NUSANTARA BIOSCIENCE

ISEA JOURNAL OF BIOLOGICAL SCIENCES

| Nusantara Biosci | vol. 13 | no. 1 | pp. 1-145 | May 2021 |
| ISSN 2087-3948 | E-ISSN 2087-3956|

Cordia africana photo by Max Seyfried

Society for
Indonesian Biodiversity

Sebelas Maret University
Surakarta

Published semiannually

PRINTED IN INDONESIA

ISSN 2087-3948 E-ISSN 2087-3956

NUSANTARA
BIOSCIENCE
| Nusantara Biosci | vol. 13 | no. 1 | pp. 1-145 | May 2021 |
| ISSN 2087-3948 | E-ISSN 2087-3956|
ISEAJournal of Biological Sciences

NUSANTARA
BIOSCIENCE
ISEAJournal of Biological Sciences
| Nusantara Biosci | vol. 13 | no. 1 | pp. 1-145 |May 2021 |
|ISSN 2087-3948| E-ISSN 2087-3956|

PUBLISHER: Society for Indonesian Biodiversity

CO-PUBLISHER: School of Graduates, Sebelas Maret University, Surakarta

FIRST PUBLISHED: 2009

ADDRESS:
Bioscience Program, School of Graduates, Sebelas Maret University
Jl. Ir. Sutami 36A Surakarta 57126, Indonesia. Tel. & Fax.: +62-271-663375, email: editors@smujo.id

ONLINE: smujo.id/nb

List of reviewers: https://smujo.id/nb/reviewers

Society for Indonesian Biodiversity **Sebelas Maret University Surakarta**

Published by Smujo International for The Society for Indonesian Biodiversity and Sebelas Maret University Surakarta

GUIDANCE FOR AUTHORS

Aims and Scope ***Nusantara Bioscience*** (Nusantara Biosci) encourages submission of manuscripts dealing with all aspects of biological sciences that emphasize issues germane to biological and nature conservation; especially for the research conducted in the Islands of the Southeast Asian reign or Nusantara, but also from around the world.

Article types The journal seeks original full-length: (i) **Research papers**, (ii) **Reviews**, and (iii) **Short communications**. Original research manuscripts are limited to 8,000 words (including tables and figures) or proportional to articles in this publication number. Review articles are also limited to 8,000 words, while Short communications should be less than 2,500 words, except for pre-study.

Submission: The journal only accepts online submissions through the open journal system **(https://smujo.id/nb/about/submissions)** or, for login problems, email the editors at unsjournals@gmail.com (or editors@smujo.id). Submitted manuscripts should be the original works of the author(s). Please ensure that the manuscript is submitted using the template, which can be found at (https://biodiversitas.mipa.uns.ac.id/D/template.doc). The manuscript must be accompanied by a cover letter containing the article title, the first name and last name of all the authors, and a paragraph describing the claimed novelty of the findings versus current knowledge. Please also provide a list of five potential reviewers in your cover letter. They should come from outside your institution and better from three different countries. Submission of a manuscript implies the submitted work has not been published (except as part of a thesis or report, or abstract) and is not being considered for publication elsewhere. When a group writes a manuscript, all authors should read and approve the final version of the submitted manuscript and its revision; and agree on the submission of manuscripts for this journal. All authors should have made substantial contributions to the concept and design of the research, acquisition of the data and its analysis, drafting the manuscript, and correcting the revision. All authors must be responsible for the work's quality, accuracy, and ethics.

Ethics Author(s) must be obedient to the law and/or ethics in treating the object of research and pay attention to the legality of material sources and intellectual property rights.

Copyright If the manuscript is accepted for publication, the author(s) still hold the copyright and retain publishing rights without restrictions. For the new invention, authors must manage its patent before publication.

Open Access The journal is committed to free-open access that does not charge readers or their institutions for access. Readers are entitled to read, download, copy, distribute, print, search, or link to the full texts of articles, as long as not for commercial purposes. The license type is CC-BY-NC-SA.

