of earlier studies conducted by Hadikusumah (2003) and Prihartini (2004), showing that vegetable crops were the dominant species. In conclusion, it can be said that the commercial crops have been predominantly planted in Sukapura Village for the last several decades because they have high economic value, but the cultivation of commercial crops has caused local environmental problems, including soil erosion and pesticide pollution (cf. Iskandar et al. 2017).

Index of plant species diversity of the homegardens

The traditional homegardens had species diversity index (**H'**) of 4.16, much higher than that of the commercial homegardens, i.e., 1.71, which is considered low (Shannon-Wiener 1949 cited by Krebs 1985). The low diversity index in the commercial homegardens was caused by the high dominance of commercial crops, including Welsh onion (*Allium fistulosum* L), carrot (*Daucus carota* L) and cabbage (*Brassica oleracea var capitata*). Although they provide some economic benefits for the farmers, having low species diversity, the commercial homegardens need high external inputs, including seeds, inorganic fertilizers, and synthetic pesticides and are subject to vulnerable market economic factors, including drastically changes of both the inputs and output prices. In addition, ecologically they are less resistant to environmental changes, including pest attack and climatic changes (cf. Iskandar 2017).

The positive and negative impacts of the conversion of the traditional homegardens into the modern ones

The conversion of traditional homegardens into the commercial ones has caused positive and negative impacts. According to perception of informants, the traditional homegardens provide some benefits, including protection of local plant varieties, maintenance of soil fertility, and provision of healthy food production. In addition, because the traditional homegardens have been predominantly planted with various perennial plants, including trees, they may provide appropriate wildlife habitats, particularly for species of birds.

Table 5. Species composition similarity between homegardens of Sukapura Village, West Java, Indonesia in 2004 and 2018 and between commercial and traditional homegardens in 2018

Communities being compared	Sørensen similarity index (%)
Homegardens in 2004 and 2018	72.13
Commercial and traditional homegardens in 2018	56.22

Table 6. Plant species having high SDR value in traditional and commercial homegardens of Sukapura Village, West Java, Indonesia

Traditional gardens			Commercial gardens		
Local names	**Species**	**SDR**	**Local names**	**Species**	**SDR**
Bawang daun	*Allium fistulosum* L	9.12	*Bawang daun*	*Allium fistulosum* L	33.59
Wortel	*Daucus carota* L	4.40	*Wortel*	*Daucus carota* L	8.30
Anyelir	*Dianthus caryophyllus* L.	1.88	*Kol*	*Brassica oleracea* var. *capitata*	6.25

Table 10. Net income from the traditional homegardens in Sukapura Village, West Java, Indonesia in a year in 2004 (Prihatini 2004)

Fruits	Vegetables	Starchy/additional staple food	Another crop	Total net income (Rp.)
Banana (16 m^2)	Pumpkin (112 m^2)	Cassava (7 m^2)	Coffee (158 m^2)	81,710
Orange (113 m^2)	Welsh onion (14 m^2)	Corn (14 m^2)		
Pomegranate (3 m^2)		Sweet potato (20 m^2)		
Net income = Rp 22,170	Net income = Rp 22,600	Net income = Rp 23,780	Net income = Rp 13,160	

Table 11. Gross income from the commercial homegardens in Sukapura Village, West Java, Indonesia in a year in 2004 (Prihatini 2004)

Planting season	Main crops and area of planting (m^2)	Production (kg)	Price of selling (Rp.)	Gross income (Rp.)
I	Welsh onion (62)	90	950	85,500
	Carrot (108)	120	750	90,000
	Potato (56)	50	1,500	75.000
	Pumpkin (63)	130 items	250	32.500
Gross income (I)				283,000
II	Welsh onion (117)	150	800	120,000
	Carrot (91)	175	550	96,250
	Potato (81)	50	1,600	80,000
Gross income (II)				296,250
III	Welsh onion (118)	130	900	117,000
	Carrot (69)	85	900	76,500
	Potato (52)	30	2,500	75,000
	Pea (50)	13	7,000	91,000
Gross income (III)				359,500
Total gross income (I +II+III)				938,750

The traditional homegardens also provide some socio-economic benefits for the owners. The traditional homegardens function as the living barn, particularly during 'the famine season' (*musim paceklik*) when rice as staple food is lacking, so some produce, including starchy food, spices, and fruits may be provided by the homegardens. Because the traditional homegardens have been commonly planted by a variety of food crops, they provide daily needs of the households, including spices and vegetables, for fulfilling the subsistence of the villagers, so the farmers do not have to buy food produce from village food stalls. As a result, the traditional homegardens have also been popularly known as the life barns (*lumbung hidup*) or life shops *(warung hidup*). In addition, the traditional homegardens also provide medicinal plants, including lemon (*Citrus aurantifolia* Swing), turmeric (*Curcuma longa* L), sand ginger/*kencur* (*Kaempferia galanga* L), ginger (*Zingiber officinale* Roscoe), and round cardamon/*kapulaga* (*Amomum compactum* Soland), so they are also called "living pharmacies" (*apotek hidup*).

The traditional homegardens also have social-cultural functions. For example, the front yard of a house (*buruan*) has traditionally been used for playing for children, performing traditional ceremonies, and chatting for the parents. Because villagers need some plants for traditional rituals, some traditional ritual plants have been traditionally planted in the traditional homegardens. In addition, since the traditional homegardens have been planted with ornamental plants, including jasmin (*Gardenia augusta* Merr), evergreen maidenhair (*Adiantum venustum* D.Don), and dahlia (*Dahlia x hybrida* Huber), the traditional gardens also have esthetical function.

It can be inferred that because the traditional gardens have been planted with a high diversity of plants, they provide various ecological, socio-economic and cultural benefits, including genepool conservation, subsistence, and commercial produce, and esthetical benefits (Arifin 2003; Suhartini et al. 2013; Hidrawati et al. 2017).

The conversion of homegardens from the traditional into the modern ones in Sukapura Village has caused changes of structure and functions of the village homegardens. Because of the homogenization of commercial vegetable plants and the high external inputs, including seeds, an-organic fertilizers, and pesticides, the commercial homegardens have lower number of individual plants of vegetables and the plant species diversity than the traditional ones (Hadikusumah 2003).

Beside causing negative impacts, the commercialization and the homogenization of the homegardens in Sukapura Village have provided advantages too, including the increase of economic production. However, although the total gross income of the commercial homegarden system in Sukapura is high, the cost of inputs, including vegetable seeds, organic fertilizer, inorganic fertilizer, fungicide, and

pesticides is also high. Conversely, the production of the traditional homegarden system in Sukapura Village is low, but it also needs low or zero inputs. For example, based on the homegarden research conducted in 2004 on analysis of inputs and outputs or crop production of the traditional homegardens in Sukapura Village planted by various plants, including banana (*Musa x paradisiaca* L), orange (*Citrus* sp), pomegranate (*Punica granatum* L), pumpkin (*Cucurbita pepo* L), Welsh onion (*Allium fistulosum* L), cassava (*Manihot esculenta* Crantz), corn (*Zea mays* L), sweet potato (*Ipomoea batatas* L), and coffee (*Coffea arabica* L), the net income was Rp 81,710 per year, without any costs (Table 7). While the commercial homegardens in Sukapura Village planted with commercial vegetable plants, including Welsh onion (*Allium fistulosum* L), carrot (*Daucus carota* L), potato (*Solanum tuberosum* L), and pea (*Vigna* sp.) resulted in the gross income of Rp 938,750 per year (Table 10) (Prihatini 2004).

Tables 10 and 11 show that the net income from the traditional homegardens (Rp 81,710) is lower than that of the commercial one (Rp 938,750); however, the input of the traditional homegardens is very low or zero, while inputs of the commercial homegardens are very high. The field research in 2018 showed that total input costs of farming Welsh onion and carrot in the commercial homegardens in Sukapura Village approximately 78% and 35% (Tables 12).

In addition, the monoculture of commercial vegetable crops in Sukapura Village has a high risk of drastic changes of input and output prices (Jalurdi et al. 2011). For instance, according to informants, many farmers of Sukapura Village who planted commercial vegetable crops in the homegardens in the main planting season of 2018 suffered financial loss due to the low selling price of vegetables. For example, the selling price of Welsh onion in early 2018 was Rp 25,000/kg, but a couple months later drastically dropped to Rp 2,000/kg because the supply of the Welsh onion increased.

Table 12. The gross income of Welsh onion (*Allium fistulosum* L.) cultivation in the commercial homegardens in Sukapura Village, West Java, Indonesia in 2018

The size of the homegarden is 400 m^2 (1 *patok*)

Welsh onion (*Allium fistulosum* L.)

Inputs:
Seeds: 150 kg x Rp 3,000 = Rp 450.000
Organic fertilizer: animal dung 10 sack (*karung*) = 10 x Rp 10,000 = Rp 100,000
Inorganic fertilizer: NPK Phonnska = Rp 160,000
Fungicide (*Kanon*) = Rp 40,000
Pesticide (*Roke*r , *Bitan*, and *Dakotil*) = Rp 180,000 + Rp 85,000 + Rp 90,000
Total inputs = Rp 450,000 + Rp 100,000 + Rp 160,000 + Rp 40,000 + Rp 180,000 + Rp 85,000 + Rp 90,000 = Rp 1,105,000

Outputs:
After 4 months of planting, the production of Welsh onion in 3 times of harvesting = 3 x 700 kg x Rp 2,000 = Rp 1,400,000

Gross income:
Cultivation of Welsh onion for one season (4 months) = Rp 1,400,000 – Rp 1,105,000 = Rp 295,000, not included labor costs, including land preparation, planting, and harvesting.
Percentage of total input costs to total outputs is approximately 78 %.

Carrot (*Daucus carota* L)

Inputs:
Seed of carrot 1 liter = Rp 50,000
Organic fertilizer of animal dung = 10 sacks x Rp 10,000 = Rp 100,000
Inorganic fertilizer (NPK Phonska) = Rp 160,000
Fungicide (Kanon) = Rp 40,000
Inputs for 3 times of planting season = 3 x (Rp 50,000 + Rp 100,000 + Rp 160,000 + Rp 40,000) = Rp 1,050,000

Output:
Farming carrot of 400 m^2 per year (3 season of 4 times of harvesting)
Production of carrot for 4 times of harvesting = 4 x 500 kg = 4 x (Rp. 1.500,00 x 500 kg) per 400 m2 per year = Rp 3.000.000

Gross income:
Farming of carrot in 400 m2 of three planting seasons in one year = Rp 3,000,000-Rp 1,050,000 = Rp1,950,000, without labor costs
Percentage of total input costs to total outputs is approximately 35%.

According to the informants, although the commercial homegardens provided some advantages, including the increase of gross income and household income increased, and job opportunity in the commercial vegetable crop farming, they also brought some disadvantages, including the disappearance of local species and varieties of plants, and higher input dependence from market or outside (cf. Iskandar et al. 2018). In addition, according to informants, it also had negative effects on local environment. For example, the soil fertility decreased and a lot of fertilizers must be added to the soil, and the soil has been intensively contaminated with poison of pesticides and fungicides. The soil erosion has also occurred due to the simplification of vegetation structure, including the loss of trees, and intensive weeding of terrestrial weeds. The simplification of vegetation stratification has drastically changed the habitat of wild animals, particularly terrestrial birds. Indeed, intensive use of pesticides has brought negative effects on wild birds in the village ecosystems due to pollution.

In conclusion, initially the traditional homegardens in Sukapura Village have been predominantly cropped with various annual and perennial crops. However, due to market economic development, the traditional homegarden systems have drastically changed. For example, the commercial vegetable crops, including Welsh onion (*Allium fistulosum* L), carrot (*Daucus carota* L) and cabbage (*Brassica oleracea* var *capitata*) have been predominantly cultivated in the commercial homegardens. Consequently, the economic production of the commercial homegardens has increased. However, some disadvantages of the commercial homegardens have occurred, including disappearance of local species and varieties of plants, and higher dependence of inputs from market or outside. This study showed that the rural homegardens have not been static but dynamically changing caused by ecological and socioeconomic and cultural factors, including intensive market economic penetration to village ecosystems. We suggest that to develop the sustainable village homegardens for the future, the diversity of plants must be maintained to provide ecological function or ecosystem services and the economic production must be improved to improve income for the rural people.

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Leunca (*Solanum americanum* Mill.): The uses as vegetable in two villages in Upper Citarum Area, Bandung, West Java, Indonesia

DEDE MULYANTO[1], JOHAN ISKANDAR[2], OEKAN S. ABDOELLAH[1], BUDIAWATI S. ISKANDAR[1], SELLY RIAWANTI[1], RUHYAT PARTASASMITA[2,♥]

[1]Department of Anthropology, Faculty of Social and Political Science, Universitas Padjadjaran. Jl. Raya Bandung-Sumedang Km 21, Jatinangor, Sumedang 45363, West Java, Indonesia

[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran. Jl. Raya Bandung-Sumedang Km 21, Jatinangor, Sumedang 45363, West Java, Indonesia. Tel./fax.: +62-22-7796412, ♥email: ruhyat.partasasmita@unpad.ac.id; rp2010rikkyo@gmail.com

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Abstract. *Mulyanto D, Iskandar J, Abdoellah OS, Iskandar BS, Riawanti S, Partasasmita R. 2018.* Leunca (Solanum americanum *Mill.*)*: The uses as vegetable in two villages in Upper Citarum Area, Bandung, West Java, Indonesia. Biodiversitas 19: 1941-1954. Leunca* is known as botanical name as *Solanum americanum* Mill, Family of Solanaceae. In recent years, academic interest has been increasing. After so long studied as weeds, today *leunca* has also studied because of its important meaning as crop that has high nutritional and economic value in relation to food resilience of developing countries, as because of its chemical substances with its medicinal properties. *Leunca* was recorded in colonial period by botanists or agricultural scientists' report as local vegetable in rural of West Java also in modern time by anthropologist or ecologists who studying rural population. In the recent time in Indonesia, *leunca* studies almost all have been focused on its pharmacological, agronomic, and economic aspects. The aspect that is related to Sundanese sociocultural system was almost neglected. This paper presents the finding of research on ethnobotany of *leunca* includes landraces, agronomical and utilization, traditional institutional aspect, and culinary culture food habits of *leunca* in rural Sundanese people. Method used in this study mixed-method of qualitative and quantitative was applied in this study, while some techniques including observation and semi-structured interviews were carried in the field research. The result of study showed that based on informants it has revealed that 7 kinds of plant that are named as *leunca*, however, only 3 kinds of *leunca* that are grown in their village. Among 7 kinds of *leunca*, *leunca biasa* (*S. americanum*) has been predominantly consumed both fruits and leaves. There is various food dishes are consumed fresh or cooked. Various dishes of *leunca biasa* have been culturally integrated everyday life of people and culturally as part of people identity of Sundanese people (*urang Sunda*). Other kinds of *leunca*, including leaves of *leunca manuk* (variety of *S. americanum*) have been consumed *leunca* as cooked vegetable, and its leaves have been used as traditional medicine of pet chicken disease.

Keywords: Ethnobotany, habitus, *leunca*, vegetable, socio-cultural identity, *Solanum americanum*

INTRODUCTION

Leunca is included into species complex of *Solanum americanum* Mill, family of Solanaceae (Siemonsa and Grubben 1996; Edmond and Chweya 1997; Samuel 2015). In recent years, there have been increasing academic interest to *leunca*. After so long studied as weeds, now *leunca* also has been studied because of its important meaning as a crop that has high nutritional vegetable of Sundanese people in relation to food resilience issue in developing countries, and because of its chemical substances with its medicinal properties (Sarma and Sarma 2011).

In West Java, research on *leunca* was initiated by Fortuin and Omta (1980) who at the end of 1970 studying *leunca* in Lembang, Bandung, West Java, concerning *leunca* photosynthetic characteristics as function of light intensity. Their choice on Lembang, Bandung, West Java as site of research was related to the fact that although *leunca* also grows in other places in Indonesia (see Silalahi and Nisyawati 2018), but this plant has predominantly consumed as fresh as vegetable and hot shrimp/fish paste (sambel), planted in homegarden, and commonly traded in traditional market of Bandung (see Iskandar et al 2018). In the past, it was revealed that *leunca* was an integral part of rural West Java's picture of biodiversity. In the description of rural life in the colonial period, Dutch scholar almost always mentioned that *leunca* was local vegetable that is cultivated and consumed by '*Soendalanden*' or '*Preanger*' rural population. In his article published in 1845, in famous Dutch botanist, Justus Karel Hasskarl, mention *leuntja* as: a crop that belongs to Family Solanaceae, just like a plant, which is used mainly in the Sunda region as a snack, both raw and cooked or steamed, is named as the *leuntja*. It is scarcely planted on rice field (*sawah*), more on tile grounds and to small expanses. The land requires little processing. When plants have ripened, as seen many ripe fruits, they can be harvested. Then the plants are pulled out of the soil, the roots are cut off and bind together to bunches. While the fruit is enjoyed as *terong*, the leaves and young stem parts of *leuntja* are used. Mainly it is mixed with fish, *leuntja* is eaten (Hasskarl 1845).

Another agronomist, van der Burg (1904) also documented the presence and utility of *leunca* in rural of West Java, as mentioned as follow: "The raw leaves are usually eaten and the whole plant is boiled, the fruits which

are the fresh fruits, sometimes raw, but mostly cooked, and mixed with meat, especially lamb and fish, for the European table. They are also mixed with minced meat and then fried. Tomato jelly is also made from this fruit. The leaves and the young sprouts are cooked in steam, eaten as vegetables".

Contemporary rural studies in West Java also mentions that *leunca* as local vegetable that was planted whether in home garden (Maten and Abdoellah 1988) or both in home garden and dry land agroforest (Abdoellah and Marten 1986; Soemarwoto 1987), in wet rice paddy field's dike or in agroforestry land (Wiyanti 2016). In some rural area of West Java some places, *leunca* is also cultivated semi-intensively as crop to supply local market (Santosa et al. 2015).

Related to role played by *leunca in* consumption pattern of rural peasant in upland West Java, special intensive case study on food ecology, carried out micro study by Igarashi (1985) in a village of Cigentur, Paseh sub-district, Bandung plain of upper Citarum, also found that from 35 types of food that was regularly consumed by villagers in a month, *lalab leunca* ranked in 16th place, the highest rank among *lalaban* vegetables category, surmounting "*seupan daun sampeu* (*Manihot esculenta* Crantz), *selong* (*Leucaena leucocephala* (Lam) de With, *peuteuy* (*Parkia speciose* Hassk), "*terong* (*Solanum melongena* Linn) , or *kangkung* (*Ipomoea aquatic* Forsk). As *angeun leunca or* soup of *leunca*, its rank also recorded in the third place (Igarashi 1985).

Similar picture was also found by Abdoellah (1985). In his study on food ecology in same village as undertaken by Igarashi, Cigentur Village, in Upper Citarum revealed that according to its presence frequency as a part of daily menu during a month observation, *leunca* appeared as much as 66.7% in rich peasant family's menu list, 63.3% in middle peasant family, and 36.7% in poor family's daily menu list. That is to say that in middle to upper class, *leunca* almost always presence in more than half of daily menu list during a month.

In addition to description of *leunca* appearance in West Java rural everyday life, the record from colonial period and contemporary micro studies also emphasize *leunca* local character in production and consumption. That is true that until recently, together with other vegetables with limited market niche, *leunca* is categorized as minor or underutilized (Soetiarso 2010b), indigenous (Putrasamedja 2005), or local vegetables (Susanti 2015): plants that have been adapted to or fully expressed in certain area and utilized from generation to generation by local people with relatively limited market niche from broader context.

In the last decade, researches on *leunca* in Indonesia, have mainly focused on its three aspects, mainly pharmacological (Rumiyati et al. 2015; Istiadji 2010), agronomical (Santosa et al. 2015), economical (Soetiarso 2010b; Yurlisa 2016), and its medicinal utilization aspect by local people (Putri et al. 2016). Although *leunca* is mentioned earlier as one of important vegetable crops in some villages of Bandung, West Java, research on ethnobotany of *leunca* as vegetable has rarely carried out in rural areas of West Java.

MATERIALS AND METHODS

Study area

The field research was conducted in two villages, namely Sukapura Village and Tarumajaya Village of upper Citarum, Kertasari Sub-district, Bandung District, West Java, Indonesia (Figure 1). Two villages of upper Citarum were selected for case study on ethnobotany of *leunca*, because based on ecological history, in the past both villages were known as traditional village, with several traditional agroforestry systems, including homegarden, perennial mixed-garden, and bamboo garden, were predominantly found. Nowadays, however, due to introduction of commercial crops, such as cabbage, potato, and spring onion, some homegardens, perennial mixed gardens, and bamboo gardens have been converted to commercial monoculture gardens. As a result, ecological aspects of agroecosystems and socio-cultural aspects of rural people in both villages, Sukapuran and Tarumajaya have dramatic changes. Based on study on ethnobotany revealed that modernization of agricultural system and consumption changes of rural people have caused changes of traditional diversity of plants (Brush 2000; Iskandar, Iskandar and Partasasmita 2018). Both villages Sukapura and Tarumajaya were predominantly resided by Sundanese people. Therefore, these villages have considered as ethnobotany on *leunca* in rural areas of West Java.

Sukapura and Tarumajaya villages are located in the high land with have topography of slopes or ridges of hills in the vicinity of Mount Wayang. The total is of Sukapura and Tarumajaya recorded approximately 633 ha and 2,744 ha, respectively. Both Sukapura and Tarumajaya villages are located in 1,200 m above sea level, which have daily temperature 17-22°C.

Based on environmental history, Sukapura and Tarumajaya were formed first half of the twentieth century. Since the decade of 1960s, both villages have been recorded as the villages of network of the Kertasari-Pangalengan complex, which is recognized as vegetable agricultural center production. Some vegetable crops have been predominantly recorded, including potato (*Solanum tuberosum* L), carrot (*Daucus carota* L), spring onion (*Allium fistulosum* L), and cabbage (*Brassica oleracea* var. *capitala* L.). In terms of land use type, the dry land has been predominantly found in Tarumajaya Village, which has 2,145 ha of the agricultural land use types, including vegetable garden, coffee garden, and community forest. Unlike Tarumajaya Village, Sukapura Village which is located more below, recorded not extensive the rice field of 15 ha planted by rice, and other land use types namely home garden and vegetable garden (Figure 2). In addition, approximately 199 ha of Sukapura Village have been predominantly used for agricultural land, and approximately 423 ha have been used for non-agricultural sectors, including market, shops, and small-scale industries. *Leunca* has commonly in homegarden and garden in mixed-cropping system other crops instead of commercial monoculture system as predominantly practiced in present time.

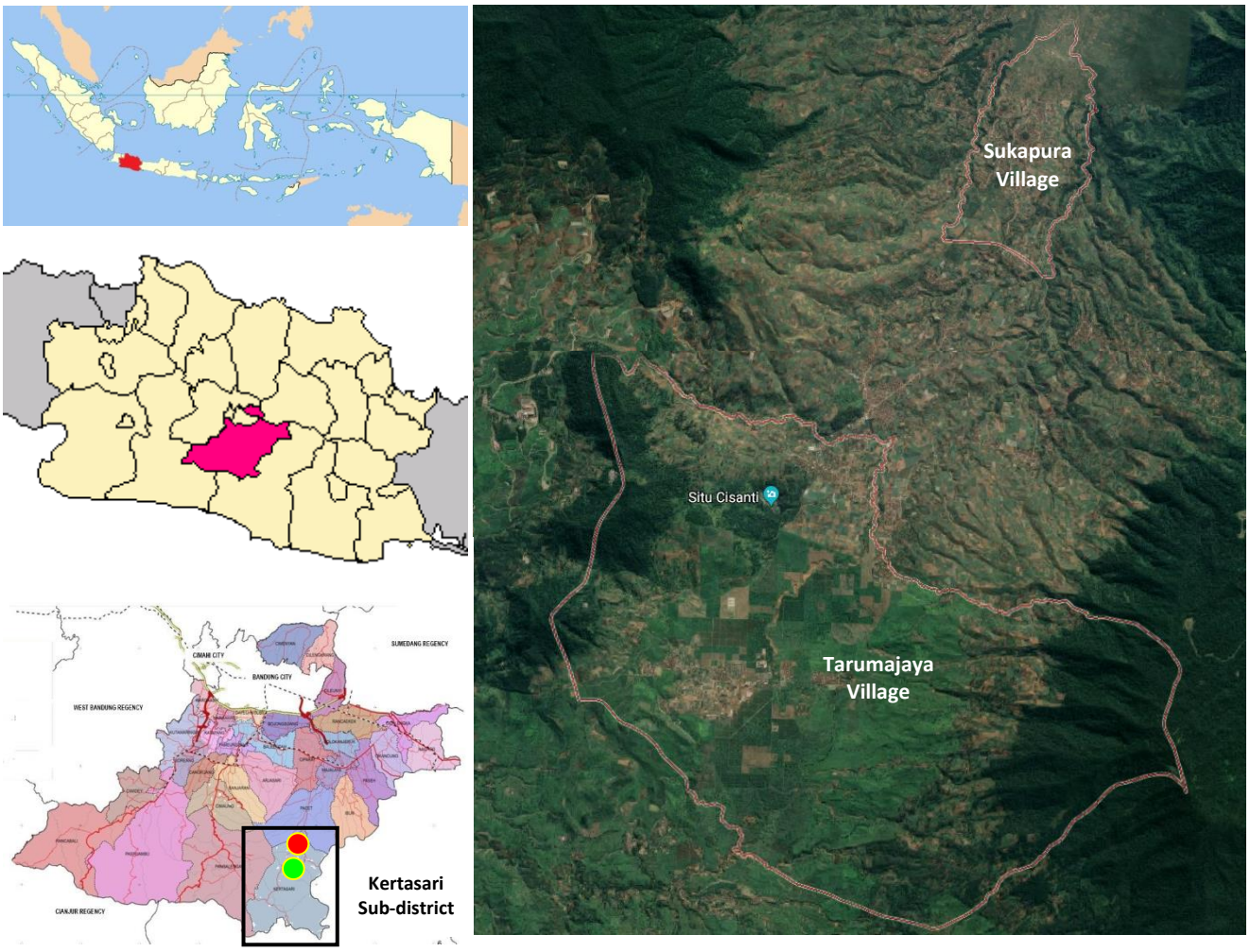

Figure 1. Study area of Sukapura Village ( )and Tarumajaya Village ( ), Kertasari sub-district, Bandung district of upper Citarum, West Java, Indonesia

Figure 2.A. Land use types consist of vegetable garden, homegarden, and forest in the study area of Tarumajaya Village, upper Citarum, West Java, Indonesia. B. The rice field and vegetable garden in the study area of Sukapura Village, upper Citarum, West Java, Indonesia

Based on village statistical data, in 2007 population of Sukapura Village was recorded 8,900 people belonging to 3,297 households, consisting of 3,297 neighborhoods (RT). While, Tarumajaya Village was resided by at least 15,000 people consisting of 2,623 households, among them 34% of population of Sukapura were recorded as classified as productive age. The proportion of productive age in Tarumajaya Village was recorded approximately 70%. Most of the productive age of people in both villages Tarumajaya and Sukapura have been involved in the agricultural sector. Vegetable productions of those villages have been predominantly recoded namely *bawang daun*/ spring onion (*A. fistulosum*), *tomat*/tomato (*Lycopersicon esculentum* Mill), *sawi*/Indian mustard (*Brassica juncea* (L) Czern & Coss), *kentang*/potato (*S. tuberosum*), and *kubis* or *kol*/cabbage (*B. oleracea* var. *capitala*). Because Tarumajaya Village is located in higher land of the high land, since the Dutch colonial period, some areas of Tarumajaya Village has been established as plantation areas, including tea/teh (*Camelia sinensis* L) and quinine/kina plantation (*Chincona* sp.) (Kurniawan et al. 2018). After the Dutch colonial period, the tea plantation has been managed by PT Perkebunan Nusantara VIII. While some areas of Sukapura have, it has directly bordered with the forest that has been managed by PT Perhutani. In addition to involve in vegetable garden, small scale of dairy farm has been undertaken by farmers of both Tarumajaya and Sukapura villages.

People of Tarumajaya and Sukapura have been considered as Sundanese people (*Urang Sunda*). It has been also recorded in Sukapura Village non-Sundanese people. For example, some Javanese families wander and have wives, and live in the research village. They communicate with each other use local language, Sundanese language (*Bahasa Sunda*). Family families who were born and their ancestors all lived in the village and still follow various customs that apply to the Sundanese people (see Ekadjati 1995; Mustapa 1996).

Procedure

Method used in this study was mixed-method that is dominant qualitative and less dominant quantitative, with ethnobotanical approach (Cresswell 1994; Martin 1995; Alexiades and Sheldon 1996; Cotton 1996; Cunningham 2001; Iskandar 2018). Several techniques, namely observation and semi-structured interview were applied in this study. Observation was done to carefully observe various *leunca* variations which are cultivated and are grown in different agroecosystem types, including homegarden, garden, and rice field, and widely grown as weeds in the dry land and forest. The observations in the field were accompanied by informants of rural people in the study area. If we have found certain *leunca* variation in natural habitats and man-made ecosystems of the study area, it was discussed with informants on various aspects of local knowledge or traditional ecological on *leunca*, including local names, distinctive characteristics, folk classifications, ecology, agronomy, and utilization. Moreover, *leunca* variations were taken of pictures and specimens or herbariums were also collected to analyze in the Ethnobiological Laboratory of the Department of Anthropology and the Department of Biology, Universitas Padjadjaran, Sumedang, West Java, Indonesia. Observation was also undertaken on sales and consumption of *leunca*. The aim of the observation of *leunca* was to get more detail information of its morphological structure and utilization of *leunca* among rural people and also selling *leunca* that was carried out in the Sundanese rural small restaurants (*warung*) and traditional markets in both in rural areas and urban areas. Some literature of plants, particularly vegetables, including Burkill (1935), Backer and Bakhuizen (1968); Heyne (1987); and Siemonsa and Grubben (1996) were also used to analyze botanical characteristics of *leunca* variations.

The semi-structured interview or deep interview was undertaken with competent informants, including informal leaders, village formal leaders, village small shop traders and vendor traders of traditional markets to obtain detail local knowledge on *leunca*. Each individual informant gives expensive to a series of general questions on variations, folk classification, botanical characteristic, ecology, agronomy, and utilization of *leunca*, some of which have been prepared in advance and some of which arise naturally during the course of the conversation. While structure interview, it was undertaken with 80 respondents of both village that is randomly selected of Sundanese households. Each respondent was interviewed by using questionnaire. Each respondent was asked with the same set questions, such as various side dishes of vegetables were consumed in breakfast, lunch, and dinner.

Data analysis

Various qualitative data obtained from the deep interviews with informants and direct observation of researchers during field research were analyzed by cross-checking with triangulation techniques, namely validation of databased information from different informants, and crosschecking data obtained from interview results and personal observation of researchers in the field, including conducting data validation with photo documented. All information was summarized, synthesized, and narrated with descriptive analysis and evaluative (cf. Martin 1991; Newing et al. 2011; Iskandar 2018). To analysis quantitative data obtained by questionnaire, interview was calculated by simple statistical analysis mainly percentage of answers (%) = (n / N) x 100%, where n= number of respondents who choose a particular answer, and N= total number of respondent's answers, moreover make narration of descriptive analysis.

RESULTS AND DISCUSSION

Botanical and ecological knowledge

Based on deep interview it has been revealed that all informants knew on plant of *leunca*. However, concerning detail local knowledge of plant of *leunca*, is not the same. In general old informants (age more than 70 years) know more variations of *leunca* or various kinds of plant named *leunca* compared to that of young informants (age of 17-70 years) All elderly informants at least know six variations of

leunca, namely: *leunca komir*, *leunca bonglot*, *leunca beureum*, *leunca monyet*, *leunca badak*, *leunca manuk*, *leunca hayam*, and *leunca biasa* (Table 1; Figure 3). Unlike elderly informants, young informants, in general, know only three variations or kinds of plant named *leunca*, namely *leunca manuk*, *leunca hayam*, and *leunca biasa* instead of 6 variations or kinds. This result is similar to that of undertaken by other scholars that local knowledge of community varies greatly person to person due to factors such as age, subsistence practice, gender, and bilingualism. Generally, people older have more knowledge than younger people (Lizararralde 2004).

Empirically, based on personal direct survey or observation in the field, it has been revealed that only 3 variations (landraces) or kinds plant named *leunca* in two villages of the study area. Landrace can be defined as local category for grouping cultivated plants, in this case, *leunca* according to common characteristics reflected in specific vernacular name (Iskandar and Ellen 1999). Kind of plant (landraces) that has been locally named by elderly informants as *leunca badak* and predominantly named by rural community as *takokak* (based on *emic*). Moreover, this kind of plant, based on botanical name is known as *Solanum torvum* Sw (based on *ethic*s). Another kind of *leunca* is locally named by informants as *leunca komir* or predominantly called by rural community as *tomat leutik* (emic). According to Botanical name (*ethic*), it has been revealed that *tomat komir* or *tomat leutik* is named as *L. esculentum*. In addition, some elderly informants recognize a kind of *leunca* named as *leunca beureum* that is based on emic view recognized as has flower petals purple, fruit red, and wild grow in the forest area, however, based on the botanical analysis it has been precisely known species named, which is considered as genera of *Solanum* or unknown species *Solanum* sp., due to did not find any sample or specimen in the field. While, *leunca bonglot* that is based on informants has distinctive morphological characteristics, including leaves are larger than leaves of other kinds of *leunca*, it has not been identified of scientific name, due to did not find any sample in the field as a result, it can be named as *Solanum* sp. Indeed, among various kinds of *leunca*, *leunca biasa* is popularly known by informants, based on botanical identification is named as *Solanum americanum* Mill. This species has an English name as the Black Shadenight (Samuel 2015). *S. americanum* has various local name in different ethnic groups in Indonesia, namely *leunca* (Sundanese), *ranti* (Javanese), *terong meranti, terong paracicit, terong perat, kelampong puyuh* (Burkill 1935; Siemonsa and Grubben 1996).

Unlike Western knowledge or botanical classification, based on classification of informants (folk classification), three predominant *leunca*, namely *leunca biasa*, *leunca hayam*, and *leunca manuk* are classified based both morphological and its edible or not-edible plant. Traditionally, informants well-known various kinds of *leunca* based on individual experiences on intensive interrelationship between the rural people and the local environment or local ecosystems (Iskandar 2018). These kinds of *leunca* are traditionally classified by informants based on morphological characteristics, including fruit of *leunca biasa, leunca manuk,* and *leunca hayam* has a round, around and shiny smooth skin, and round and shiny when still young and becomes rather wrinkled when ripe, respectively. In terms of leaves, the leaves of *leunca biasa* are similar in that thin and green, while leaves of *leunca hayam* are thick, oval, with clear veins and dark green.

Another morphological classification, *leunca* can be classified based on fruit size. According to informants, *leunca biasa* was described has a fruit size between two or three times higher than a fruit of *leunca hayam* and *leunca manuk*. In addition, fruit skin of *leunca biasa is* thicker and not easily brittle compared to that of *leunca manuk* and *leunca hayam.* Conversely, the skin fruit of both *leunca manuk* and *leunca hayam* is recognized as thin and easily broken. The characteristics of morphological differences between fruit of *leunca hayam* and *leunca* were mentioned by informants. In terms of fruit size, the fruit size of *leunca hayam* is similar to that of *leunca manuk*. However, the ripe fruit color of *leunca manuk* is black similar to that of *leunca biasa*, while the *leunca hayam* is bright bluish with small array of bright yellow.

Regarding habitat of *leunca*, three variation or kinds of *leunca, leunca biasa, leunca manuk,* and *leunca hayam* have similar habitats, including roadside, hilly garden dyke, among the trees of young coffee trees, around uninhabited house, cemetery complex, near water channels, home-garden, public bathing place or ritual ablution place of mosque, and village small river. In addition, those kinds of *leunca* were found in forest that is directly adjacent to gardens and settlements.

Some areas of Tarumajaya are owned by the plantation of the PTN VIII. Since the end of the first decade of the twenty-first century, the PTPN has managed the abandoned plantation planted by coffee trees. Some locations of the coffee garden have been planted by *leunca* plants understory of coffee trees. Because of the flowers of *leunca* plant can be considered as distracting of coffee flower disrupting (cf. Withaningsih et al. 2018).

Based on informants (emic analysis), it has been recognized 6 variations of *leunca* (*landraces*), namely *leunca biasa, leunca manuk, leunca hayam, leunca badak or takokak, leunca bonglot, leunca beureum*, and *leunca komir*. After all kinds of *leunca were* scrutinized and analyzed by literature (ethic analysis), it can be identified those kinds *leunca* consist of 3 species belong to two families, namely *leunca biasa* as *S. americanum*, *leunca hayam* as *Lantana camara* L, *leunca badak* or *takokak* as *S. torvum*, and *leunca komir* or *tomat kecil* as *L. esculentum*, while *leunca bonglot* and *leunca beureum* are considered as *Solanum* sp., but at the present time did not precisely identified due to did not find any sample in the field (Table 1). In other words, *leunca biasa*, *leunca badak* or *takokak*, and *leunca komir* or *tomat kecil* are different species at the same genera, *Solanum* of family Solanaceae, and *leunca hayam* are different species, *L. camara*, while *leunca bonglot* and *leunca beureum* have not been able to identify due to lack of samples. Therefore, both *leunca bonglot* and *leunca beureum* have been predicted the same genera, *Solanum*.

Table 1. Various plants that were named *leunca* based on informants of two villages of upper Citarum (emic analysis) and botanical science (etic analysis)

Local name	Characteristics based on informants (emic analysis)	Botanical name (Siemonsa and Grubben 1996).	Main characteristic based on botanical science (etic analysis) (Siemonsa and Grubben 1996)
Leunca biasa	Tree height approximately between 1,200 cm and 1,500 cm. The fruit is a globular berry bigger than other *leunca* variations. The fruits are rather bright green rather bright green when it is ripe bright green and black purple. The leaves are slightly oval with edges flat, rather bright green.	*Solanum americanum* Mill), Vernacular name: Glossy nightshade. Local names: *leunca* (Sundanese); *ranti* (Javanese), *kampai*; Malaysia: *ranti*, *terong meranti*, *terong perat*.	Glossy nightshade is an erect and short—lived perennial herb, up to 1.5 m tall, unarmed, dark green or flushed with purple, glabrous or sparsely hairy with curved simple hairs. Stem terete, angular or narrowly winged, sometimes warty. Leaves arranged spirally to almost opposite, variable in size. Fruit a globular berry, 0.5-1 cm in diameter, from green turning glossy bluish-black or purplish-black at maturity, readily shed when ripe; flesh with 0-4(-8) sclerotic granules and 40-100 seeds. Seed discoid, 1-1.5 mm long, creamy.
Leunca manuk	Shrub of 500-1,200 cm tall. Fruit a very small globular in a group, unripe fruit light green and turning dark black when ripe. The tape of leaf has a pointed shape.	A variety of *Solanum americanum* Mill	-
Leunca hayam	Tree high is approximately 1,000-1,500 cm. Fruits are small in group purple color with yellowish glow. Leaves are rather round at the base and taper at the end and thick, dark green.	*Lantana camara* L.; Family *Verbenaceae.* Local names: *saliara* (Sundanese); *temblekan*, *kembang telek* (Javanese)	Shrub of 1-2 m. Branches usually acculate, with sessile glands when young. Leaves opposite, rarely ternately whorled, ovate, contracted into the petiole, densely hispid on upper surface shortly pubescent beneath. Young flower pale, turning with age to pink or red, frequently with an orange eye, tube curve, inside coated with obliquely erect hair.
Leunca badak or *takokak*	Shrub of individual mature has height 2,000-2,200 cm, stem and leaf twig have thorns. Leave is an oval with the base of leaf rather round with its tip not pointed. Branch short flower stalk. Fruit a globular green not shiny similar to that of *leunca* biasa but higher.	*Solanum torvum* Swartz., Vernacular names: Devil's fig, Plate brush. Local names: *takokak*, *pokak* (Javanese); *terong pipit* (Sumatra); *terong pipit*, *terong rembang* (Malaysia).	Shrub with up to 3 m tall, pubescent with stellate hairs. Prickles scattered on stem, branches, and leaves, especially in younger growth, 3-7 mm long, slightly hooked. Leaves alternate, solitarily or in pairs. Fruit a globular berry, 1-1.5 cm in diameter, yellowish, glabrous, produced in clusters of few to 10. Seeds 300-400 per fruit, flat, 1,5-2 mm long, brownish.
Leunca bonglot	Annual shrub of individual mature 600-1,000 cm tall. Jagged leaves with sharp edge. Fruit a globular beery shiny green separated not grouping similar that of *leunca biasa*, *leunca manuk*, and *leunca hayam*.	*Solanum* sp*), Family Solanaceae	-
Leunca beureum	Herb of individual mature approximately 1,000-1,500 cm high. The leaves are rather long similar to that of the potato. Flower petal is purple, and red fruit when ripe. In the past, it was predominantly grown in forest bordering of settlement.	*Solanum* sp*), Family Solanaceae	-
Leunca komir or *tomat kecil*	Mature annual herb of approximately 200-250 cm tall. Stem and leaves have feathers. Flower is yellow. Fruit a berry green when young and red when ripe. It is usually grown in homegardens and edge of the forest.	*Lycopersicon esculentum* Mill, Syn. *Solanum lycopersicum* L; *Lycopersicon lycopersicum* (L) Karsten, Family Solanaceae. Vernacular names: tomato, love apple Local names: *tomat* (Indonesia); *tomato* (Malaysia).	Variable annual herb, up to 2 m or taller. Stem solid, coarsely hairy and granular. Leaves spirally arranged with 2/5 phyllotaxy. Fruit a berry, flattened, globular or oblate, smooth or furrowed, 2-15 cm in diameter, green and hairy when young, glorious and shiny, red, pink, orange or yellow when ripe.