Acceptance Only articles written in US English are accepted for publication. Manuscripts will be reviewed by editors and invited reviewers (double-blind review) according to their disciplines. Authors will generally be notified of acceptance, rejection, or need for revision within 1 to 2 months of receipt. Manuscripts will be rejected if the content does not align with the journal scope, does not meet the standard quality, is in an inappropriate format, or contains complicated grammar, dishonesty (i.e., plagiarism, duplicate publications, fabrication of data, citations manipulation, etc.), or ignoring correspondence in three months. The primary criteria for publication are scientific quality and significance. **Uncorrected proofs** will be sent to the corresponding author by system or email as .doc or .docx files for checking and correcting typographical errors. The corrected proofs should be returned in 7 days to avoid publication delays. The accepted papers will be published online in chronological order at any time but printed at the end of each month.

Charges Publishing costs waiver is granted to foreign (non-Indonesian) authors who first publish the manuscript in this journal. However, other authors are charged USD 150 (IDR 2,100,000). **Reprint** Authors or other parties may freely download and distribute. However, a printed request will be charged, especially regarding postal charges.

Manuscript preparation Manuscript is typed on A4 (210x297 mm^2) paper size, in a single column, single space, 10-point (10 pt) Times New Roman font. The margin text is 3 cm from the top, 2 cm from the bottom, and 1.8 cm from the left and right. Smaller lettering sizes can be applied in presenting tables and figures (9 pt). Word processing program or additional software can be used; however, it must be PC compatible, use the template, and be Microsoft Word based (.doc or .rtf; not .docx). **Scientific names** of species (incl. subspecies, variety, etc.) should be written in italics, except in italicized sentences. Scientific names (genus, species, author) and cultivar or strain should be mentioned completely for the first time mentioning it in the body text, especially for taxonomic manuscripts. The genus name can be shortened after the first mention, except in early sentences, or where this may generate confusion; name of the author can be eliminated after the first mention. For example, *Rhizopus oryzae* L. UICC 524 can be written hereinafter as *R. oryzae* UICC 524. Using trivial names should be avoided. **Biochemical and chemical nomenclature** should follow the order of the IUPAC-IUB. For DNA sequences, it is better to use Courier New font. Standard chemical abbreviations can be applied for common and clear used, for example, completely written butilic hydroxyl toluene (BHT) to be BHT hereinafter. **Metric measurements** should use IS denominations, and other systems should use equivalent values with the denomination of IS mentioned first. A dot should not follow abbreviations like g, mg, mL, etc. Minus index (m^{-2}, L^{-1}, h^{-1}) suggested being used, except in things like "per-plant" or "per-plot." **Mathematical equations** can be written down in one column with text; in that case, they can be written separately. **Numbers** one to ten are written in words, except if it relates to measurement, while values above them are written in number, except in early sentences. The fraction should be expressed in decimal. In the text, it should be used "%" rather than "percent." Avoid expressing ideas with complicated sentences and verbiage/phrasing, and use efficient and effective sentences.

The title of the article should be written in compact, clear, and informative sentence, preferably not more than 20 words. Name of author(s) should be completely written, especially for the first and the last name. **Name and institution** address should also be completely written with street name and number (location), postal code, telephone number, facsimile number, and email address. We choose local names in Bahasa Indonesia for universities in Indonesia. The mention of "strata" program, should be avoided. Manuscript written by a group, author for correspondence along with address is required (marked with "♥"). **The title page** (first page) should include title of the article, full name(s), institution(s) and address(es) of the author(s); the corresponding authors detailed postage and e-mail addresses (P), and phone (O) and fax numbers (O).

Abstract A concise abstract is required (about 200 words). The abstract should be informative and state briefly the aim of the research, the principal results and major conclusions. An abstract is often presented separately from the article, thus it must be able to stand alone (completely self-explanatory). References should not be cited, but if essential, then cite the author(s) and year(s). Abbreviations should be avoided, but if essential, they must be defined at their first mention. **Keywords** are about five words, covering scientific and local name (if any), research themes, and special methods used; and sorted from A to Z. **Abbreviations** (if any): All important abbreviations must be defined at their first mention there. **Running title** is about five words.

Introduction is about 600 words, covering the aims of the research and provide an adequate background, avoiding a detailed literature survey or a summary of the results. **Materials and Methods** should emphasize on the procedures and data analysis. **Results and Discussion** should be written as a series of connecting sentences, however, for a manuscript with long discussion should be divided into subtitles. Thorough discussion represents the causal effect mainly explains why and how the results of the research were taken place, and do not only re-express the mentioned results in the form of sentences. **Concluding** sentence should be given at the end of the discussion. **Acknowledgements** are expressed in a brief; all sources of institutional, private and corporate financial support for the work must be fully acknowledged, and any potential conflicts of interest are noted.