Note: *) This plant cannot be identified because of did not find plant sample in the field, only based on information of the informants

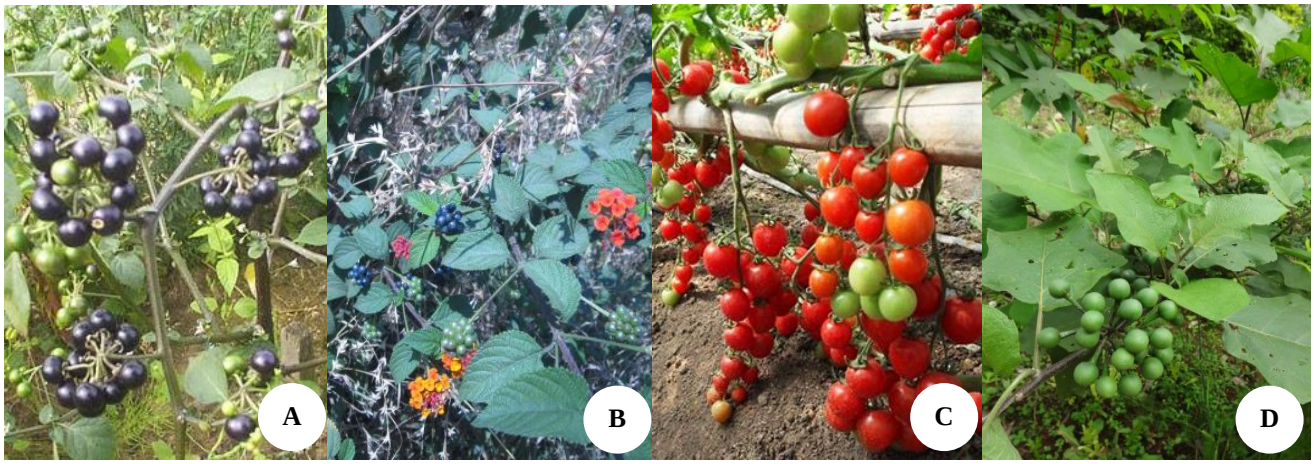

Figure 3. A.*Leunca biasa* (*Solanum americanum* Mill). B. *Leunca hayam* (*Lantana camara* L). C. *Leunca komir* or *leunca kecil* (*Lycopersicon esculentum* Mill). D. *Leunca badak* or *takokak* (*Solanum torvum* Swartz)

Agronomical knowledge and uses of *leunca biasa*

Leunca has been traditionally cultivated by rural people in homegarden (Table 2). In addition, sometimes, leuca has planted in garden or mixed-garden. Unlike other commercial vegetables, *leunca* has cultivated by mixed-cropping with other annual as well as perennial crops of homegarden system. While in the garden, *leunca* is predominantly cultivated as mixed-cropping with other annual crops. *Leunca* is traditionally propagated by seeds. It is usually sown in seed-beds or pots in the homegrden. Moreover, approximately several weeks after sowing, when the plants are about 5-10 cm tall are planted in the homegarden or garden.

In Tarumajaya Village, for example, it has been recorded at least 4 households has cultivated *leunca biasa* in their homegardens (Table 2; Figure 4). Those *leunca biasa* has been planted in area of no more 1-2 m^2 of the home garden with 8-16 individuals. According to informants who commonly planted *leunca biasa* cultivation of *leunca biasa* is similar to that of tomato (*L. esculentum*). It is mainly cultivated by making nursery of ripe seeds. Traditionally, cultivation of *leunca biasa* consists of some stages. Firstly, seeds are sown on the soil that has been hoed (*dipacul*) and loosened (*dilaci*) previously. Secondly, after seedlings grow between 4 weeks and 6 weeks that have approximately 10 cm tall, are selected. Thirdly, good individuals are planted in the garden with the spacing of one individual with another individual approximately 40 x 40 cm.

According to farmers of Tarumajaya Village, since the agricultural land has been considered as fertile, the planting *leunca biasa* is not necessarily provided by fertilizer. However, it is deemed and fertilizer availability, these crops are given fertilizers of chicken dungs put surrounding plants (*disaeur*). The fertilizers are mainly obtained from residual fertilizer for their vegetable garden. Unlike common vegetable, the *leunca* garden (*kebun leunca*) has rarely weeded regularly (*dikaramas*) or sprayed by herbicides (*diobat*).

Forth, when *leunca* biasa has grown approximately of between 10 weeks and 12 weeks, both fruits and leaves have been readily harvested. Fruits that are nearly mature owning characteristics, including green fruit mixed several purplish have been considered as appropriate time to be harvested.

This traditional practice of cultivation of *leunca* is rather similar to that of commonly undertaken by farmers of Soreang, South Bandung, the *leunca* crops are mainly planted in garden of the *kebun-talun* system, by providing manure of predominant bamboo biomass burning ash and livestock manure and without spreading herbicides. In addition, the *leunca* crops are predominantly planted mixed with other annual crops, and generally under shading other crop canopies, including corn crops (Iskandar and Iskandar 2011; Iskandar and Iskandar 2013).

According to informants, if farmers want to harvest leaves, the *leunca biasa* plants must be planted in the shading area. On the basis of the framer experiences, the *leunca* crops planted in the shading area, producing wide leaves, while planted in the full sun area of non-shading canopy of other crops producing small wide leaves that has close relation with their photosynthetic characteristics as function of light intensity (cf. Christanty et al 1978). Based on some observations, the *leunca biasa* have been planted in shading area producing leave size approximately between four and five times of that of planted in the full sun area without shading of other plant canopies. As a result, one harvest from each trunk can be harvested approximately 20-28 grams of leaves, and its leaves can be commonly harvested at least for time before trunks are being cut. In addition, one of reasons the *leunca biasa* has been planted in full-shade or half-shade, the taste of the fruits is not so bitter compare to that of plant in the full-sun area. In addition to wider leaves, the leaves of *leunca biasa* planted in full-shade have a sweeter taste.

Conversely, if the main purpose of planting *leunca biasa* to harvest a lot of fruits, the *leunca* crops are better planted in the full-sun area by getting direct sunlight. The

leunca biasa planted in full-sun area may produce smaller leaf size. Because the farmers have not planted *leunca biasa* in the full-sun area, we direct comparing between planted *leunca biasa* planted in the full-sun area of the garden and *leunca biasa* wild grown in the full-sun area. The result showed that the farmer ecological knowledge and their perception were confirmed that leaves of *leunca biasa* grown in the full-sun area producing smaller size, approximately 10-20% smaller size of the leaves of *leunca* crops planted in the full-shade area. Indeed, based on direct measurement, one harvest each individual trunk can be harvested about 300-350 gram of fruits of *leunca* without harvesting leaves. However, if individual *leunca biasa* that was harvested leaves, each individual can be harvested approximately 180-200 grams only. This production is much lower compared to that of production based on experience undertaken by Fortuin and Omta (1980) in horticultural garden in Lembang, Bandung, Indonesia. According to Fortuin and Omta, each individual *leunca* after planting 113 days, can be harvested about 1,070 grams of fruits.

In addition, based on informants, the fruit production of *leunca biasa* can be determined by season conditions, range of up-down of daily temperature. Generally, *leunca* produced a lot of fruits on the dry season when average of daily temperature is higher than that of the rainy season. Traditionally, *leunca* has not been cultivated in emphasized as commercially cultivated. As a result, *leunca* has predominantly planted with other crops in homegarden or mixed-garden. For example, the mixed-garden or *kebun-talun* system of approximately 0.5 ha, in Karamat Mulya Village, Soreang, Bandung planted by *leunca* (*S. americanum*), ketimun (*Cucumis sativus*), cable rawit (*Capsicum frutescens* L.), bamboo, fruit, and woods, *leunca* harvested 200 quintals of sold Rp. 200.000 or only 3% of the total mixed-garden or *kebun-talun* system productions (Rp. 6,471,600/0.5 ha) (Nuryani 2002). Therefore, because of *leunca* has predominantly cultivated as small scale of farming system in homegarden system and commonly sold on local market, no-production figures are available in local as well national level or international level. Only figures from one experiment in Indonesia, *leunca* plants were harvested until 4 months after planting in the field; mean fruit yield was 30 kg per 10 m^2 (30 ton/ha) when leaves were not harvested, and 16 kg per 10 m^2 (16 ton/ha) when also 0.8 kg edible leaves per 10 m^2 (0.8 ton/ha) were harvested (Siemonsma and Piluek 1994).

Leunca can be classified into two categories, namely edible and not edible *leunca*. According to informants both fruit and leave of *leunca hayam* (*L. camara*) have never been eaten due to consider as poison. However, leaves of *leunca hayam* can be used as traditional medicine for poultry, namely leaves are boiled and the boiled water mixed with water drunk on sick chicken (cf. Partasasmita at a. 2017). The leaves of *L. camara* has predominantly used as poultry medicine (*boat hayam*). Therefore, this plant is locally named as *leunca hayam*. In addition, leaves of *leunca hayam* can be used as cure against abscess, colic, nausea, as diaphoretic, against tumefaction, rheumatism. While root this plant can be used as traditional medicines, including against gonorrhea, syphilis, depurative, and leucorrhea (PT Eisai 1986).

Leunca manuk (a variety of *S. americanum*) is most distinctive characteristic a very small globular in a group, has predominantly consumed only leaves as boiled vegetable (*lalab kuluban*) or cooked mixed with fishes. Since fruits of this kind of *leunca* have been predominantly eaten by bird (*manuk*), the local name of this *leunca* is called *leunca manuk* (bird *leunca*). Traditionally, *leunca manuk* has been usually eaten by children only as a snack while are plying.

Among various plants named *leunca*, only *leunca biasa* (*S. americanum*) has predominantly consumed by rural people of Upper Citarum, West Java. It has been traditionally consumed both fresh fruits and leaves (Table 2).

Traditional institutional aspects of *leunca*

'Institutional aspect of *leunca*' in this article may be defined as social rules that apply in social daily life in the community some kind of guidance in interaction between people with distribution of *leunca*.

All farmers in two villages of the study area, namely Tarumajaya and Sukapura are vegetable farmer that in village or district statistical data is classified as farmer of 'horticultural strategies', including farming of kentang (*S. tuberosum*), tomat (*L. esculentum*), bawang daun (*A. fistulosum*) and kol (*B. oleracea* var *capitala*). Although many farmers have engaged in farming commercial vegetables, *leunca* has not been cultivated as the garden monoculture, but traditionally cultivated as mixed-cropping with other crops in homegarden or *kebun-talun* system (Nuryani 2002: Iskandar and Iskandar 2011). However, based on interview and observation on selling and consumption of *leunca*, it was revealed that *leunca* is predominantly sold in the vegetable stalls of local traditional market and small shops (*warung*) that selling good daily need. In addition, *leunca* whether as fresh vegetable or as part of main component of the dishes has not only been widely recognized but also consumed by rural people at least between 2 times and 4 times each week.

Leunca in both villages, Tarumajaya and Sukapura has been commonly distributed until household consume as last consumers mainly through institutional market channels. It has been recorded two marketplaces in Sukapura Village recognized in that place there was a sale and purchase transaction. On one place it has permanent building, and another one busy trading once a week. *Leunca* has been predominantly sold only in the permanent market of Cibeureum (Figure 5). As a result, most people have commonly gone to Pasar Baru of Cibeureum located in Cibeureum Village. It has been recorded one trader who especially trading *leunca* as main commodity.

Leunca fruits were sold in level of traders of Sukapura market and Pasar Baru of Cibeureum between Rp 10,000 and 12,000 per kg. However, in level of small shop (*warung*), *leunca* was commonly sold between Rp 3,000 and Rp 3,500 per package (about 180-200 grams). In commonly sell daily needs of rural people, only one

warung has not sold *leunca* due to not selling vegetables. In Sukapura Village has been recorded 46 *warung* that is similar that of in Tarumajaya, only 14 *warung* not sold vegetables, including *leunca*.

Table 2. Cultivation of *leunca* planted in homegarden system and tradition of *leunca* consumption as vegetable dishes

***Leunca* planted in homegarden**	**Sukapura Village**		**Tarumajaya Village**	
	Number of respondents	**Percentage of the total**	**Number of respondents**	**Percentage of the total**
Leunca planted in homegarden	8	20	4	10
Consumed *leunca* as vegetable dishes	40	100	40	100
Not consumed *leunca* as vegetable dishes	0	0	0	0

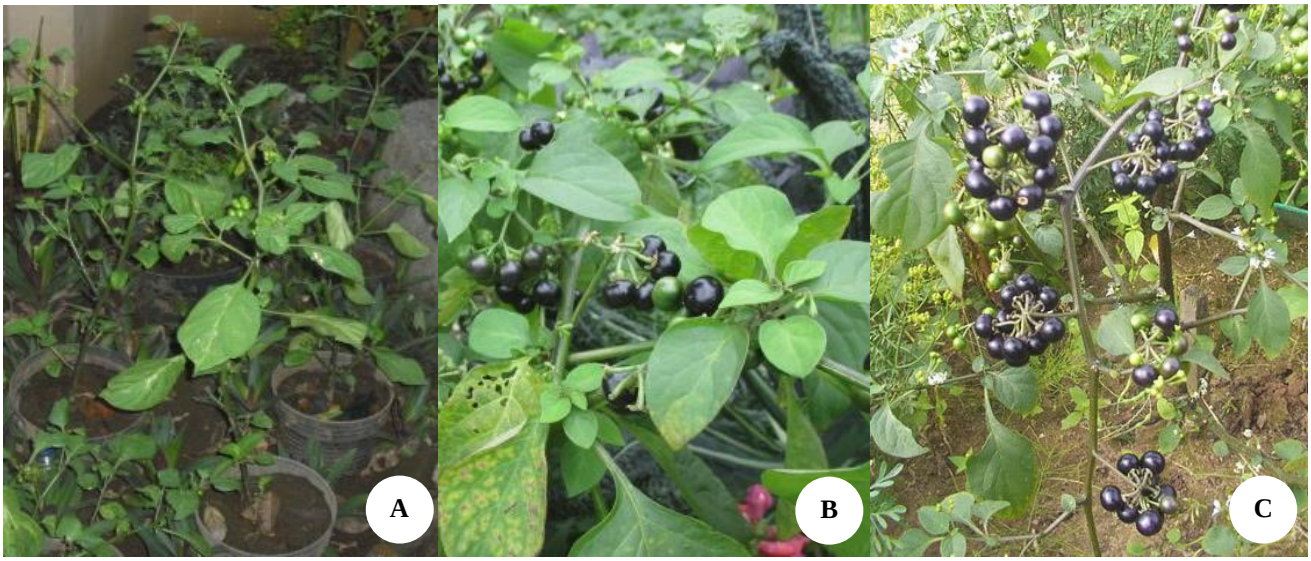

Figure 4. A. Nursery of *leunca biasa* (*Solanum americanum* Mill) in the home garden. B. *Leunca biasa* planted in the homegarden of Upper Citarum, West Java. C. *Leunca biasa*) planted in a garden (*kebun*) of Upper Citarum, West Java, Indonesia

Figure 5. A. *Leunca* has been predominantly sold in Pasar Baru of Cibeureum, Cibeureum Village, West Java, Indonesia. B. *Leunca* has been predominantly sold in the vegetable stall traders

Approximately *leunca* fruits have been predominantly sold in every day recorded between 8 packages (14-16 ons) and 12 packages (2.1-2.4 kg). If it is calculated based minimum number used as standard, average *leunca* was daily consumed by rural people of Tarumajaya Village estimated 35 kilograms, and 44.8 kg in Sukapura Village. Based on consumption habit of respondents, at least *leunca daily* consumed by respondent households estimated 1,050 kg/per month (Rp. 10.500.000-Rp. 12,600,000) of Tarumajaya households, and 1,344 kg/month (Rp. 13,440.000-Rp. 16.128.000) of Sukapura households with assumption each household consumed *leunca* as vegetable dishes in everyday menu. Although based on economic value *leunca* is not too high as other vegetable crops, including *bawang daun*/spring onion, *tomat*/tomato, *sawi*/Indian Mustard, *kentang*/potato and *kubis* or *kol*/cabbage, and since both villages have not been recognized as place of *leunca* cultivation, average level of consumption of *leunca* has been considered as high. Indeed, it can be seen that *leunca* has been daily part of rural people live.

In addition to through relations based on market mechanism, distribution of *leunca* can also be undertaken through neighboring and kinship relations. On the basis of information from four households who semi-intensively cultivated *leunca* in their homegarden, it can be revealed that asking (*nyuhunkeun*) and borrowing (*nambut* or *nginjeum*) of *leunca* among neighboring and kinship relations with the *leunca* owners. The relationship of asking *leunca* is different from borrowing *leunca*. In the relationship of lending and borrowing, if a neighboring or relatives borrow a small amount of *leunca* fruits, the borrower must return the same amount as *leunca* she borrowed at another time; even though the return transaction is always accompanied by words that indicate that the lender feels ashamed to receive the return the size she requested. While in relationship of asking and giving, the recipient is not obliged to request the return of a number of *leunca* fruits to the giver. However, there is a kind of norm that requires the recipient of *leunca* at other times to provide certain amounts of things other than *leunca* to the giver *leunca*; even though usually the giver or the person who behalf of the giver while the requestor is replying to his gift always mention that the requester does not need to do that.

Whether relation of asking and giving of *leunca* or borrowing and returning of *leunca* is both it is considered as in the context of neighboring social relations. It means both relationships are based on the showing of close relation of physical, personal or social closeness or a combination of the three kinds of relationships among parties involved. Physical closeness can be fulfilled considering that the event of borrowing and asking can only occur between those who live in an affordable space by just a few steps (*salangkah*). However, because the closeness of space often also causes conflicts, therefore, the personal proximity requirement must also be fulfilled. People who are involved in both relationships are usually people who know each other well. As a result, this has to do with the third prerequisite, namely social closeness. The people involved not only know each other, but also understand the reasons behind being asked for requests or loans; because they are in the same social situation either because they are both poor, both work for the same farmers or also because they have patronage and kinship relationships.

Various things that occur behind the relationship of borrowing and giving *leunca* are usually related to unexpected events, such as the arrival of guests who are considered entitled to be served meals, unplanned activities, such as special cooking in a pan (*ngaliwet*) or the events when the required *leunca* are not available in the nearest small shop (*warung*).

Leunca in local culinary culture

People of two villages of the study area consume both fruit and leave of *leunca*. Leaves of *leunca manuk*, *leunca hayam*, or *leunca biasa* are usually presented as parts of dish mixed with fish, wrapped in banana leaf and burned with burning ash (*dipepes*) or consumed as fresh vegetable after cleaned by water or soaked in hot water for a while (*dileob*) or steamed (*seupan*) with other vegetables, particularly leaves of cassava and *paria* (Table 3).

Unlike *leunca manuk*, fruits of *leunca biasa* are usually consumed as part of daily dishes of the households. In addition, the leaves of *leunca biasa* are usually served limited, while fruits of *leunca biasa* are usually served with dish varieties. The *leunca* biasa fruits are most popularly consumed as fresh vegetable.

According to Suriawiria (2006), fresh vegetable has been part of life and culture of Sundanese people, in West Java. In two villages of research area, the fresh vegetable has also embedded in local culture. Based on survey with structured interview with 80 households, all respondent (100% of respondents) mentioned that they have also always dished up fresh vegetable as part of daily menu, particularly for lunch menu, and eat interlude between breakfast and lunch, also between lunch and dinner, that is called as *ngawadang*. Based on respondents, it has been revealing that daily menu of lunch and *ngawadang* during one-month, average *leunca* used as dish food was recorded between 16 and 18 times as fresh vegetable. Rating of *leunca* was presented as a food dish 23 times of the months, under ranking of cucumber/bonteng nearly every day of the month. However, *leunca* is more presented as fresh vegetable compared to that other vegetable, including *salada bokor* , *kacang Panjang*, *surawung*, *terong hejo*, *kol*, and *tespong*.

In local culinary structure, *leunca* was commonly served with rice and fried foods, including freshwater fish, salted fish, soybean *tempe*, chicken meat and tofu/soybean *tahu*, *leunca* and other fresh vegetables, and traditionally mixed with *sambel* (Sundanese sauce). It has been popularly known special *sambel*, namely *sambel terasi* (chili shrimp paste) is strongly combined dish with the fresh *leunca* fruits. Traditionally, *sambel terasi* is made of *terasi* or *balacan* (chili shrimp paste or fish paste), *cabe rawit/cengek*, *cabe merah*, salt, sugar/brown sugar, *bawang merah*, and soaking water of *asem koak/asam Jawa* or substituted by *jeruk sambel* or *jeruk limau* or *jeruk purut* (Table 3).

Another the food dish of fresh fruit *leunca* was served as *pencok leunca* and *karedok*. Both food dishes have basic structure not only *leunca* fruit but also with other spices. *Pencok* is kind of food dish as *sambal* (souce) was made of *bawang merah, bawang putih terasi, cikur* (*Kaempferia galanga* L), *gula Jawa*/brown sugar, *surawung*, and salt. All basic materials, except *surawung* were ponded (*tumbuk, ulek* or *rendos*). After processing resulted half smooth, *hiris* was put mixed with *surawung* and stirred. In food dish of *pencok leunca*, all basic materials are similar to that of *karedok*, except *hiris* is replaced by *leunca* fruits.

It has also been known in Sundanese food dish that *karedok* is not always consisting of *leunca*. Similarly, *pencok* is principally as *karedok* namely is a kind of sauces with has more constituent elements. The constituent elements of *karedok* namely *cabe rawit/cengek, bawang merah bawang putih, cikur* (*Kaempferia galanga* L), *surawung* (*Ocimum bacilicum* L), *kacang tanah* soaking water of *asam Jawa, gula merah, teras*i, and salt. All materials, except *surawung*, are pounded. After processing it has been resulted in a kind of paste, *surawung* and other vegetables are put together. A dish food is named *karedok leunca*, if one kind of vegetable is used mixing with the paste namely *leunca* fruits. Like food dish of *pencok*, *leunca* is not fixed component. *Leunca* fruits can be replaced by *kacang panjang* or terong.

There is one kind of special food dish of Sundanese people that *leunca* is known as main identity, namely *ulukutek*. Principally the *ulukutek* is categorized as food dish of '*tumis-tumisan'* (stir fry) or is cuisine is made of by stir frying (*ditumis*) with using little cooking oil in kettle with low hot frying. Unlike other food dishes, *ulukutek* is the only one that has strong association with *leunca*. Indeed, without the name suffix of *leunca*, everybody knows that *ulukutek* is food dish with main component consists of *leunca* fruit and *oncom* (fermented *kacang tanah/suuk*). Main components of *ulukutek* consist of *cabai hijau* and/or *cabai merah, cabe rawit*, *bawang merah* (*Allium cepa* var. *ascalonicum*), *bawang putih, tomat terasi,* leaves of *salam, sereh, bawang daun, cikur,* salt and brown sugar (Tabel 3). Processing of making *ulukek* is undertaken by several stages. Firstly, main basic components are cooked in a kettle with little cook oil (*dioseng*). Secondly, after frying produces fragrant is indicated ready and put pounded *oncom*. Thirdly, after all spices have properly mixed with *oncom*, finally *oncom* is put it. Forth, before *leunca* fruits are being overcooked (*genjur*), food dish removed from the kettle and ready to be served at the dining table.

In addition to *ulukutek*, there is two food dish of *leunca*, namely *angeun leunca* (*leunca* soup) and *oseng leunca* (stir frying of *leunca*). Based on the questioner, although all respondent knew '*angeun leunca'*, all of them claimed have not consumed the *angeun leunca* anymore. Based on informants, there are two kinds of *angeun leunca* namely it was cooked with *tauco* and also cooked with coconut milk. The firstly, cooked with *tauco* is similar with *ulukutek* but has broth, while the secondly, cooked with coconut milk, is similar to *sayur lodeh*. Unlike *ulukutek*, in this food dish, *leunca* is overcooked (*genjur*). As a result, element of its crispness has totally disappeared.

Oseng leunca is principally known as one kind of '*tumis-tumisan'* (stir fry). Like *ulukutek*, main spice of *oseng leunca* is oncom or often also replaced by *tempe*, and *leunca* fruits. Basic cooking ingredient *oseng* consists of *bawang putih, bawang merah, cabai hijau besar, cabe rawit/cengek* (*C. frutescens*), *cabe merah besar* (*Capsicum annuum* L), leaves of *salam* (*Syzygium polyanthum* L), *bawang daun, tomat* salt, and sugar. Like all kind of *tumis-tumisan* (stir fry), *oseng leunca* was cooked with little cook oil on a kettle with frying medium fire. After all basic spices were cooked by stir frying (*ditumis*), *oncom* or *tempe* that has been cut into small pieces were put. After all spices were properly mixed with *oncom*, later on *leunca* was put it, stirred, and before *leunca* was overcooked, the *oseng leunca* was lifted from a kettle. On this basis, it can be inferred that at least 23 crop species and its parts have been predominantly used for traditional dish food with have relation with *leunca* (Table 3).

Leunca and Sundanese food menu habit

On the basis of most people who have ever been consumed *leunca* fruits, if they have tried to consume *leunca* fruits, they may be do not like to consume *leunca* fruits. For example, this study was carried on rural Sundanese community, but there is one household who has identified as a Javanese and is predominantly called by local people as "*urang Wetan*" (Eastern people) is documented in this study area. When a household leader asked his perception on *leunca* whether as fresh vegetable or as dish food it has been revealed that he mentioned that do not so like to consume *leunca* due to he has not commonly consumed *leunca*. He has known *leunca* which is called in his initial hamlet as '*ranti*'. In Javanese of Central Java, unlike in rural people of West Java, *ranti* has commonly used based on old people in hamlets recognized as traditional medicines, including stomach ache. Of traditional medicines, it has been confirmed by Eisai (1986) that *leunca* which is known as *ranti* in Javanese leave and fruit are recognized as traditional medicines, including exophthalmia, dysuria, dropsically swelling, hypertension, anemia, and constipation. Similarly, according to Burkill (1935), *leunca* (*Solanum americanum* L) is locally named as *ranti, terong meranti, terong paracicit*, *terong perat*, *kelampong puyuh*, and *leunca* recognized as vegetable. This plant is very distributed plant, found as weed in temperate regions, and there are reputed to be poisonous, but in the tropics, throughout which it occurs, it is used as a pot-herb. In the Malay Peninsula it is found all down the west side, but singularly its occurrence is not recorded for the east side. It is brought to market, sometimes as '*daun ranti'*. The tender shoots are boiled as spinach in India, Indo-china and through Malaysia. Usually, they are much liked, but the plant is not cultivated at all. The alkaloid solanine has been detected in seeds examined in Europe. In China the leaves, stalk, and roots are applied to wound and sores, and, again, the young shoot, like spinach, is considered tonic. In India the berries and the juice are medicinal and the plant is considered beneficial when taken as spinach. Apparently, its action is laxative and diuretic.

Table 3. Various plant species that have relation with *leunca* in local culinary structure

Local name/ Indonesia	Common name	Scientific name	Part that has relation to *leunca*	In culinary context as
Bonteng/mentimun	Cucumber	*Cucumis sativus* L.	Fruit	Fresh vegetable
Salada bokor	Lettuce	*Latuca sativa* L.	Leaf	Fresh vegetable
Tespong	Java water dropwort	*Oenante javanica* L.	Leaf	Fresh vegetable
Surawung/kemangi	Holi basil	*Ocimum sanctum* L.	Leaf	Fresh vegetable, raw material of *pencok leunca*
Terong hejo/ terong hijau	Eggplant	*Solanum melongena* L.	Fruit	Fresh vegetable
Kacang panjang	Cowpea	*Vigna unguiculata* L. Walp.	Fruit	Fresh vegetable
Kol/kubis	Cabbage	*Brassica oleracea* var. *capita* L	Leaf	Fresh vegetable
Asem koak	Tamarind	*Tamarindus indica* L.	Fruit	Spices of *sambal terasi*
Jeruk sambel/ jeruk purut	Kaffir lime	*Cytrus hystrix* L.	Fruit	Spices of *sambal terasi*
Cengek/cabe rawit	Chili pepper	*Capsicum frutescens* L.	Fruit	Spices of *karedok* and *pencok leunca*, component of *sambal terasi*
Cabe beureum/ cabe merah	Red pepper	*Capsicum annuum* L.	Fruit	Spices of *oseng leunca*, *ulukutek*
Cabe hejo/ cabe hijau	Green pepper	*Capsicum annuum* L.	Fruit	Spices of *oseng leunca*, *ulukutek*
Tomat	Tomato	*Lycopersicon esculentum* Mill.	Fruit	Component of *sambal terasi*
Sampeu/ singkong	Cassava	*Manihot esculenta* L.	Leaf	Boiled vegetable
Bawang daun	Spring onion	*Allium fistulosum* L.	Leaf	Component of *ulukutek* and *oseng leunca*
Cikur/kencur	Aromatic ginger	*Kaempferia galanga* L.	Rhizome	Spices of *karedok leunca*, *pencok leunca*, and *ulukutek*
Kawung/aren	Sugar palm	*Arenga pinnata* L.	Arenga juice/ *nira*	As brown sugar for using spices of *ulukutek* and *karedok leunca*
Bawang beureum/ bawang merah	Onion	*Allium cepa* var. *ascalonicum* (L) Back)	Bulb	Spices of *ulukutek*, *oseng leunca*, and *karedok leunca*
Bawang bodas/ bawang putih	Garlic	*Allium sativum* L.	Bulb	Spices of *oseng leunca*, *pencok leunca*
Sereh/serai	Citronela grass	*Cymbopogan nardus* L.	Stem	Spices of *ulukutek*
Salam	Indonesia bayleaf	*Syzygium polyanthum* L.	Leaf	Spices of *ulukutek*
Kalapa/kelapa	Coconut	*Cocos nucifera* L.	Fruit	Spices of *angeun lodeh leunca*
Suuk/kacang tanah	Groundnut	*Arachis hypogaea* L.	Fruit	Spices of *karedok leunca*

Moreover, based on the Javanese informants, who tasted of *leunca* or *ranti* he has not been liked as bitter and sensation as little bit hot in the mouth, like chewing tobacco. Therefore, they were rather surprised that why Sundanese people eat *leunca*, it seems like don't feel bitter, even willing to buy it for Rp. 3,500 in a small shop only for a handful (180-200 g) of *leunca* fruits.

The amazement of '*urang Wetan'* (Javanese people) on Sundanese customer to consume *leunca* and they did not like taste of *leunca* is not something natural. The assessment comes from what anthropologists call "habitus" or habits that are internalized from a very early age about what is permissible/not permissible and delicious unpleasant.

On the basis many social structures that exist around the lives of individuals, the nuclear family is a structure that has the most influence on the formation of individual food tastes and preferences. According to respondents, generally, they claimed known *leunca* since childhood. Their first information on *leunca* was obtained from seeing their mother serving *leunca* as fresh vegetable (*lalab*) as one of the daily menus in the household. Moreover, they obtained information when they saw their family eating *leunca* fruit on many occasions and in a variety of dishes, either raw or cooked. By imitating, some experiments in the family shaped personal preferences which then evolved from merely an understanding that *leunca* could be eaten. Moreover, they understood *leunca* could be eaten in various ways served and delicious taste.

Since each family in study area of both villages have closed interrelationship with other families with reference similar food dish, not surprisingly, the preference for *leunca* will also be strengthened by a wider social structure other than the family. As time has changed over time, the appetite for *leunca* that is part of the collective taste is ingrained in an individual's body as if it were natural. For Sundanese people, *leunca* is not only delicious to eat but also something delicious that is natural. *Leunca* is considered something that has become part of the body unless there is a time when enculturation *leunca* is cut off from the lives of individuals before the taste of *leunca* is embodied. This is because of moving to a place that does not know *leunca* as food or because there is a prohibition to consume *leunca*.

Figure 6. *Leunca*, '*ulukutek leunca'*, and other raw vegetables are being offered in a Sundanese restaurant

The *leunca* links with the identity of the Sundanese is not only raised when '*urang Wetan'*(Javanese) is asked to assess the habits of his Sundanese neighbors to consume *leunca*. Based on survey to 20 restaurants or cook shops (*rumah makan*) that served special Sundanese food, it could be revealed that all cook shops served fresh vegetables. Composition of fresh vegetables consists of *leunca*, *mentimun*, *seladah bokor*, *kemangi*, *kacang panjang*, and *tespong* were predominantly found in cook shops. Apart from the diverse composition of fresh vegetable (*lalaban mentah*), *leunca* as fresh vegetable was found in survey of 20 restaurants.

Aside from being fresh vegetable lalab, *leunca* was also served as a processed dish. There are two processed dishes made from *leunca* which were usually offered in 20 restaurants or cook shops, namely *karedok leunca* and *ulukute*k (Figure 6). *Pencok* or *sambal leunca* was offered at around 14 restaurants. While *oseng leunca teri* was offered in 12 restaurants.

The predominant of restaurants or food shops serving 'Food Sundanese specialties' in cities, including Jakarta and Bandung which almost all serve *leunca* dishes; showed that *leunca* is not just food, but an important component of the Sundanese identity. At the very least, the existence of *leunca* in these restaurants is a symbol of the authenticity of the Sundanese.

In conclusion, based on informants it has revealed that 7 kinds of plant that are named as *leunca*, however, only 3 kinds of *leunca* that are grown in their village. Among 7 kinds of landraces of *leunca*, *leunca biasa* (*S. americanum*) has been predominantly consumed both fruits and leaves. There are various foods dishes are consumed fresh or cooked. Various dishes of *leunca biasa* have been culturally integrated everyday life of people and culturally as a part of people identity of Sundanese people (*urang Sunda*). Other kinds of *leunca*, including leaves of *leunca manuk* (variety of *S. americanum*) have been consumed *leunca* as cooked vegetable, and its fruits consumed as snack, while *leunca hayam* (*L. camara*) has not been consumed, but its leaves have been used as traditional medicine of pet chicken disease.

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Short Communication: The utilization and effectiveness test of andisol soil-bioball-*Agrobacterium* sp. toward heavy metal chrome removal

PRANOTO[1,♥], RETNO ROSARIASTUTI[2], ALFIAN PRIHANDOKO[3]

[1]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57 126, Central Java, Indonesia. Tel./fax.: +62-271-663375, ♥email: pakpranotomipa@staff.uns.ac.id

[2]Department of Soil, Faculty of Agriculture, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57 126, Central Java, Indonesia

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Abstract. *Pranoto, Rosariastuti R, Prihandoko A. 2018. Short Communication: The utilization and effectiveness test of andisol soil-bioball-*Agrobacterium *sp. toward heavy metal chrome removal. Biodiversitas 19: 1955-1959.* This study was aimed to determine chrome metal adsorption and bioremediation ability using andisol soil, bioball, and *Agrobacterium* sp. The andisol soil characterization was performed by Fourier Transform Infrared Analyzer (FTIR), X-Ray Diffraction (XRD), and Surface Area Analyzer (SAA) while the cell quantity of *Agrobacterium* sp. was calculated by hemocytometer method. Determination of adsorption and bioremediation effectiveness was done using several parameters such as effect of pH solution, ratio variation of andisol soil-bio ball-*Agrobacterium* sp, and contact time. The pH variation was ranged from 1-6, while the composition variation of andisol-bioball-*Agrobacterium* sp. were 2: 0: 0, 1, 5: 1: 5, 1: 2: 10, 0, 5: 3: 15, and 0: 4: 20 (gr: item: mL), and contact time of 30,60,90,120, and 150 minutes. The result of this research shows that the optimum adsorption and bioremediation of Cr metal at pH 5, while the optimum ratio of andisol soil-bioball-*Agrobacterium* sp was 1, 5: 1: 5 (gr: item: mL), with the contact time for 120 minutes with Cr decrease percentage of 71.3%. The adsorption isotherm followed Langmuir and Freundlich isotherm.

Keywords: Adsorption, *Agrobacterium*, bioball, bioremediation, chrome, soil andisol

INTRODUCTION

Rapid industrial growth in Indonesia on one side has a positive effect to country, but it also has a negative impact on the environment. The increasing industrial residua especially waste can be accumulated and can pollute the environment. Heavy metals are among these harmful chemicals. Most industries such as the electroplating industry, metallurgy, melting, batik, and others contribute to spreading heavy metals to the environment (Baidho et al. 2013).

One of the heavy metals is chrome that harmful to health and the environment. If chrome is used excessively, it will cause acute poisoning. Other impacts, these heavy metals could be mutagenic and carcinogenic which lead to the serious disease such as lung cancer, kidney failure, anemia, skin allergies, asthma and stomach (Kaszycki et al. 2005, Palar 2008). According to the Ministry of Environment Decree KEP-03/MENLH/I/2010 the quality standard of industrial wastewater for maximum total chrome parameters is 1 mg/L.

Many method has been reported by previous researchers to treat chrome metals in industrial wastewater such as chemical precipitation (Gheju and Balcu 2011), ion exchange (Rafati et al. 2010), electrochemical precipitation (Animes et al. 2011), coagulation-flocculation (Haydar and Aziz 2009), solvent extraction (Elbagermi et al. 2013), membrane separation (Kumar et al. 2015), electrolysis (Wu et al. 2013), bioremediation (Iye 2015) and adsorption (Mthombeni et al. 2015). Bioremediation is the use of biological materials (e.g. microorganism) in the removal of toxic compounds from the environment such as the heavy metals which are considered more cost-effective and environmentally friendly (Iye 2015). Adsorption is also heavy metal removing method, and it is efficient, environmentally friendly, cost-effective and easy treatment method (Mthombeni et al. 2015).

Andisol soil is one type of soil that can be used as an adsorbent. In andisol, allophane minerals that have high specific surface area, porosity, and ion exchange capacity are usually found. It could be applied to wastewater treatment (Pranoto et al. 2013). The Andisol surface has properties such as the exchange of cations and anions, sorption of organic and inorganic compounds, and the acidity derived from silanol (Si-OH) and aluminol (Al-OH and $AlOH_2$; -OH and single coordination - OH_2/monodental) functional groups (Sukmawati 2011).

Agrobacterium sp. is a potential bacterium that can remove toxic substances so that it can be used in bioremediation. Pramono (2013) showed that *Agrobacterium* sp. was able to reduced Cr (VI) in both cell growth and rest conditions up to 100% and 51% within 18 hours. Moreover, Wang (2009) showed that *Agrobacterium* sp. has the ability to decrease nicotine in tobacco solid waste. The use of bacteria requires a medium. Bioball can be used as a bacterial medium. The bioball has a function as a place of living bacteria to maintain water quality (Said 2005). Furthermore, the use of bioball can balance the

expenses (Yang 2003). In this research, the ability of andisol soil, bioball, and *Agrobacterium* sp. on heavy metal (Cr metal) removal was studied.

MATERIALS AND METHODS

Materials and instrumentation

The materials used in this research were andisol soil (Cemoro Kandang, Lawu Mount, Indonesia), Bioball (Depok Fish Market, Surakarta, Indonesia), isolate of *Agrobacterium* sp. (collection fo UNS Central Laboratory, Surakarta, Indonesia), Agar LB medium, distilled water, 1000 ppm of Cr standard solution (E-Merck), NaOH (E-Merck), HNO_3 p.a (E-Merck), HCl p.a (E-Merck), KCl (E-Merck), $C_6H_8O_7$ (E-Merck), $Na_3C_6H_5O_7$ (E-Merck), CH_3COOH (E-Merck), and CH_3COONa (E-Merck).

The instruments and apparatus used in this research were atomic adsorption spectrometer (AAS, Perkin Elmer Analyst 700), Fourier transform infrared spectroscopy (FTIR, Shimadzu 8201 PC), x-ray diffraction (XRD, Shimadzu 6000), Surface Area Analyzer (SAA, Nova 1200e), Hemocytometer (Assistant), Microscope (Olympus CX21), analytical balance (Sartorius BP 110), pH meter (Eutech Instrument pH 700), shaker (Mitamura Riken), and glass tools (Pyrex and Duran).

Preparation of andisol soil and *Agrobacterium* sp.

Andisol soil was cleaned to remove impurities, washed with water and dried with aerated in the open air. Afterward, Andisol was crushed until smooth. Andisol soil was then sieved with a 150 mesh sieve. The powder that passed 150 meshes were soaked in distilled water and filtered, which was then dried at a temperature of 105°C for 4 hours. The subsequent andisol soil was mixed in 250 mL of 3 M sodium hydroxide solution for 5 h at 70°C. It was then washed with distilled water until neutral condition and calcined for 3 h at 400°C. The final product was then characterized using FTIR, XRD, and SAA.

Agar LB medium was placed into a sterile reaction tube. Then *Agrobacterium* sp. isolate was inserted into the test tube containing agar LB medium and incubated for 2x24 hours. After 2x24 hours, the culture of *Agrobacterium* sp. was transferred into a reaction tube containing 100 mL agar LB medium and incubated for 2x24 hours. After 2x24 hours, the culture of *Agrobacterium* sp. was transferred into a reaction tube containing 1L agar LB medium and incubated for 2x24 hours. The cell number of the final product was calculated by hemocytometer method.