Figures and Tables of a maximum of three pages should be clearly presented. The title of a picture is written down below the picture, while the title of a table is written above the table. Colored figures can only be accepted if the information in the manuscript can lose without those images; the chart is preferred to use black and white images. The author could consign any picture or photo for the front cover, although it does not print in the manuscript. All images property of others should be mentioned the source. Author is suggested referring to Wikipedia for international boundaries and Google Earth for satellite imagery. If not specifically mentioned, it is assumed to refer to these sources. **There is no appendix**, all data or data analysis is incorporated into Results and Discussions. For broad data, it can be displayed on the website as a supplement.

References Preferably 80% of it comes from scientific journals published in the last 10 years. In the text, give the author names followed by the year of publication and arrange from oldest to newest and from A to Z; in citing an article written by two authors, both of them should be mentioned; however, for three and more authors only the first author is mentioned followed by et al. For example, Saharjo and Nurhayati (2006) or (Boonkerd 2003a, b, c; Sugiyarto 2004; El-Bana and Nijs 2005; Balagadde et al. 2008; Webb et al. 2008). Extent citation should be avoided, as shown with the word "cit." Reference to unpublished data and personal communication should not appear in the list but should be cited in the text only (e.g., Rifai MA 2007, pers. com. (personal communication); Setyawan AD 2007, unpublished data). In the reference list, the references should be listed in alphabetical order. Names of journals should be abbreviated. Always use the standard abbreviation of a journal's name according to the **ISSN List of Title Word Abbreviations** (www.issn.org/2-22661-LTWA-online.php). Please include DOI links for journal papers. The following examples are for guidance.

Journal:

Saharjo BH, Nurhayati AD. 2006. Domination and composition structure change at hemic peat natural regeneration following burning; a case study in Pelalawan, Riau Province. Biodiversitas 7: 154-158. DOI: 10.13057/biodiv/d070213.

The usage of "et al." in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al. 1999. Future of health insurance. N Engl J Med 965: 325-329. DOI: 10.10007/s002149800025.

Book:

Rai MK, Carpinella C. 2006. Naturally Occurring Bioactive Compounds. Elsevier, Amsterdam.

Chapter in the book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds.). Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta*. 50th annual symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.). Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

Information from the internet:

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. DOI: 10.1038/msb.2008.24. www.molecularsystembiology.com.

THIS PAGE INTENTIONALLY LEFT BLANK

NUSANTARA BIOSCIENCE
Vol. 13, No. 1, pp. 1-10
May 2021

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n130101

Potential of *Candida glabrata* from ragi as a bioethanol producer using selected carbohydrate substrates

MICKY VINCENT♥, QUEENTETY JOHNNY, DAYANG SALWANI AWANG ADENI, NURASHIKIN SUHAILI
Faculty of Resource Science and Technology, Universiti Malaysia Sarawak. 94300 Kota Samarahan, Sarawak, Malaysia.
Tel.: +60-825-82985, Fax.: +60-825-83160, ♥email: vmicky@unimas.my

Manuscript received: 16 September 2020. Revision accepted: 1 December 2020.

Abstract. *Vincent M, Johnny Q, Adeni DSA, Suhaili N. 2021. Potential of* Candida glabrata *from ragi as a bioethanol producer using selected carbohydrate substrates. Nusantara Bioscience 13: 1-10.* The flexibility and efficiency of fermenting microorganisms to convert substrates to ethanol are important factors in achieving high bioethanol yields during ethanolic fermentation. In this study, *Candida glabrata*, a common yeast found in fermented food, was evaluated in terms of its capability to produce ethanol using different types of carbohydrates, which included simple saccharides (glucose, maltose, sucrose), polysaccharides (starch and cellulose) and complex carbohydrates (total sago effluent, TSE). Our results indicated that *C. glabrata* was able to efficiently produce ethanol from glucose at 79.84% TEY (Theoretical Ethanol Yield). The ethanol production from sucrose was low, which was only 6.44% TEY, while no ethanol was produced from maltose. Meanwhile, for complex carbohydrate substrates such as starch and cellulose, ethanol was produced only when supplementary enzymes were introduced. Simultaneous Saccharification and Fermentation (SSF) of starch dosed with amylases resulted in an ethanol yield of 55.08% TEY, whilst SSF of cellulose dosed with cellulases yielded a TEY of 31.41%. When SSF was performed on TSE dosed with amylases and cellulases, the highest ethanol production was recorded within 24 h, with a yield of 23.36% TEY. Lactic acid and acetic acid were found to be at minimal levels throughout the fermentation period, indicating an efficient ethanol conversion. A notable increase in *C. glabrata* biomass was observed in cultures fed with glucose, starch (with supplementary amylases), and TSE (with supplementary amylases and cellulases). The current study indicates that *C. glabrata* can be used for bioethanol production from glucose, polysaccharides, and complex starchy lignocellulosic substrates such as TSE via SSF.