Optimization of pH solution

Andisol soil: bioball: *Agrobacterium* sp. (gr: item: mL) 1: 2: 10 were placed into beaker containing 100 mL of 6 ppm Cr solution with buffer pH variation of 1, 2, 3, 4, 5, and 6. The solution was given an aerator and shaken for 60 minutes. After 60 minutes, the solution was diluted 5x and analyzed using AAS.

Optimization of ratio and contact time

Andisol soil: bioball: *Agrobacterium* sp. at ratio of 2: 0: 0, 1,5: 1: 5, 1: 2: 10, 0,5: 3: 15, 0: 4: 20 (gr: item: mL) and 20 mL *Agrobacterium* sp. without bioball were placed into beaker containing 100 mL of 6 ppm Cr solution (pH optimum). The solution was given an aerator, and shaken for 30, 60, 90, 120 and 150 minutes. Afterward, the solution was diluted 5x and analyzed using AAS.

Adsorption isotherm

Andisol soil-Bioball-*Agrobacterium* sp. ratios at best conditions were added to 10 mL of varied Cr solution (2, 4, 6, 8, 10 and 12 ppm). The solution was given an aerator and shaken at optimum contact time. Afterward, the solution was diluted 5x and analyzed using AAS.

RESULTS AND DISCUSSION

Characterization of andisol soil

FTIR Analysis

The IR spectra of natural andisol soil and active andisol soil was shown in Figure 1. It can be observed that Si-OH/Al-OH peak at 3445 cm^{-1}, H-OH peak at 1642 cm^{-1}, Si-O-Si peak at 1004 cm^{-1}, and Si-O/Al-O peak at 573-473 cm^{-1} was found in natural andisol soil. The found to peak at 2305 cm^{-1} was the peak of impurities contained in andisol soil (Silverstein and Webster 2005). Moreover, Si-OH/Al-OH peak at 3427 cm^{-1}, H-OH peak at 1654 cm^{-1}, Si-O-Si peak at 1002 cm^{-1}, and Si-O/Al-O peak at 557-465 cm^{-1} was found in active andisol soil. The active andisol soil did not reach the peak at 2305 cm^{-1} because the activation process is not capable for removing impurities. The loss of impurities after the activation process caused pores of andisol soil surface was opened.

XRD analysis

The XRD diffractograms of natural andisol soil and active andisol soil are shown in Figure 2. This figure presents allophane minerals appeared at (2θ) 8.03o and 26.99o. There are also other minerals such as montmorillonite at 10.04o; 21,92o; 29.35o and 35.45o, kaoliniteat 12,37o; 15,63o; 28,29o; 34,43o; 36,12o; 52,48o and 63,74-64,53o, gibsitat 27,99o and 78,76o, as well as feldspar at 23,85o and 34,41o. The comparison of diffractogram after activation indicates that some peak shifted and the intensity, as well as appearance of a new peak, decreased. Structural damage at soil minerals andisol resulted in the decreased intensity of the diffractogram.

SAA analysis

The SAA analysis of natural andisol soil and active andisol soil was shown in Table1. It shows that the surface area of andisol soil increases after the activation process. The impurity on the andisol soil surface has been dissolved during the activation process so that the opening of pores of the andisol soil and the value of the surface area increased. Adsorbents with larger surface area will provide more active sites (Hartopo 2014). The surface area will give the area of exposure to the surface of the andisol soil in the adsorption process to the chrome metal.

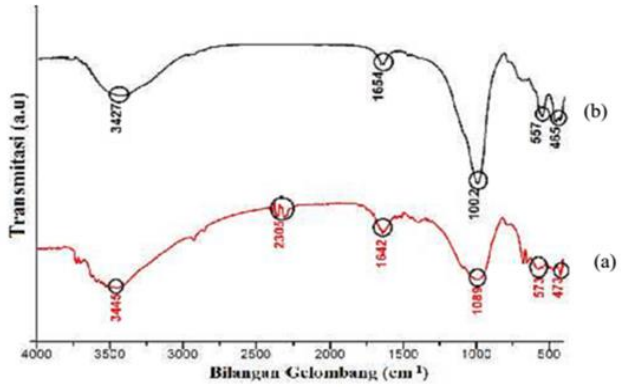

Figure 1. FTIR spectra of (a) natural andisol soil, and (b) active andisol soil

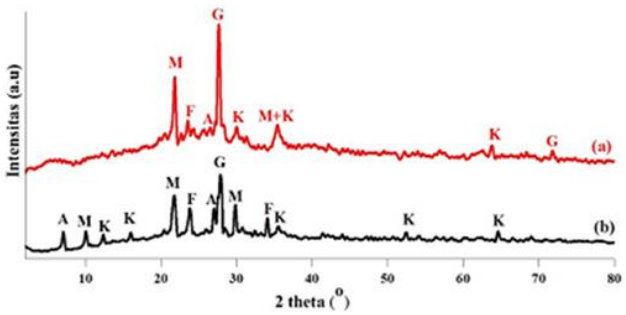

Figure 2. XRD diffractograms of (a) natural andisol soil, and (b) active andisol soil

Table 1. Result of surface area at natural andisol soil and active andisol soil

Name	Surface area (m^2/g)
Natural andisol soil	24.8
Active andisol soil	54.36

Table 2. Results test cell of *Agrobacterium* sp.

Name	Value (cell/mL)
Cells quantity before bioremediation	7.75×10^6
Cells quantity after bioremediation	2×10^6

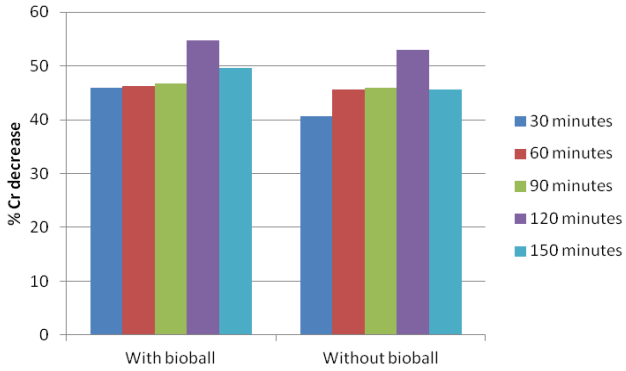

Figure 3. Graphic of bioball effect in *Agrobacterium* sp.

Identifications of *Agrobacterium* sp.

Test cell of Agrobacterium *sp. before and after bioremediation*

The quantity of *Agrobacterium* sp. cells after bioremediation became less (Table 2). Differences in the quantity of *Agrobacterium* sp. cells before and after bioremediation process was less because after the bioremediation process. Most of the *Agrobacterium* sp. cells died. Bacterial growth phase could be categorized into 4 phases, i.e., lag phase, logarithmic phase (exponential), stationary phase and death phase. The lag phase is the phase of bacteria to adjust to the new environment. The exponential phase is the process of a period of rapid growth of bacteria. The stationary phase is when the rate of bacterial growth is equal to the rate of death, so the quantity of bacterial will remain. This stationary phase is followed by a death phase that increases the rate of death than the rate of growth (Volk and Wheeler 1988). This indicates that the quantity of *Agrobacterium* sp. cells is reduced due to the death phase.

Effect of bioball in Agrobacterium sp.

The effect of bioball in *Agrobacterium* sp. is shown in Figure 3. The use of bioball medium in *Agrobacterium* sp. caused a larger percentage of Cr decrease, which is 54.74% than without bioball (Figure 3). This indicates that the addition of bioball medium is able to give maximum result to *Agrobacterium* sp. in bioremediation process. The giving bioball could reduce the cells death and increase the ability of cells to give maximum results (Ng et al. 2011).

Determination of optimum conditions

Effect of pH

The test results of the pH effect toward adsorption and bioremediation of Cr metal were presented in Figure 4. Figure 4 shows that at pH conditions 1-5, the percentage of Cr adsorption increased. However, at pH 6 conditions, the percentage of Cr adsorption decreased. At low pH (<5), the protonation will occur resulting in the formation of H_3O^+. This will cause the competition between H_3O^+ and metals. The lower of pH, the more H^+ ions are formed so that the lower percentage of Cr decreased. While at pH>5, hydroxide ion will be formed so that the deposition of Cr occurred lead to the formation of precipitated hydroxide causing their percentages decreased. At pH 5, a process of deprotonation on andisol soil occurred so that a negative site of OH^- ions was formed to have effective free electrons to bind Cr metals. While on *Agrobacterium* sp., it will produce maximum reductase enzyme at pH 5.

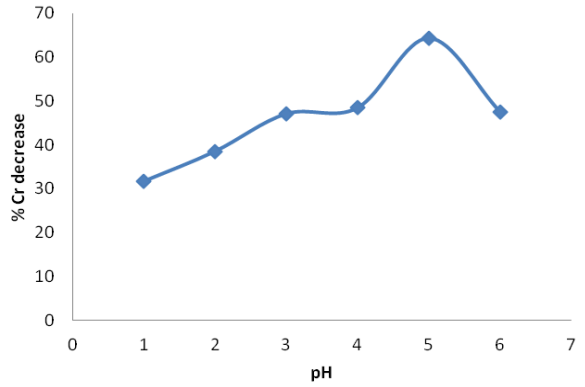

Figure 4. Graphic of the pH effect toward adsorption and bioremediation of Cr metals

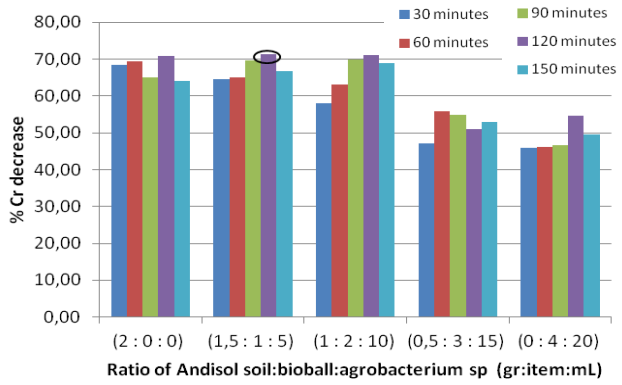

Figure 5. Graphic of ratio and contact time effect toward adsorption and bioremediation of Cr metals

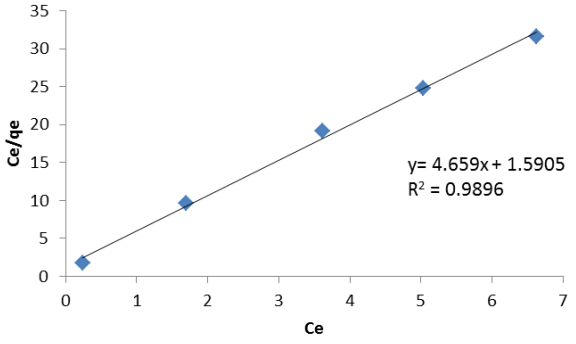

Figure 6. Langmuir isotherm model

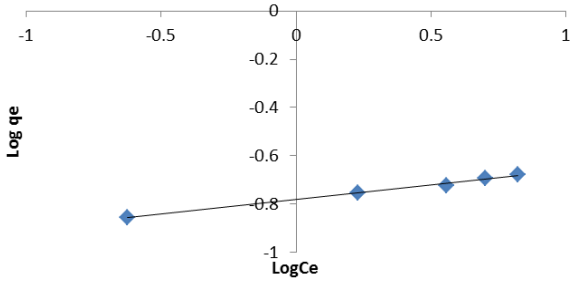

Figure 7. Freundlich isotherm model

Table 3. Langmuir and Freundlich Isotherm Value

Isotherm adsorption	Value
Langmuir	
Adsorption capacity (mg/g)	0.2146
K_L	2.9298
R^2	0.9896
Freundlich	
Adsorption capacity (mg/g)	0.1651
N	8.2713
R^2	0.9915

Effect of ratio and contact time

The test result of the ratio and contact time effect toward adsorption and bioremediation of Cr metal was presented in Figure 5. It can be seen in Figure 5 that the optimum condition of ratio of andisol soil: Bioball: *Agrobacterium* sp. (gr: item: mL) was 1,5: 1: 5 at contact time for120 minutes with the percentage of 71.3%. At that ratio the active site of soil andisol possibly had ability to absorb Cr metal greatly. The reductase enzyme produced by *Agrobacterium* sp. Could also help andisol soil in absorbing Cr metal.

The optimum contact time occurred at 120 minutes. When the contact time absorbing Cr metal was longer, the number of absorbed Cr metal will also greater until the optimum conditions. However, the concentration of Cr metal absorbed will decrease when it has passed the optimum contact time. At that condition, the saturation point, which means that Cr metal is no longer acceptable by andisol soil and *Agrobacterium* sp. and the absorbed Cr will be released back to the solution.

Isotherm adsorption

Langmuir and Freundlich isotherm graphics for Cr metal can be seen in Figure 6 and 7. Langmuir isotherms show that the adsorption process between adsorbent and adsorbate chemically occurred to form a monolayer (Bentahar et al. 2016). From Figure 6, the value of R^2close to 1, precisely the R^2value is 0.9896 indicating that the adsorbent active group interacts with Cr metal through chemical bond. While at Freundlich plot graph (Figure 7), the value of R^2close to 1 by 0.9915. This indicates that the adsorption of Cr metal also physically happened through van der Waals forces. When the Van Der Waals force occurs, the surface of the electronegative adsorbent interacts with the electrolytic Cr metal, although the interaction was weak. This weak repulsive force caused the adsorbate to move from one point of adsorbent surface to another surface forming a multilayer (Pranoto et al. 2013).

Based on the calculation at Table 3, the adsorption capacity of adsorbent on Langmuir isotherm was 0.2146 mg/g and Freundlich isotherms of 0.1651 mg/g in the Cr solution concentration ranged of Cr2-10 ppm.

In conclusion, soil andisol, bioball and *Agrobacterium* sp. could absorb the best chrome (Cr) metal at pH 5, with ratio soil andisol: Bioball: *Agrobacterium* sp. (gr: item: mL) 1.5: 1: 5, and contact time for 120 minutes with Cr decrease percentage of 71.3%. Type of adsorption isotherm

against chrome metal (Cr) solution was following by isotherms Langmuir and Freundlich.

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Predicting impacts of future climate change on the distribution of the widespread selaginellas (*Selaginella ciliaris* and *S. plana*) in Southeast Asia

AHMAD DWI SETYAWAN[1,2,3,♥], JATNA SUPRIATNA[3], NISYAWATI[3], SUTARNO[2], SUGIYARTO[2], ILYAS NURSAMSI[4]

[1]Department of Environmental Science, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Jend. Urip Sumoharjo No. 179, Surakarta 57 128, Central Java, Indonesia. Tel./fax.: +62-271-663375, ♥email: volatileoils@gmail.com

[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57 126, Central Java, Indonesia

[3]Program of Conservation Biology, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok 16424, West Java, Indonesia

[4]Bird Conservation Society (BICONS). Bandung 40184, West Java, Indonesia

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Abstract. *Setyawan AD, Supriatna J, Nisyawati, Sutarno, Sugiyarto, Nursamsi I. 2018. Predicting impacts of future climate change on the distribution of the widespread selaginellas (*Selaginella ciliaris *and* S. plana*) in Southeast Asia. Biodiversitas 19: 1960-1977.* The current global climate is moving towards dangerous and unprecedented conditions that have been seen as a potentially devastating threat to the environment and all living things. *Selaginella* is a fern-allies that needs water as a medium for fertilization, hence its distribution is presumed to be affected by climate change. In Southeast Asia (SEA), there are two widely distributed selaginellas, namely *Selaginella ciliaris* and *S. plana*. *S. ciliaris* is a small herb (up to 4 cm), annual, abundant during the rainy season, and found in the middle-high plains, whereas *S. plana* is a stout large herb (up to 80 cm), perennial, and mainly found in the lowlands. The purpose of this study was to determine the potential niche distribution of *S. ciliaris* and *S. plana* under current climatic conditions, and to predict its future distribution under the impacts of climate change. We used Maxent software along with bioclimatic, edaphic, and UV radiation variables to model the potential niche distribution of those two selaginellas under current and future predictions climate conditions. We generated future predictions under four detailed bioclimatic scenarios (i.e., RCP 2.6, RCP 4.5, RCP 6.0, and RCP 8.5) over three times intervals (2030, 2050, 2080). The results showed that future climatic conditions in the SEA had been predicted to significantly disrupt the distribution of suitable habitat of *S. ciliaris* and *S. plana*, and alter their geographic distribution patterns. Although some areas were predicted to become suitable habitat in the early period of future climate change, the overall projections show adverse effects of future climate conditions on the suitable habitat distribution of *S. ciliaris* and *S. plana*, as estimated losses of suitable habitat will be higher than the gains.

Keywords: Climate change, distribution, *Selaginella ciliaris*, *Selaginella plana*, Southeast Asia, widespread selaginellas

INTRODUCTION

Global climate is currently moving toward dangerous and unprecedented condition which has been viewed as a potentially devastating threat to the environment and all life within it (Beckage et al. 2008; Fitzpatrick et al. 2008; Hasanuzzaman et al. 2013). The Intergovernmental Panel on Climate Change (IPCC) in the Fifth Assessment Report (AR5) developed predictive scenarios on the future of global climate condition. In this report, IPCC projecting a further increase in global mean surface temperature by 2.6-4.8°C above pre-industrial levels, spatial and temporal changes in precipitation patterns, and increased incidence of floods and droughts in the year 2100 (IPCC 2014). These predictions presenting scientists with serious challenges in forecasting the impact of future climate projection on the sustainability of biodiversity (Fitzpatrick and Hargrove 2009). In the last decade, many scientists have been trying to measure the ecological impact of an ongoing climate change combined with continuous destructive human activities and to predict the response of biodiversity to different drivers of change (e.g. Dillon et al. 2010; Gilman et al. 2010; Pereira et al. 2010; Salamin et al. 2010; Beaumont et al. 2011; Dawson et al. 2011; McMahon et al. 2011; Alice et al. 2012; Bellard et al. 2012; Belgacem and Louhaichi 2013). In order to gain a deeper understanding of biodiversity responses to climate change, it may be more convenient to conduct the assessment on the regional scale, which is spatially heterogeneous, rather than assessing on the global scale (Walther et al. 2002; Bonebrake and Mastrandrea 2010). Currently, among all of the five global climate domains (i.e., tropical, subtropical, temperate, boreal, and polar regions), the tropical biome has been expected to become more vulnerable to the impact of climate change.

Myers (1988, 1990, 2000) initially defined 14 hotspots in the tropical biome and four in Mediterranean bioclimates. One of the defined hotspots of diversity and endemicity in tropical biome is Southeast Asia (SEA) (Sodhi et al. 2010). Climatically, Southeast Asia is monsoonal region with summer-dominant rainfall and a large-scale seasonal reversal of the wind regimes (Loo et al. 2015). However, SEA region has been experiencing a change on its climate condition. Average annual surface

temperature has increased by 0.5-1.1ºC during the period 1901-2005 (NIC 2009). Furthermore, climate model projection shows the average temperature will increase by approximately 1ºC until 2030 and will keep increasing through the rest of the 21st century (IPCC 2014). Although there is no clear projection in precipitation patterns in this region, climate model suggests that net precipitation rates will increase across the region, but there will likely be a local decrease of precipitation rates in some areas that will vary geographically and temporally (NIC 2009). An acceleration of annual rainfall, a significant increase of mean temperature, and extreme climate events such as floods, drought, and cyclones are several projected negative impacts of climate change in SEA region (IPCC 2014; Loo et al. 2015). The increase of mean temperature also has several impacts on the future climate condition, such as frequent changes and shifts in monsoon precipitation up to 70% below normal level and the delayed of monsoon by up to 15 days (Schewe and Levermann 2012). Along with the human-induced environmental degradation, climate change is believed to negatively affects the current plant diversity patterns (Belgacem et al. 2008). These threats are expected to lead to low emergence of annual species, change the life cycle of plants, changes in phenology and the timing of reproduction and finally reduced plants biodiversity (Thuiller et al. 2008; Belgacem et al. 2008; Hilbish et al. 2010; Hill and Preston 2015).

A number of plants species have been reported affected by recent climatic change (e.g. Bertin 2008; Skelly et al. 2010; Chen et al 2011; Agnihorti 2017; Evans and Brown 2017). However, this substantial development of assessing the ecological impact of climate change have been conducted almost exclusively on vascular plants, while only a few studies addressed the presumptive impact of future climate on cryptogams (Cornelissen 2007; Ellis et al. 2007). Autotrophic non-vascular cryptogams, such as spike-mosses, are also expected to be one of the earliest groups to be highly affected by the climate change (Cornelissen 2007; Bellard et al. 2012). Examining the impact of future climate condition on this group of species, which has been previously neglected, may be beneficial in acquiring a wider understanding of potential future risks of climate change, and serves as a crucial step in the development of effective management and conservation of biodiversity.