Keywords: Bioethanol, *Candida glabrata*, *Metroxylon sagu*, simultaneous saccharification and fermentation, total sago effluent

INTRODUCTION

The interest in producing renewable fuels has increased tremendously over recent years due to the instabilities of fossil fuel supplies and increasing global demands (Wei et al. 2015; Wong and Vincent 2019; Mohammad et al. 2020). Alternative liquid biofuel, such as bioethanol, is seen as the current choice of such renewable fuel to supplement and substitute petroleum-based fuel, due to its sustainability and carbon dioxide neutrality (Vincent et al. 2015; Hung et al. 2018). Compared to conventional gasoline, bioethanol is highly attractive as it offers cleaner combustion that is friendlier towards the environment. Economically, the bioethanol production and supply chain are also desirable as it creates many jobs and financial opportunities for both urban and rural areas (Ștefănescu-Mihăilă 2016).

In mass bioethanol production, substrate selection plays a major role. It is one of the main cost factors for the ethanol industry (Vincent et al. 2015). There are currently many feedstock sources that are used as substrates for bioethanol production (Techaparin et al. 2017; Ahorsu et al. 2018; Mohammad et al. 2020). When substrates such as lignocellulosic biomass are used to produce bioethanol, this type of bioethanol is termed as second-generation bioethanol (Zhang et al. 2016). It is projected that in the future, second-generation bioethanol will replace first-generation bioethanol, which is mostly produced from food-based materials, because of its low cost and feedstock abundance (Vincent et al. 2015; Ștefănescu-Mihăilă 2016). In bioethanol production, the typical bioprocess engaged is Simultaneous Saccharification and Fermentation (SSF) as this procedure offers higher reaction rates, higher yields, and greater ethanol concentrations compared to its closest counterparts such as Separate Hydrolysis and Fermentation.

The efficiency of ethanol production is also influenced by the species of microorganisms used. The desired microorganism should be robust and capable of converting substrates to ethanol effectively. The most common examples of ethanol producers are *Saccharomyces cerevisiae*, *Zymomonas mobilis* and *Fusarium oxysporum* (Ali et al. 2016; Vincent et al. 2018; Mohammad et al. 2020). Among these, *S. cerevisiae* has been mostly used in alcoholic fermentation due to its ethanol productivity. However, this particular species has several limitations. For example, poor stress tolerance and incapability to ferment xylose and arabinose, the main sugars released from hemicelluloses. Therefore, several genetic engineering studies to improve sugar utilization, ethanol production, and other applications have been explored (Carrasco et al. 2013; Pagliardini et al. 2013; Wong and Vincent 2019). Another approach is to search for new fermenting yeasts that may perform better or are more flexible than *S. cerevisiae* (Vincent et al. 2018).

Another yeast species that has similar characteristics to *S. cerevisiae* is *Candida glabrata*, which can be commonly