Selaginella Pal. Beauv. is the single remaining genus of vascular plants from the order Selaginellales (family Selaginellaceae), which can be found widely distributed in SEA region. This genus contains about 750 known species with a wide range of characters (Christenhusz and Byng 2016) and about 200 species found in SEA (Camus 1997; Hassler dan Swale 2002). *Selaginella* can be found in both very dry and very humid environments; and in open and shaded habitats (Setyawan et al. 2017). Therefore, the high humidity and tropical-hot characteristics of SEA's climate condition are highly suitable for the wide distribution of *Selaginella*. *Selaginella ciliaris* (Ritz.) Spring. and *Selaginella plana* (Desv. ex Poir.) Hieron are two examples of widespread selaginellas in the SEA region. The capability of these species to spread widely in the vast variety of microclimatic, physiographic, topographic, and edaphic conditions of SEA region, represent their presumed broad eco-physiological niche. Therefore, it is important to predict how the projected future climate affects the survival and the geographical distribution of these species.

Selaginella is relicts from ancient times and has survived almost unchanged in appearance for hundreds of millions of years (Banks 2009). To avoid extinction, *Selaginella*, like any other plant groups, may develop micro-evolutionary mechanisms as a response to climate change condition by reducing photosynthetic rates, growth rates, mineral absorption, tissue regeneration, and by increasing concentrations of secondary metabolites (Jochum et al. 2007; Wiens et al. 2009), or more likely, responding by shifting distribution to follow changing environments (e.g., Philips et al. 2006; Wiens et al. 2009; Minteer and Collins 2010; Chen et al. 2011; Morueta-Holme et al. 2015). Recently, attention has been shifted toward understanding more about the redistribution mechanism of species to cope with the change in climate condition. To project how the climate change affects the species distribution, Ecological Niche Modeling (ENM), which frequently called as Species Distribution Models (SDM) has become especially popular (Lawler et al. 2009; Merow et al. 2013; Fourcade et al. 2014). Peterson and Soberon (2012) have cautiously overviewed the conceptual considerations in terminology related to ENM and SDM. The authors found that there are a variety of differences in biogeographic and ecological basis of the two terms wherein each term has its own conceptual framework and its basis application. Following this overview, subsequent to reviewing our conceptual framework, we deliberately use the tern ENM in this study. Such models were built by using information on the environmental features that define the current ecological niche of species (Wiens et al. 2009). One of the most developed approaches of ENM/SDM is through the use of Maximum Entropy or Maxent algorithms (Belgacem and Louhaichi 2013). Maxent is a general-purpose machine learning method with a simple and precise mathematical formulation, for characterizing probability distribution from presence-only data, as well as a set of environmental predictors across a user-defined landscape (Phillips et al. 2006; Merow et al. 2013). Maxent has the ability to utilize different climatic scenarios to estimate the extent of occurrence of species (Beaumont et al. 2015). Therefore, allowing the evaluation of the impact of climate changes on geographical distribution of species' suitable habitat (e.g. Rondini et al. 2006; Botkin et al. 2007; Randin et al. 2008; Engler and Guisan 2009; Garavito 2015).

Here in this study, by utilized Maxent software along with bioclimatic, edaphic, and UV radiation variables, we tried to model the potential geographic distribution of *S. ciliaris* and *S. plana*'s suitable habitat under present climate condition, and predict the impacts of projected climate change on their potential distribution. We generate future predictions under four detailed bioclimatic scenarios (i.e., RCP 2.6, RCP 4.5, RCP 6.0, and RCP 8.5) over three-time intervals (2030, 2050, 2080). Quantifying the potential impacts of various climatic scenarios offers opportunities

to develop understanding the plant response to climate change and develop mitigation strategies under all projected scenarios of climate change to effectively conserve biodiversity.

MATERIALS AND METHODS

Study area

The study was conducted in an attempt to predict the impacts of future climate change on the distribution of *Selaginella ciliaris* and *Selaginella plana* in Southeast Asia (SEA). SEA is a sub-region of Asia, consist of countries that are geographically located in south of China, east of India, west of New Guinea and north of Australia (Kastle 2013). This region consists of eleven political countries that can be categorized into Mainland SEA (i.e., Cambodia, Laos, Myanmar/Burma, Peninsular Malaysia, Thailand, and Vietnam) and Maritime SEA (i.e., Indonesia, Philippines, Malaysian Borneo, Brunei, Singapore, and East Timor) (United Nations 2012). The geographic scope of this study includes the region of approximately 23.5 °N to 10 °S latitude and 97 °E to 141 °E longitude (Figure 1), covers approximately 4,687,481 km^2 of lands. The highest peak of Southeast Asia is Mount Hkakabo at roughly 5,881 m asl. (meters above sea level), situated in Northern Myanmar (Burma) and on the border with China and Tibet (Leinbach and Frederick 2015). The wide areas and vast altitudinal range of SEA create a wide variation in physiographic, topographic, edaphic, and climatic conditions resulting in rich biodiversity in this region.

The climate condition in Southeast Asia is mainly humid and tropical-hot all year round with high degree of rainfall variability and its climate generally can be characterized as monsoonal (i.e., marked by wet and dry periods) (Leinbach and Frederick 2015), hence, SEA region has only two seasons (i.e. wet and dry season). The only areas that feature a subtropical climate are in Northern Vietnam and the Myanmar Himalayas, featuring a cold winter with snow. These areas are in high altitudes which lead to milder temperatures and drier landscape (NIC 2009).

Materials

Selaginella ciliaris (Retz.) Spring. (Figure 2.A)

Annual herb, small, creeping, ascending, or sometimes fan-shaped, 4-15 cm. *Stems* recumbent, without significant main stem, 4-5 mm wide (incl. leaves). *Rhizophores* present at intervals, mostly near the base, from the lateral side of branching stem, ca. 0.3 mm in diam. *Leaves* dimorphic, composed in 4 lanes (2 lateral, 2 median), vein single; *lateral leaves* ovate-lanceolate, more or less symmetrical, 1.5-2 mm long, 0.6-1 mm wide, base subcordate or rounded, apex acute or acuminate, margin ciliate or serrulate, single vein reaching the apex, keeled, pointing outwards; *median leaves* ovate to falcate, asymmetrical, 2-2.5 mm long, 0.6-1.5 mm wide, base rounded, apex acute, cuspidate or attenuate, margin serrulate but laciniate at basal part, pointing upwards, minutely toothed, ciliate, midrib prominent, single vein reaching or nearly reaching the apex; *axillary leaves* lanceolate to ovate, bisymmetrically, 1.8-2.5 mm long, 1-1.5 mm wide, single vein reaching or nearly reaching the apex, base subcordate to rounded, ciliate, apex acute, margin toothed, laciniate at basal and serrulate at apical. *Strobilus* terminal, solitary or twin, complanate, flattened, up to ca. 1.5-2 cm long (Setyawan et al. 2013).

Habitat: Steep cliff, banks of irrigation water, ditches, small tributaries, and waterfalls, cliff edge of road, only abundant in the rainy season (Setyawan et al. 2013).

Distribution: Java, Sulawesi, Maluku (e.g. Ternate), Myanmar, Thailand, Vietnam, Philippines, New Guinea, Solomons, Northern Australia, Marianas, Palau, Micronesia, India, Sri Lanka, Southern China (Guangdong), Taiwan, (Hassler and Swale 2002).

Selaginella plana (Desv. ex Poir.) Hieron. (Figure 2.B)

Perennial herb, stout. *Stems* sub-erect with stoloniferous rhizome, without branches on the lower part, ascending from subterranean trailing base, up to 80-100 cm long, 3-10 cm wide (incl. leaves); rhizome (subterranean stems) shallowly radiating. *Rhizophores* sometimes at the branching stem, from the dorsal side of stem at the branch site, ca. 1-1.5 mm in diam. *Leaves* on the lower part and main stem monomorphic, well spaced, upper part slightly spreading, appressed, 1.5-3 mm long, 1-2 mm wide, ovate, apex acute or acuminate, but rounded tip, asymmetrical, margin translucent, entire. Leaves on the branches dimorphic, arranged in 4 lanes (2 dorsal, 2 ventral), loosely arranged at lower stem, closely arranged at branches; *lateral leaves* ovate to oblong, asymmetrical, 2-4.5 mm long, 2-3 mm wide, apex acute to acuminate, rounded tip, sessile, vein single, obscure, not reaching the apex, base truncate and rounded, upper base with spur-like lobe which overlaps the stem, margin entire, transparent; *median leaves* ovate to oblong, asymmetrical, 1.5-3 mm long, 1-2 mm wide, apex acuminate to acute, rounded tip, sessile, vein single, obscure not reaching the apex, base rounded and truncate, margin entire, transparent; *axillary leaves* ovate, asymmetrical, 2.5-3.5 mm long, 1.5-2.5 mm wide, apex acute, minutely ciliate, base rounded, margin entire. *Strobilus* terminal, solitary, tetragonal, up to more than 3 cm long (Setyawan et al. 2013).

Habitat: Steep cliffs above small irrigation channel, tributary, and waterfall, remaining abundant in the dry season (Setyawan et al. 2013).

Distribution: Sumatra, Java, Bali, Flores, Sumbawa, Solor, Timor, Sulawesi, Maluku (Ambon, Banda, Buru, Ceram, Kei, Ternate), Malay Peninsula (Hassler and Swale 2002).

Procedures

The occurrence data of Selaginella ciliaris and Selaginella plana

General information relating to the occurrence of *S. ciliaris* and *S. plana* across its whole range in SEA region was obtained from several literatures (Spring 1843; Mishra et al. 2001; Rachata and Boonkerd 2001; Beukema and

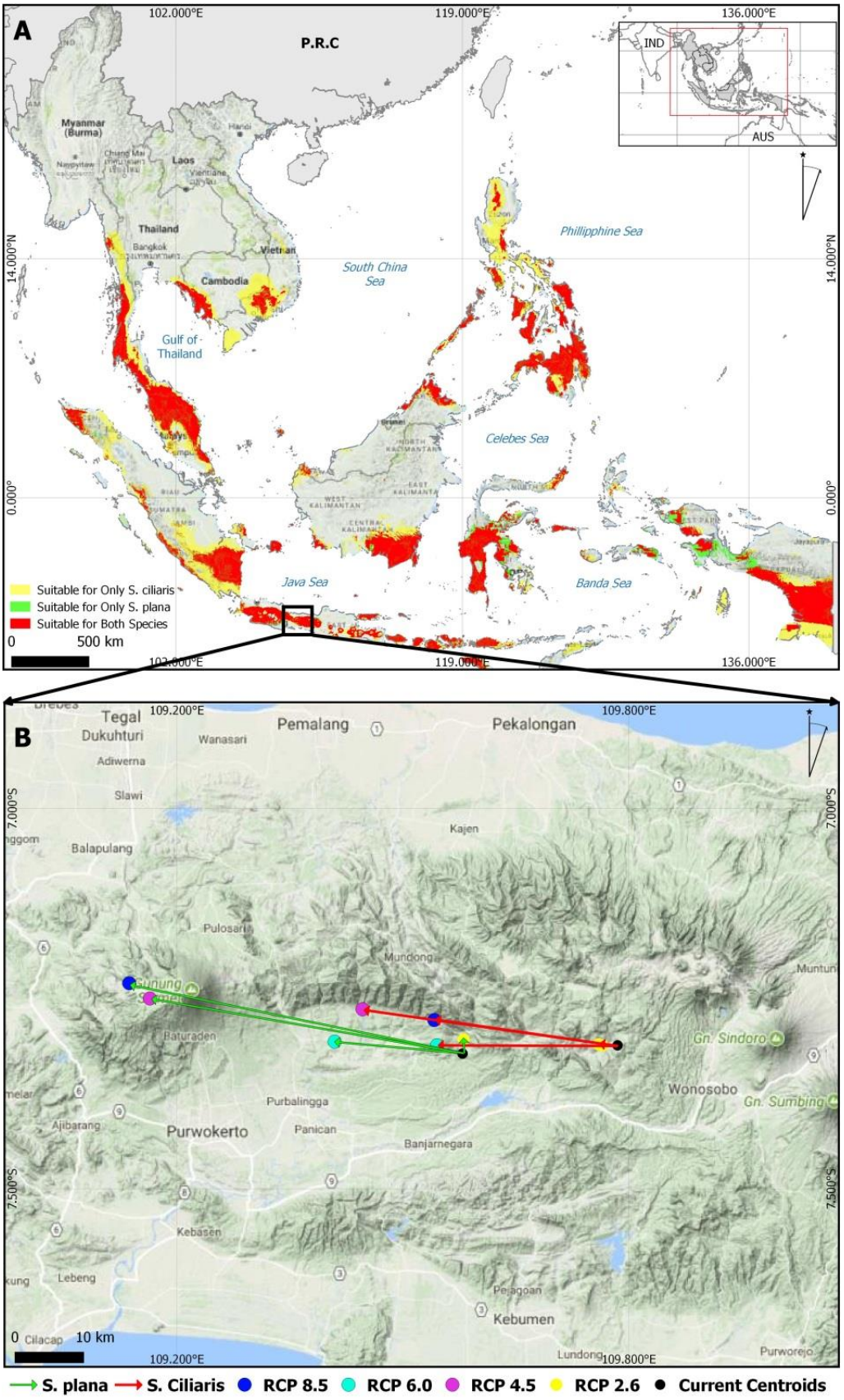

Figure 1. A. Predicted distribution of potential habitat for *Selaginella ciliaris* and *Selaginella plana* under current climate condition. B. Core distributional sifts under different climate scenarios in the year 2080. Black dot indicates the geometric center of suitable area under current climate condition. Colored dots indicate the new geometric centers. The arrows depicting magnitude and direction of predicted change (Basemap source: Google Physical Maps 2014)

Figure 2. Selaginella species used in research. A. *Selaginella ciliaris*, B. *Selaginella plana*

Noordwijk 2004; Ali et al. 2006; Setyawan et al. 2015a,b,c; Setyawan and Sugiyarto 2015), as well as Global Biodiversity Information Facility (http://www.gbif.org). Authors collected the occurrence data for *S. ciliaris* and *S. plana* from two main sources, i.e., field survey and GBIF database. Field survey aiming to collect the locality data for both species was conducted in all province across Java island between July 2007 and January 2014. The island of Java was chosen because of its diverse habitat and easy access; as well as both species are distributed widely and evenly throughout Java. All specimens founded were identified using several references on *Selaginella* of the Malay Archipelago and adjacent regions (Alston 1934, 1935a,b, 1937, 1940; Wong 1982, 2010; Tsai and Shieh 1994; Li and Tan 2005; Chang et al. 2012; Zhang et al. 2013) to ensure the high-confidence level of species identification.

During the occurrence data collection, we tried to cover as wide area as possible while attempted to cover the possible climatic variability of Java island as an attempt to minimize bias in sampling intensity (Elith et al. 2006; Yackulic et al. 2013). Using Garmin eTrax GPS series, we collected 379 occurrence points of *S. ciliaris* and 384 occurrence points of *S. plana* which were found distributed in Java island. We conducted none of error-correction method for the data as we ensuring the level of telemetry error on modern GPS (normally between 0.01 km and 0.05 km), which is smaller than the resolution of predictor variables, has a little effect on the accuracy of models (Montgomery et al. 2011). Global Biodiversity Information Facility (GBIF 2016) database, which provides freely accessible occurrence points in its website (http: //www.gbif.org), was the second source of locality points for both species. All of the occurrence record acquired from GBIF were carefully verified and errors that may occur were corrected using Google Earth software (Google Earth Pro 2017). Biogeomancer Workbench (http: //www.biogeomancer.org) was used as a tool to geo-reference data record which lacks latitudinal and longitudinal value, guided by locality descriptions on each datum (Guralnick et al. 2006), and then data record that does not have specific locality description and cannot be geo-referenced were removed. The remaining 369 locality points of *S. ciliaris* and 214 points of *S. plana* were compiled with the occurrence points collected from the field survey.

The increasing number of regional to continent-wide ENM/SDM study was mainly induced by the availability of biodiversity and environmental datasets globally (Hijmans et al. 2005; Kozak et al. 2008). Nevertheless, a strong geographic bias often contained in such datasets derived from opportunistic observation and/or collection of records (Stolar and Nielsen 2015). Sampling bias correction is highly important and strongly advised to be conducted to minimize the strong influence of sampling bias on modeling prediction ability and later interpretation (Kramer-Schadt et al. 2013; Fourcade et al. 2014). Fourcade et al. (2014) proposed five option methods of sampling bias correction which carefully designed to overcome or minimize the effect of four types of bias that might occur when collecting observation. Subsequently, after we identified the type of sampling data bias contained in the sampling data used for this study, we conducted two out of five sampling bias correction methods, i.e., (i) We conducted spatial filtering by creating a grid of 2 km x 2 km cell size and randomly select only one point of occurrence per grid cell. Nevertheless, it should be noted that the size of this grid is not the representation of approximate species' dispersal capabilities, but rather as a result of modifying the 10-km radius rule of spatial filtering proposed by Kramer-Schadt et al. (2013) and Boria et al. (2014). The grid creation and points selection were conducted using QuantumGIS software ver. 2.18.14 (QGIS Development Team 2017). (ii) Bias file was created and included it into Maxent modeling process through setting options (Dudik et al. 2005; Elith et al. 2010; Phillips

et al. 2017). Bias file is a probability surface represented by cell value that reflects the intensity of sampling effort across the area of study and provides a gradual weight to random background data used for modeling (Fourcade et al. 2014). Bias file can be artificially estimated using the aggregation of occurrences from closely related species (Phillips et al. 2009). Nevertheless, in real modeling situation, this information is limited. Therefore, by following Elith et al. (2010), we produced a Gaussian kernel density map of the occurrence locations, then rescaled it from 1 to 20 to be derived as bias file instead of using our knowledge to create artificial bias file (Fourcade et al. 2014). As the distribution of both species occurs in different countries (of different areas), we used the political state boundary extracted from Global Administrative Areas website (www.gadm.org/), to limit the background areas for the models.

Current environmental and bioclimatic variables

Environmental and bioclimatic variables to build the models in this study were selected based on the model-driven selection process. Model-driven selection is a selection process that will use all possible predictors and choose those with greatest importance in the model to be considered as the main factor influencing the distribution of species, rather than expert-driven selection where the expert priory will choose the predictors expected to directly affect the species distribution (Fischer 2011). For this study, on the basis of earlier screenings of related variables (Soria-auza 2009; Hu et al. 2015; Mod et al. 2016; Setyawan et al. 2017; Velazco et al. 2017), we collected 19 bioclimatic, two edaphic variables, and five environmental variables, which are expected to have direct effect on plant growth. We collected 19 bioclimatic layers ver 2.0 plus one altitude layer from WorldClim Bioclimatic datasets website (www.worldclim.org). The bioclimatic datasets were generated through interpolation of average monthly climate data from about 9,000 to 60,000 weather stations on a 30 arc-second resolution grid (often referred to as "1 km^2" resolution) (Fick and Hijmands 2017). We collected Global UVB radiation layers (UVB1, UVB2, UVB3, UVB4) from the glUV database (http: //www.ufz.de/gluv/) (Beckmann et al. 2014). Additionally, we collected global Soil pH (SpH) and soil organic carbon (SOC) datasets from the International Center for Tropical Agriculture (https://dataverse.harvard.edu). All of these layers were processed through several steps including resampling data, image cutting, and type file converting by using Qgis Software Ver. 2.18.14 (QGIS 2017). Variables that considered related to the occurrence of species, i.e., land use/land cover changes, human disturbances, and species dispersal or biotic interaction changes were not included as the availability of these data were limited.

Bioclimatic layers are highly correlated with each other, and although including all of the bioclimatic layers into modeling process will not affect the predictive quality of model greatly (Elith et al. 2011), it does, nonetheless, will significantly limit any inference of the contribution of any correlated variables since Maxent often excludes all other highly correlated variables from being incorporated (Van Gils et al. 2012, 2014). Therefore, we decided to remove highly correlated variables to minimize the effect of autocorrelation of climatic variables. SDM toolbox Ver. 2.0 (Brown 2014) in ArcGIS ver. 10.3 was used to perform the autocorrelation calculation and then we omitted the bioclimatic variables yielding correlation values above 0.95 (Spearman's rho coefficient) in the pairwise cross-correlation matrix of each dataset (intra-dataset correlations) (Bedia et al. 2013). The remaining six bioclimatic variables (i.e., bio_1, bio_2, bio_3, bio_4, bio_12, and bio_19), two edaphic variables (Soil pH and Soil Organic Carbon), plus five environmental variables (i.e., altitude, UVB1, UVB2, UVB3, and UVB4) were then compiled to be used as predictor variables in Maxent (Table 1).