found in fermented foods (Tsuyoshi et al. 2005) and starters, such as *ragi*, a traditional fermented food starter (Kofli and Davaon 2010; Hajar et al. 2012; Vincent et al. 2018). In this study, *C. glabrata* was isolated from the samples of ragi in Sarawak and was identified by using PCR and commercial identification kit. According to Kwon-Chung and Bennett (1992), *C. glabrata* was firstly classified under the genus *Torulopsis* due to its lack of pseudohypha production but in 1978, it was confirmed that the ability to produce pseudohyphae was not a reliable distinguishing factor for members of the genus *Candida*; and thus it was classified as a member of *Candida* genus, along with over 200 species. The *C. glabrata* cell size was reported to be small compared to other *Candida* species and is in the range of 1 to 4 µm (Fidel et al. 1999). In terms of ethanol production, *C. glabrata* is reported to be suitable for bioethanol fermentation as it possesses higher stress tolerance to acid and high temperatures in addition to impressive ethanol conversion capabilities (Watanabe et al. 2010). According to Merico et al. (2007), *C. glabrata* was reported to yield high ethanol percentages when grown under anaerobic conditions. Furthermore, *C. glabrata* is suitable for SSF since the optimum temperature for amylolytic and cellulolytic activities is above 40 °C.

Presently, limited information is available in the systematic studies of the ethanolic fermentation involving *C. glabrata*, especially when biomass is used as substrates. Biomass such as total sago effluent (TSE) can be found abundantly in Sarawak, Malaysia. TSE is a waste or residue from sago (*Metroxylon sagu*) industries that are usually disposed of into nearby rivers. According to Vincent et al. (2020), about 7 tons of TSE is discharged into the rivers per day from typical sago starch factories. This waste needs to be handled properly to avoid water pollution (Mohammad et al. 2020). TSE consists of sago hydrolysate, which is the liquid part while the solid component is called sago hampas. It is reported to contain high amount of lignocellulosic materials, which can be found in the solid sago hampas, making it suitable to be used as a substrate for bioethanol production (Hung et al. 2018). The hydrolysate can also contribute to bioethanol production as it contains starch that can be converted into fermentable sugars (Vincent et al. 2015). Therefore, the main objective of this study is to investigate the abilities of *C. glabrata* in producing ethanol from a variety of carbohydrates, ranging from common simple carbohydrates (glucose – monosaccharide, sucrose, and maltose – disaccharides) to polymeric carbohydrates (starch and cellulose) and a complex carbohydrate mixture (total sago effluent).

MATERIALS AND METHODS

Experimental setup

Figure 1 shows an overview of the experimental procedures in this study.

Collection of total sago effluent

TSE was collected from Herdsen Sago Mill Sdn Bhd in Pusa, Sarawak (Figure 2). The TSE samples were stored at -20 °C prior to its usage.

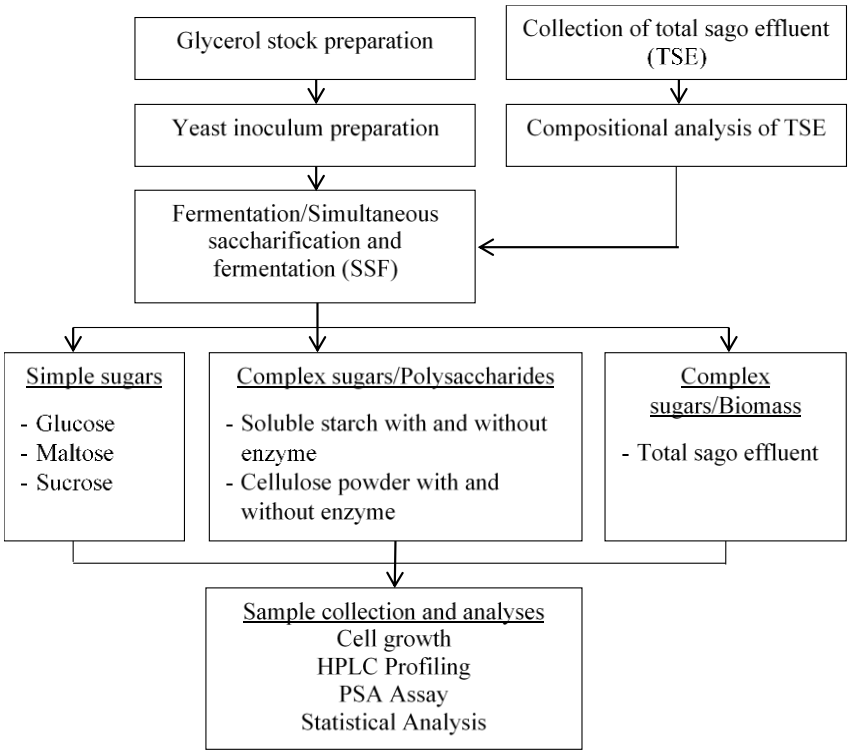


Figure 1. Overall experimental setup

Figure 2. Total sago effluent (TSE) released from the sago mill in Pusa, Sarawak, Malaysia