Future climate scenarios

Future climate scenarios used to predict the impact of future climate change on the redistribution of *S. ciliaris* and *S. plana*'s suitable habitat, were acquired from CGIAR Research Program on Climate Change, Agriculture, and Food Security website (www.ccafs-climate.org). For this study, the HadGEM2-CC (Hadley Global Environment Model-2 Carbon Cycle) global circulation model, which was developed by the Hadley Center, United Kingdom was selected to build the models (Collins et al. 2011). HadGEM2-CC model has been used to perform all the CMIP5 (Coupled Model Inter-comparison Project Phase 5) centennial experiments including ensembles of simulations of the RCPs (Shrestha and Bawa 2014). We selected four future greenhouse gas (GHG) trajectories, which were represented by Representative Carbon Pathways (RCP), namely RCP 2.6, RCP 4.5, RCP 6.0, and RCP 8.5 for three different periods of time (2030, 2050, and 2080). RCP 2.6, the most optimistic projection, assumes that global GHG will increase slowly to reach its peak at 3.1 W/m^2 in between 2010-2020, with the emissions declining substantially thereafter to 2.6 W/m^2 by the year 2100 (van Vuuren et al. 2007; Moss et al. 2010). Emissions levelin RCP 4.5 is assumed to be stabilized at 4.5 W/m^2 by the year 2100 due to the variety of technology and strategies which predicted will be implemented to reduce GHG emissions level (Clarke et al. 2007). Likewise, the emissions level in RCP 6.0 is projected to reach its peak

Table 1. Environmental parameters used to build the models

Code	Name	Unit
Alt	Altitude	m asl
bio_1	Annual Mean Temperature	°C×10
bio_2	Mean Diurnal Range	°C×10
bio_3	Ishotermality	×100
bio_4	Temperature Seasonality	×100
bio_12	Annual Precipitation	mm
bio_19	Precipitation of Coldest Quarter	mm
soil_carbon	Soil Organic Carbon	
soil_ph	Soil pH	
UVB1	Annual Mean UVB	J m^{-2} day^{-1}
UVB2	UVB Seasonality	J m^{-2} day^{-1}
UVB3	Mean UVB of Lightest Month	J m^{-2} day^{-1}
UVB4	Mean UVB of Lowest Month	J m^{-2} day^{-1}

around 2080 and stabilizes in 2100 at 6.0 W/m^2. In RCP 8.5, emissions levelcontinue to increase throughout the 21st century, reaching around 8.5 W/m^2 as the highest level by the end of the century (Riahi et al. 2011). As the availability of future projection of environmental variables is currently limited, the six environmental variables (Soil pH, Soil Organic Carbon, UVB1, UVB2, UVB3, and UVB4) remained unchanged for the following ENM analysis under future climate projection. Furthermore, the same altitude layer was used since this variable is a static variable that does not change with time.

Global Climate Models (GCMs) have become the fundamental resource of information for constructing future climate scenarios and for developing impact assessments of climate change from local to global scale. Nonetheless, these climate models exhibit systematic error (biases) due to the simplified physics and thermodynamic processes, limited spatial resolution, and numerical schemes or incomplete knowledge of climate system processes (Ramirez-Villegas et al. 2013). Consequently, we implemented the bias correction data provided by CGIAR-CCAFS under three different calibration approaches : (i) Bias Correction, this approach revise the projected raw GCM output using the differences in the mean and variability between observations and GCM, in a reference period (Hawkins et al. 2013). (ii) Change Factor (CF): in this approach, the raw GCM outputs current values are subtracted from the future simulated values, resulting in "climate anomalies" which are then added to the present day observational dataset (Tabor and Williams 2010). (iii) Quantile Mapping (QM), this approach removes the systematic bias in the GCM simulations and account the biases in all statistical moments, however, like all statistical downscaling approaches, it is assumed that biases relative to historical observations will be constant in the projection period (Thrasher et al. 2012).

Model development

Developing the model of potential distribution of climatically suitable habitat for *S. ciliaris* and *S. plana* under current climate condition and assess its redistribution under the impact of projected future climate change scenario was conducted by using MaxEnt software ver. 3.4.1 (Phillips et al. 2017). Certainly, there is no "silver bullets" in correlative ecological niche modeling (Qiao et al. 2015), which means that there is no single algorithm approach that can provide robust, reliable, and acceptable results under all circumstances. Maxent software, however, utilized in this study as it has been proved to give the best results among other modeling algorithms available on the basis of presence-only (PO) data (Philips and Dudik 2008; Summers et al. 2012). Further, consideration to utilize Maxent in this study was the aim of this study which met the capability of Maxent to performs well in estimating the effect of climate change on the potential shifting range of species (Kou et al. 2011; Johnston et al. 2012; Duan et al. 2016), whereas more than 1000 published distribution modeling study has been conducted by using Maxent software since 2005 (Merow et al. 2013; Fourcade et al. 2014).

ENM/SDM using Maxent software are often confronted with a wide variety of modeling options, from choosing appropriate input datasets to choosing the right multiple settings available in the software package (Merow et al. 2013). As the aim of this study is beyond simple exploratory analysis, we tried to ensure that the modeling setting decisions are adjusted to our specific hypothesis, study aims, and species-specific considerations and reflect our intended a priori assumptions (Peterson et al. 2011; Araujo and Peterson 2012; Merow et al. 2013). The adjusted parameter values were: maximum iterations which were set to 5,000 for each run to allow the model to have adequate time for converging. Convergence threshold was set to 1 x 10^{-6}. The number of replicated runs was set to ten times (the averaged value is the one used as the result) using “cross-validate” as the replicated run type. Using "cross-validate" means to split the data ten times (10% per partition), train the model ten times on 90% of the data, and test it each time on the 10% partition alternately. To avoid over-fitting and assuming that both selaginellas are responded directly to the predictors (vs to correlated factors), we decided to "smooth" the model by choosing only hinge features to model both *S. ciliaris* and *S. plana*. Considering that we used a large collection of occurrence from diverse regions to be projected to different climate condition, we doubled the default "regularization multiplier" value to accommodate aforementioned type of data and aim of study (Elith et al. 2006; Merrow et al. 2013; Radosavljevic and Anderson 2013). We used the "projection" feature to extrapolate the model into different climate projection to predict the impact of projected future climate condition to the redistribution of climatically suitable habitat for both species (van der Wall et al. 2009).

Core distributional shifts

We tried to further examine the trend of suitable area changes by calculating and comparing the centroids of current and future suitable areas. We utilized a python-based GIS toolkit, SDM tool-box (Brown 2014) to summarize the core distributional shifts of the ranges of suitable habitat for both species in between two binary models (i.e., current and future SDMs). The tool will produce the centroids by calculating the average of latitude and longitude of binary input pixels, then depict their magnitude and direction of change (based on centers of the species ranges-the centroids). Assessment of core distributional shifts was conducted only on Java island for the following reasons: (i). SEA region has very wide areas, consists of several big archipelagic countries separated by seas, hence it is impractical to conduct core distributional shifts assessment in the whole region. (ii). Java island closely represents the vast variations in physiographic, topographic, edaphic, and climatic conditions of SEA region, therefore the results will closely depict the projected core shifts in the whole region. Furthermore, we used only projected future climate condition in the year 2080 to represent maximum shifts of the geometric distribution center.

Data analysis

The main output of Maxent software is predictive map which represents the distribution of potential ecology niche of species across the study area. The degrees of potential suitable are linearly scaled between 0 (lowest) to 1 (highest) probability (Philips and Dudik 2008). Additionally, Maxent software will calculate the variables' relative contribution to the model and quantify the degree of these variables affect the prediction. We also retrieve the alternate estimation of variable importance by running the jackknife test. Jackknife test show which variable appears to have the most information that is not present in the other variables and which variable have the most useful information by itself (Phillips et al. 2006). The predictive maps, which by default are in ASCII format, were further analyzed using QuantumGIS software ver. 2.18.14 (QGIS Development Team 2017). To allow us to compare and quantify the geographical distribution of predicted suitable habitat, we applied the binary calculation, categorized the value into two categories (i.e. suitable vs unsuitable) using the selected threshold rule. Selecting the threshold rule is one of the many sources of bias that should be minimized by Maxent user (Phillips and Dudik 2008; Nenzen and Araujo 2011; Bean et al. 2012; Syfert et al. 2013). In the process of selecting threshold rule, one should avoid arbitrariness and should consider the relative importance difference between commission and omission error (Phillips and Dudik 2008; Nenzen and Araujo 2011; Bean et al. 2012). Norris (2014) in his study proposed the "minimum training presence" or "fixed cumulative value 1" to be the most appropriate threshold rule, considering that reducing omission error is more important determinant than reducing commission error. However, Liu et al. (2016) stated that the threshold rule proposed by Norris (2014) may be more convenient for rarer species, but when considering a more common species, commission error should be weighted more than omission error. Accordingly, we selected "maximum training sensitivity plus specificity" threshold rule since this rule will produce lower commission error.

To evaluate model performance, as used by several studies (e.g., Pearson and Dawson 2003; Pearson et al. 2007; Jiménez-Valverde 2012), Maxent software will calculate an area under the receiver operating characteristic (ROC) Curve (AUC). AUC value is ranged between 0 (lowest value) to 1 (highest value), wherein value between 0-0.5 represents that the model is no better than random prediction, value below 0.7 is low, value between 0.7-0.9 is good, and value above 0.9 is indicating high discrimination or means that the model is far better than random prediction. However, studies conducted by Lobo et al. (2008); Bahn and McGill (2013); and Aguirre-Gutiérrez et al. (2013) proved that AUC value does not provide useful information to assess and/or to evaluate the SDM performance. Therefore, for this study, we conducted True Skill Statistic (TSS) (also known as the Youden index) calculation as an additional measurement to evaluate the performance of the model (Youden 1950; Allouche et al. 2006).

RESULTS AND DISCUSSION

Contribution of the variables and model evaluation

Based on our known occurrences of *S. ciliaris* and *S. plana* combined with climatic, topographic, edaphic, and UVB radiation data, we generated geographic distribution maps predicting areas wherein both species can live in concordance with all the aforementioned variables. Our models demonstrated that the variable which provides the highest relative contribution to explain the predicted geographic distribution of both *S. ciliaris* and *S. plana*'s suitable habitat in SEA region is similar (Table 2). Isothermality (bio_3) was the highest relative contributor to the distribution pattern of the models, with a contribution of 28.5% and 39.4% for *S. ciliaris* and *S. plana* respectively. Combined variables of soil organic carbon, UVB2, and temperature seasonality (bio_4) explained in total of 36.5% of the variation in the distribution pattern of *S. ciliaris*' suitable habitat, whereas the remaining variables, each contributed less than 10% to the model. Another variable significantly contributed to the model of *S. plana* were temperature seasonality (bio_4), UVB2, and soil organic carbon which in total had a relative contribution of 36.9%. Others, appeared to had a little contribution to this model with only less than 25% contribution in total (Table 2).

Additionally, we retrieved the alternate estimation of variable importance through the utilization of jackknife test. The results showed that for both *S. ciliaris* and *S. plana*'s model, the environmental factors with the highest gain when used in isolation is isothermality (bio_3), which therefore appears to have the most information by itself (Phillips et al. 2006). These results confirmed to the previous result that the same bioclimatic factor has the highest relative contribution to the models. Nevertheless, the results of jackknife test showed a different finding of which factor which will reduce the gain the most when it is omitted. Annual precipitation (bio_12) appears to have the most information that is not present in the other variables, thus, omitting this variable will decrease the fitness of *S. ciliaris*' model. For *S. plana*'s model, isothermality (bio_3) variable was both the highest gain when used in isolation and decrease the gain highest when it is omitted from the model, which indicates that bio_3 variable has the most useful information which is not present in the other variables (Figure 3).

To assess predictive performance and statistical significance of the models, a post-hoc evaluation of distribution models is commonly performed (Peterson et al. 2011). Despite the fundamental problems when using AUC (Area Under the Curve) for model evaluation, we retrieved the AUC value of 0.946 for *S. ciliaris* model and AUC value of 0.978 for *S. plana* model to illustrate that the predictions in this study perform better than any model with a set of random predictors (Lobo et al. 2008; Fourcade et al. 2017). Furthermore, we conducted additional evaluation of the models using True Skill Statistic, which has been proposed as an alternative metric of evaluation (e.g., Allouche et al. 2006; Hijmans 2012; Phillips and Elith 2010). The TSS value of 0.83 and 0.86 for *S. ciliaris*

and *S. plana*'s models respectively, give the impression that the models built in this study have a good degree of agreement and also have a good predictive capacity (Li and Guo 2013). Studies had also demonstrated the use of Kappa statistic for Maxent validation (e.g. Duan et al. 2014; Ali and Hossein 2016; Bagheri et al. 2017), but, regarding the use of Kappa value, it is highly correlated to prevalence of the locality points and the size of the study area (Lobo et al. 2008; Fourcade et al. 2017). Therefore, it would generate some sort of bias or misunderstanding. Moreover, due to the fact that both AUC and Kappa are weighting omission and commission errors equally (Allouche et al. 2006; Lobo et al. 2008; Jimenez-Valverde 2012, 2014; Fourcade et al. 2017), Kappa, just like AUC, is more reliable if it is applied in PA (Presence-Absence) model. Consequently, in case of this study where presence only data were used, we assume that the use of TSS is more suitable than Kappa statistic.

Predicted distribution of current potential habitat

We built the models by using 748 unique locality points of *S. ciliaris* and 598 locality points of *S. plana*, which were the remaining points after the implementation of spatial filtering to reduce bias sampling (see method). The potential present-day distribution of suitable habitat for both species, as derived from Maxent (Phillips and Dudik 2008; Elith et al. 2011; Phillips et al. 2017), are shown in Figure 1. Our models predicted roughly 26% (1,361,050.9 km^2) of the SEA region is suitable for *S. ciliaris*. In Mainland SEA area, the predicted suitable habitat spread patchily in southern part of Myanmar, Cambodia, and Vietnam, with a wide predicted suitable area in Peninsular Malaysia and Singapore. While in Maritime SEA, the predicted suitable habitat for *S. ciliaris* spread widely in all of big islands of Indonesia (Sumatra, Java, Sulawesi, Borneo, and Papua), and also appears in Lesser Sunda islands. Additionally, the predicted suitable habitat also appears in most of the Philippines archipelago. Moreover, our model predicted there are approximately 18% (871,889.51 km^2) of *S. plana*'s suitable habitat in SEA region, spread in mainland SEA almost at the same area as the suitable habitat for *S. ciliaris* (i.e. southern of Myanmar, Cambodia, Vietnam, and in most area of Peninsular Malaysia). In the maritime SEA, the predicted suitable areas spread across big islands of Indonesia (mostly in the southern part of Sumatra, Borneo, Sulawesi, Java, and Papua). Additionally, the predicted suitable habitat of *S. plana* also appears in most of the southern part of the Philippines archipelago.

Potential future changes in the distribution of suitable habitat

The predicted redistribution of suitable habitat for both species as the impact of climate change is illustrated in Figure 4. Overall, under all RCP scenarios in three different periods of time, the suitable areas were predicted to significantly decreased, even though there is also a significant increase in areas wherein predicted to become suitable for both species as a result of a warming climate condition in the future. Under the influence of RCP 2.6 climate projection (lowest GHG emission) in the year 2030, Maxent predicted roughly 2.6% gains of the currently suitable habitat area for *S. ciliaris*. Expansions in area increased with increasing latitude and elevation, and predicted will occur in the western and northern part of Sumatra, southern part of Peninsular Malaysia and Philippines archipelago, northern and southern part of Sulawesi, and southwestern part of Papua. Furthermore, for the next four decades, until the end of 2080, the predicted losses of suitable habitat area are greater than the gains. Maxent predicted a total of 0.6% and 2.1% reduction of current suitable area in the year 2050 and 2080 respectively. The losses were predicted to occur mostly in the lower altitude area of southern Vietnam and Sumatra . Likewise, the predicted suitable habitat for *S. plana*, under the same RCP 2.6 climate trajectory, will likely to increase at about 2.1% in 2030 before continuously losing its suitable area to reach a decrease of ca. 2.9% of the current suitable area by the end of 2080. The pattern of losses and gains of suitable habitat for *S. plana* is almost the same as the pattern of losses and gains of *S. ciliaris*' suitable habitat (Table 3, Figure 4).

Under the future climate scenario of RCP 4.5, Maxent software also predicted a slight gain in both suitable habitat area for *S. ciliaris* and *S. plana* at almost the same pattern. The areal extent of gains were predicted to appear in southern Peninsular Malaysia, northern part of Sumatra, and in the eastern part of Papua, which amounted to 0.24×10^5 km^2 (1.7%) and 0.06×10^5 km^2 (0.7%) for *S. ciliaris* and *S. plana*'s suitable habitat, respectively (Table 3, Figure 4). Furthermore, the predicted suitable habitat areas for *S. ciliaris* and *S. plana* in the year 2050 and 2080 were predicted to be about 1.6-2.7% less than the currently suitable habitat areas (Table 3). The predicted suitable area under RCP 6.0 was projected to be more decreased than under former RCP trajectory. Under this GHG emission trajectory, in all three different time periods (2030, 2050, and 2080), the predicted suitable area for *S. ciliaris* will gradually to decline by about 0.3-4.5% of currently suitable habitat and about 0.2-11.1% of current *S. plana*'s suitable habitat will be lost.

Table 2. Percentage of variable contribution to the final model

Variables	Description	Contribution (%)	
		S. ciliaris	*S. plana*
Alt	Altitude	1.5	3.4
bio_1	Annual Mean Temperature	0.5	0.2
bio_2	Mean Diurnal Range	7.2	5.8
bio_3	Ishotermality	28.5	39.4
bio_4	Temperature Seasonality	10.2	13.3
bio_12	Annual Precipitation	8.9	8.1
bio_19	Precipitation of Coldest Quarter	4.3	0.4
soil_carbon	Soil Organic Carbon	15.3	11
soil_ph	Soil pH	6.2	4.8
UVB1	Annual Mean UVB	0.2	0.3
UVB2	UVB Seasonality	11	12.6
UVB3	Mean UVB of Lightest Month	0.5	0.1
UVB4	Mean UVB of Lowest Month	5.4	0.7

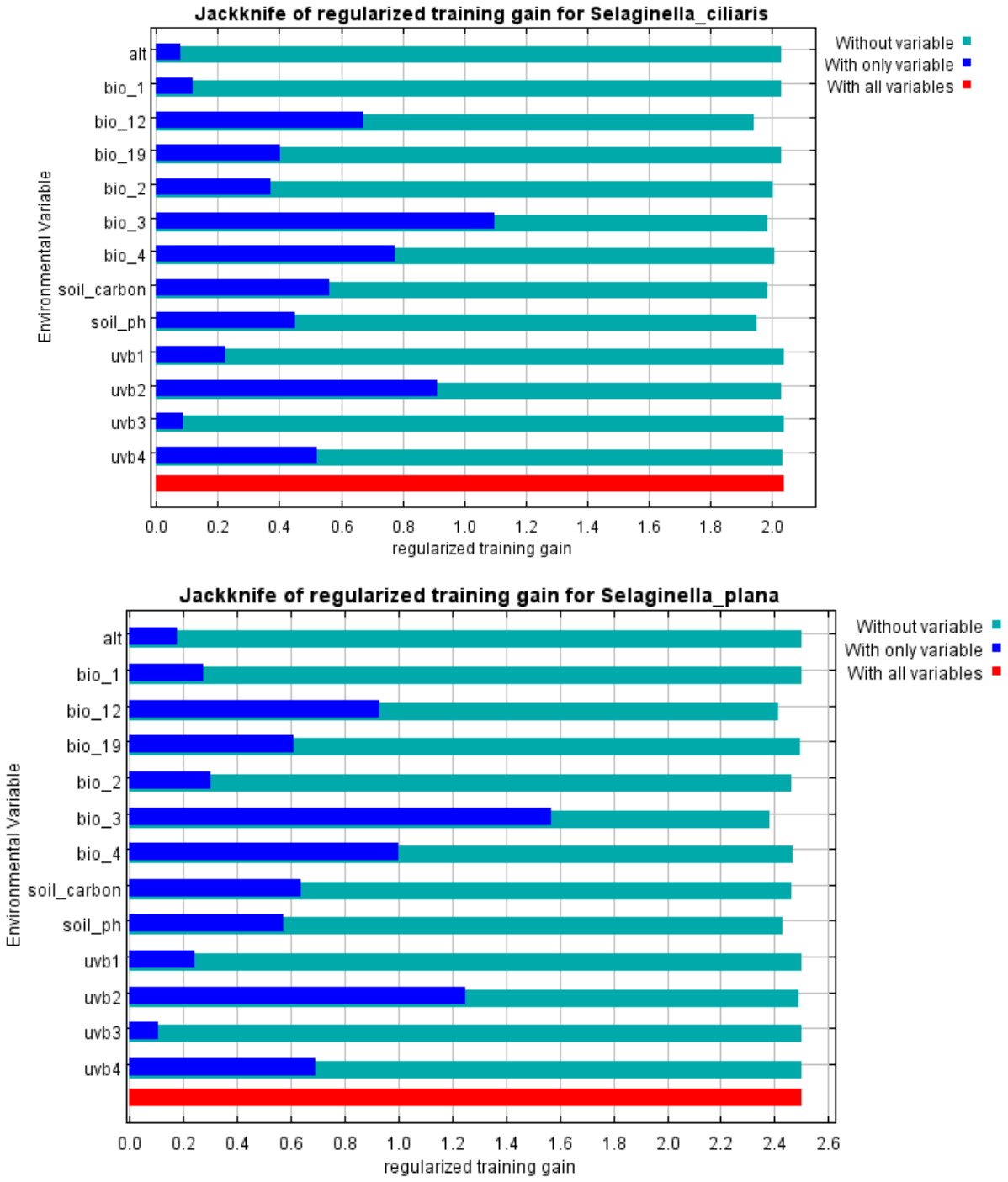

Figure 3. Results of jackknife test of relative importance of predictor variables for *Selaginella ciliaris* and *Selaginella plana*

The biggest reduction of suitable area was predicted to happen under RCP 8.5 trajectory. In 2030, there will be a significant decrease of approx. 1.2% and 0.4% of suitable habitat area for *S. ciliaris* and *S. plana* respectively. Moreover, approximately 1.6% of *S. ciliaris* and 2.3% of *S. plana*'s suitable habitat area will vanish by the end of 2050. For the next three decades, the predicted suitable habitat will continue to decrease, and predicted to affect more on the sustainability of suitable habitat for *S. plana* than for *S. ciliaris* By the end of 2080, approx. 14.4% of currently suitable habitat for *S. plana* will be lost, whereas only about 6.6% of *S. ciliaris*' suitable habitat area will vanish under the effect of this future climate trajectory. During all of the aforementioned periods of time, indeed there are also gained areas which were predicted to become suitable habitat for both species. However, the predicted losses of suitable area are greater than the gains (Table 3).

Table 3. Dynamics of changes in suitable habitat area for *Selaginella ciliaris and Selaginella plana* under four combinations of future climate scenario within three different periods of time

Year	RCP Projection	*S. ciliaris* (Area × 10^5 km^2)				*S. plana* (Area × 10^5 km^2)			
		Loss	Gain	Total	Future	Loss	Gain	Total	Future
2030	RCP 2.6	0.92	1.51	0.59	14.2	0.95	1.14	0.19	8.9
	RCP 4.5	0.9	1.6	0.7	14.31	2.25	2.31	0.06	8.77
	RCP 6.0	1.16	1.2	0.04	13.65	1.48	1.49	0.01	8.72
	RCP 8.5	1.56	1.4	-0.16	13.49	1.38	1.35	-0.03	8.68
2050	RCP 2.6	0.4	0.37	-0.03	13.58	1.06	1.02	-0.04	8.67
	RCP 4.5	0.51	0.42	-0.09	13.52	1.7	1.62	-0.08	8.63
	RCP 6.0	0.63	0.52	-0.11	13.5	0.94	0.82	-0.12	8.59
	RCP 8.5	1.06	0.83	-0.23	13.38	1.06	0.87	-0.19	8.52
2080	RCP 2.6	0.8	0.51	-0.29	13.32	1.2	0.94	-0.26	8.45
	RCP 4.5	0.78	0.41	-0.37	13.24	1.46	0.97	-0.49	8.22
	RCP 6.0	1.65	1.03	-0.62	12.99	1.9	0.93	-0.97	7.74
	RCP 8.5	2.69	1.82	-0.87	12.74	2.6	1.34	-1.26	7.45

Note: - = Negative mark indicates suitable habitat area contractions

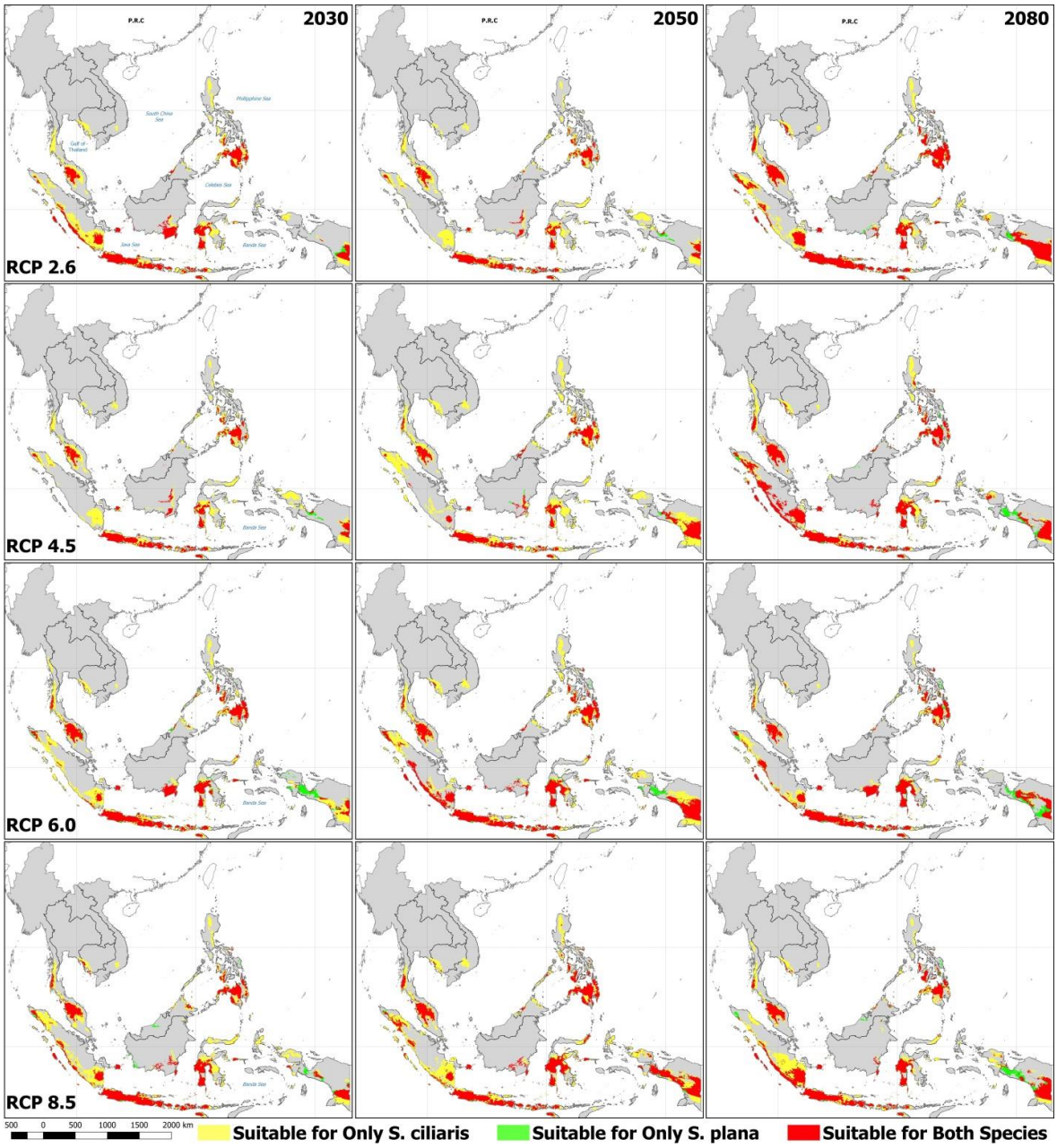

Figure 4. Redistribution of climatically suitable habitat under future climate projections

Core distributional shifts

Given the aforementioned reasons over why core distributional shifts assessment was conducted only on Java, we may look first into the predicted distribution of both species' suitable habitat in this particular island. Under current climate condition, it has been predicted that there are approx. 55,676.4 km^2 (41.5%) suitable areas for *S. ciliaris* and about 45,500.1 (33%) km^2 areas are suitable habitat for *S. plana*. These numbers were predicted to gradually decreasing as future climate change altering the habitat capability to support the survival of both *S. ciliaris* and *S. plana*. In the year 2080, under all of the GHG emission trajectories, current suitable habitat area for *S. ciliaris* and *S. plana* will decrease by up to 11% and by up to 19% respectively. Furthermore, redistribution of predicted suitable habitat for both species, under future climate condition, will also alter its geometric distribution core. The centroid of the currently suitable habitat for *S. ciliaris* was located at the position of 109.786E longitude and 7.313S latitude in Central Java (Figure 1.B). The centroid of future suitable area under RCP 2.6 was predicted to shift marginally to west direction to the position of 109.760E, 7.310S. The shift under the RCP 4.5, RCP 6.0, and RCP 8.5, show a greater extent wherein the centroid shift about 26.13 km to 37.32 km to west direction at the position of 109.446E, 7.263S under RCP 4.5, 109.547E, 7.312S under RCP 6.0, and 109.540, 7.277S under RCP 8.5. Likewise, major shift of currently suitable habitat centroid of *S. plana* has been predicted to occur under the RCP 4.5, RCP 6.0, and RCP8.5 whereas under the influence of RCP 2.6, the shift is relatively small. Under the RCP 2.6 the centroid predicted to shift to the north at the position of 109.579E, 7.300S, which is about 2.5 km from its original position at the position of 109.579E, 7.324S. Under the influence of other RCPs, the centroid shift to west direction about 18.76 to 49.78 km from its original position. The farthest shift of suitable habitat centroid is under the RCP 8.5 at the position of 109.139E, 7.231S. The new centroid position under RCP 4.5 and RCP 6.0 are 109.409E, 7.305S, and 109.165E, 7.254S, respectively. Overall, there is tendency of centroid shifting to the western side of the island under all future RCP trajectories, and the weakest shift of suitable habitat core of both species is always under the influence of RCP 2.6 (Figure 1.B).

Discussion

Recently, only a few studies attempt to model the impact of climate change on the sustainability of autotrophic non-vascular cryptogams (e.g., Cornelissen 2007; Ellis et al. 2007). The number is even less for study which focuses on the particular genus such as *Selaginella* (e.g., Setyawan et al. 2017). Nonetheless, several studies have reported that the sustainability of *Selaginella*, as a member of biotic component of vegetation, is also predicted to be affected by any measured changes in climate both in the past condition and in the projected of future condition (e.g. Muller et al. 2003; An et al. 2005; Trivedi et al. 2008; Cao et al. 2010; Xu et al. 2010). Indeed, discrepancies may occur between different climate modeling system used in the preceding studies (Cheaib 2012), but the approaches nevertheless can be functioning as an important research tool for assessing and predicting the effect of both current and future climate condition on the distribution of suitable habitat for, especially, genus of *Selaginella*.

Selaginella ciliaris is predicted to has a wide but fragmented distribution in the southern part of mainland SEA region (South Vietnam, Cambodia, Myanmar, Peninsular Malaysia, and Singapore) and in most of the big islands in maritime SEA. The model prediction is in agreement with past and recent years studies reported its occurrences in Vietnam (Thin 1997; Costion and Lorence 2012), Cambodia (Spring 1843; Zhang et al. 2013; Rundel and Middleton 2017), Myanmar (Spring 1843; Winter and Jansen 2003; Chang et al. 2012), Peninsular Malaysia and Singapore (Hanum and Hamzah 1999; Yusuf et al. 2003; Tan et al. 2014), Philippines (Barcelona 2003; Tan 2013), Sumatra (Spring 1843; Iwatsuki 1973: Wardani and Adjie 2017), Borneo (Spring 1843; Iwatsuki and Kato 1981; Said 2005). Sulawesi (Spring 1843), Java (Setyawan 2009; Setyawan 2012), and Papua (Johns et al. 2012; Gartmann 2015). Likewise, the predicted distribution of *Selaginella plana*'s suitable habitat has almost the same pattern as the predicted suitable habitat for *S. ciliaris*. Several documents and studies had also reported the occurrence of *S. plana* in Vietnam (Spring 1843; Chang et al. 2012), Cambodia (Spring 1843; Chang et al. 2012), Myanmar (Chang et al. 2012; Parveen et al. 2017), Peninsular Malaysia and Singapore (Turner et al. 1998; Chua et al. 2005; Bedawi et al. 2009), Philippines (Alston 1935; Zamora et al. 1999; Tan 2013; De Guzman et al. 2014), Sumatra (Sauerborn 2003; Beukema and Noordwijk 2004), Borneo (Said 2005; Ahmad and Holdsworth 2008; Komara et al. 2016), Sulawesi (Mansur 2003; Hidayat 2011), Java (Rahayu et al. 2012; Setyawan et al. 2013; Setyawan et al. 2015a;b; Setyawan et al. 2016; Trimanto and Hapsari 2016), and Papua (Sambas et al. 2003; Ebihara et al. 2012; Johns et al. 2012).

Based on the modeling results, constancy and stability of temperature (isothermality and temperature seasonality) are among the most important factors affecting the distribution of both *S. ciliaris* and *S. plana*. Isothermality (bio_3) is defined as the quantification of how large the diurnal temperature range oscillate with annual temperature oscillations, while temperature seasonality (bio_4) is defined as a measure of temperature change over the course of the year (O'Donnell and Ignizio 2012). Past studies confirmed the importance of stability of temperature in preserving the survival of genus *Selaginella*. Temperature, allegedly affect both the photosynthetic capability and preservation of photosynthetic apparatus of *Selaginella* (Jagels 1970, Eickmeier 1986). Additionally, water availability which was measured in annual precipitation is also among the most important factors affecting the distribution of both species. Water availability is correlated with many environmental factors that influence the biochemical and physiological processes of plants (e.g. Platt et al. 1994; Wang et al. 1998; Rusala et al. 2011). Therefore, these hydrothermal factors may have played

main roles in shaping the ecological adaptation and the distribution pattern of both *S. ciliaris* and *S. plana*. Moreover, these results also indicate that both *S. ciliaris* and *S. plana* appear to grow well in a highly isothermal environment and with low variability of temperature.

The intensity of UV radiation also predicted to have a major role in shaping the distribution range of both *S. ciliaris* and *S. plana*. Generally, UVB radiation has a great effect on the sub-aerial organs of plants (Yang et al. 1994). Plants species subjected to elevated UVB reveal that UVB radiation affects plants morphology by inhibiting leaf area expansion and stem elongation (Caldwell et al. 1998). UVB radiation also influences the protective mechanism of plants (Bellare et al. 1995; Márquez-Escalante et al. 2006) and decreases photosynthetic activity (Jagels 1970; Battaglia et al. 2000). Another environmental factors, such as increased CO_2 concentration, water stress, and availability of nutrients interact with this form of radiation (Wu et al. 2009), which in turn affect the plant response to the changes in environmental parameters (Caldwell et al. 1998; Teklemariam and Blake 2003; Qaderi and Reid 2005). Past studies on several *Selaginella* species also confirmed that net photosynthesis, stress regulation mechanism, and local distribution are closely related to the component of light source (Jagels 1970; Eickmeier 1979; Márquez-Escalante et al. 2006). However, further specific information on effects of UVB radiation on the changes in biochemistry and physiology of *Selaginella* is limited, hence future studies regarding these subjects are recommended.

Future climate condition in SEA region has been predicted will significantly disturb the distribution of suitable habitat of *S. ciliaris* and *S. plana*, and alter its geographical distribution pattern. Despite there are some gained areas which were predicted to become suitable habitat in the early period of future climate change, overall projection shows a negative effect of future climate condition on the distribution of *S. ciliaris* and *S. plana*'s suitable habitat; as the predicted losses of suitable habitat will be greater than the gains. Under the lowest and medium GHG emission projection (RCP 2.6, RCP 4.5 and RCP 6.0); wherein radiative forcing will gradually rise up before it stabilizes at the certain figure by 2100 (Meinshausen et al. 2011; IPCC 2014), annual mean temperature will rise up to about 1.7-5°C in all areas of SEA region. Unlike in the case of temperature changes, the changes in precipitation will not be equivalent in all of SEA region areas. There will be both areas wherein the amount of precipitation shows an increasing tendency by up to 15% of current annual precipitation rate (Northern Philippines, Myanmar, and Laos) and areas wherein the amount of precipitation will tend to decrease by about 10% (e.g., southern Indonesia, Thailand, Laos, and Myanmar) by the end of 21st century (IPCC 2014). This condition predicted leads to a slight increase of *S. ciliaris* and *S. plana*'s suitable habitat area by the end of 2030. The gains are mostly predicted to occur in a higher latitude area, as future climate increases its probability to support the existence of both species. However, for the next five decades, as climate continues to change, these figures will gradually to decrease. The same negative trend will also predict to occur under the worst GHG emission scenario (RCP 8.5), with no gained area will appear under this scenario in all periods of time. Core distributional shifts assessment indicates that there will be upward shifts to higher elevation area as the atmosphere warms, which is in line with certain studies that predicted a shift of forest ecosystems to a higher altitude (e.g. Walther et al. 2005; Bertrand et al. 2011). Increased temperature and occurrence of severe drought, as indicated by precipitation variability, should increase plant stress in some years (Kelly and Goulden 2008). Thus, expected to decrease the species' ability to survive in the drier, warmer, lower parts of its range (Allen and Breshears 1998; Lenoir et al. 2008a,b) and increase its competitive ability and tolerance in the wetter, cooler, upper parts of its range (Parmesan and Yohe 2003; Parmesan 2006).

Generally, plant species may migrate to higher elevations and latitude as its mechanism to cope with the changes in climate condition (Lenoir et al. 2008a; Bertrand et al. 2011). However, the trends may differ between narrowly distributed plant species and widely distributed plant species. Plants with narrow distribution usually have a constrained capability of ecological adaptation, and are more vulnerable to the impact of climate change, whereas plants with wider distribution tend to have broader adaptability and have a stronger resistibility to climate change (Hu et al. 2015). This tendency, is what the models have predicted in this study, wherein the distribution of suitable habitat for both species is increased at first, but then began to decrease as climate change intensified. Several studies have also reported the early sign of plants migration into higher altitude areas under the effect of changes in climate condition (e.g., Zhang et al. 2001; Parmesan and Yohe 2003; Root et al. 2003; Leng et al. 2008; Lenoir et al. 2008a). Additionally, an attempt of evaluating the impact of climate change on the distribution of suitable habitat for both species, should also incorporate anthropogenic factors such as deforestation activity which will be resulting in fragmentation and shrinkage of habitat area. The results of this study may suggest that both *S. ciliaris* and *S. plana* have a medium degree of vulnerability to the impact of climate changes, nonetheless, under the influence of human-induced land conversion, the loss of suitable habitat for both species will be greater than expected. Therefore, more studies are needed to quantify and qualify the future anthropogenic impacts on the sustainability of *S. ciliaris* and *S. plana*.

The maps, presented in this study, depict the predicted distribution of suitable habitat for both species, which were built by using climate, topography, edaphic, and UVB radiation variables. Nonetheless, it must be taken into account that, like most of the ENM, the "predicted" distribution of suitable habitat does not represent the "true" prediction of the distribution of species eco-physiologically, but rather the prediction of the distribution of "suitable" habitat based only on the aforementioned predictors. Therefore, in the predicted suitable area, the species may not actually exist. There are also several assumed reasons for the absence of species in the predicted

area, i.e. (i) Micro-climate variation affect the existence of species in the predicted areas, but were not included in the model as a result of limited availability of data. (ii) The weak resolution of the recorded environmental variables has not yet capable of represents the unique environmental condition that greatly drives the probability of the occurrence of species. (iii) Human-induced changes that causing the predicted areas are no longer habitable for the species (e.g. deforestation, construction activity, etc.). Moreover, omission error may also occur as a result of occurrence data which were supplied into the models did not represent all the varieties of environmental condition that can sustain the existence of species. Despite all of bias correction methods which were carefully applied to achieve greater quality of models, these possible mismatches between the models and real-life situation may still occur. Nevertheless, we may acknowledge the result of the model as an appropriate representation of how the current climate condition shapes the distribution of suitable habitat for *S. ciliaris* and *S. plana*, and its predicted redistribution under the effect of future climate change.

Building an ideal model requires the availability of multiple compounding factors which are expected to have either direct or indirect effect on the target species and its associated biota. However, such ideal packages of data are currently limited. This limitation in the availability of more detailed ecological and physiological data prevents the construction of more ideal models (Morin and Thriller 2009; Sinclair et al. 2010; Ellis 2011). Nevertheless, recent development of new climate models and the refining of current models provide opportunity to build more precise and ideal model. Further modeling attempt should also incorporate potential human-induced land use/land cover changes, biotic interactions between species in the regional ecosystems, more detailed ecological data, and better presence data which accurately represent the variability of ecological niche of species. Despite all of these limitations, this study provides the baseline of understanding the potential effect of climate change on the distribution of predicted suitable habitat for *S. ciliaris* and *S. plana*. Using different technique of species distribution modeling, such as profile technique (e.g. DOMAIN, ENFA) and Regression-based technique (e.g. GLM, GAM, and MARS), may present slightly different quantitative results. Nonetheless, we believe that by using currently available resources of data, the overall trend and projection results would be similar. Therefore, it is concluded that the sustainability of *S. ciliaris* and *S. plana* potentially will negatively be influenced by all of the scenarios of future climate condition presented in this study.

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