Characterization of sago hampas

The moisture content of TSE sample was determined by measuring the weight loss from the initial weight after oven-drying (Shel Lab, USA) at 105°C for 3 days. Prior to the characterization process, the TSE was oven-dried before it was ground to fine powder. The powdered samples were subjected to starch and fiber analysis by using the Phenol-Sulphuric Acid assay (PSA), Acid detergent Fiber (ADF), Neutral Detergent Fiber (NDF), and Klason Lignin Determination (Goering and van Soest 1970). The ash content was determined using furnace incineration, where the sample was subjected to dry ashing at 550°C for 6 hours in an ashing muffle furnace (KC, 40/13. ThermConcept, Germany).

Glycerol stock preparation

The *C. glabrata* strain used in this study was isolated from samples of ragi, collected from Kuching, Sarawak (Malaysia) (Vincent et al. 2018). Isolation of yeast single colonies was carried out on Rose Bengal Chloramphenicol Agar (RBDC) (Hi Media, India) and the single colonies obtained were grown in YM broth (Sigma, USA) by shaking at 150 rpm (NB-101MT Multi Shaker, N-Biotek, Korea) at ambient temperature. For long-term storage, the stock culture was prepared by mixing culture medium with 20% (v/v) glycerol (R&M Marketing, UK) and were stored at -20°C for preservation.

***Candida glabrata* inoculum preparation**

For *C. glabrata* inoculum preparation, the cultures were taken from the glycerol stocks. The cultures were grown overnight in YM broth at ambient temperature with constant shaking at 150 rpm. The cultures were then centrifuged (BK-1032J Low-Speed Centrifuge, Biobase, China) for 6 min at 4500 rpm. The resulting *C. glabrata* cell pellets were used as inocula for fermentations in this work.

Fermentation –using glucose, maltose and sucrose

Fermentation medium was prepared in 500 mL Schott bottles containing 150 mL of mixed solution of 1.5 g/L of yeast extract (Bacto, USA), 3.0 g/L of bacteriological peptone (Bendosen Laboratory Chemicals, Malaysia) and 5% (w/v) of either glucose (R&M Chemical, United Kingdom), maltose (R&M Chemical, United Kingdom) or sucrose (Bendosen Laboratory Chemicals, Malaysia) in 0.05 M citrate buffer, pH 5.0. The pH of the media was adjusted to the optimum pH level for fermentation at 4.8, before it was autoclaved (Model No. 25X Electric Pressure Steam Sterilizer, All American, United State) for 15 min at 121°C. Prior to the fermentation, the harvested cells, which were in the range of 1.29 x 10^8 cells/mL to 2.86 x 10^8 cells/mL were added aseptically into the sterilized fermentation media. Fermentations were carried out, anaerobically for 5 days, at ambient temperature with a constant shaking of 150 rpm. For each type of sugar, the fermentations were done in triplicates (n=3).

Fermentation –using starch and cellulose

The two sets of media consisting of polysaccharides namely 5% (w/v) starch (Bendosen Laboratory Chemicals, Malaysia) and 5% (w/v) cellulose (Nacalai Tesque, Japan) were prepared separately. After the pH adjustment to 4.8, the media were autoclaved in order to sterilize and also to gelatinize the starch. Next, the harvested *C. glabrata* cells and 75 µL of each amylase (Sunson, China), α-amylase (EC.3.2.1.1) and glucoamylase (EC.3.2.1.3) were added into the sterile starch solution, while 2.0 mL of cellulase (50 FPU) (Sunson, China) was added into the sterile cellulose solution. Cellulase enzymes used consisted of acid cellulase, beta-glucosidase, and xylanase. Another set of sterile starch and cellulose solution was prepared but no enzyme was added to those media. The fermentations were conducted under the same conditions of the fermentation with simple sugars.

Fermentation – using total sago effluent (TSE)

TSE fermentation medium was prepared by mixing 1.5 g/L yeast extract, 3.0 g/L peptone, and 0.05 M citrate buffer, with pH of 5, in a 500 mL Schott bottle and the solution was topped up to 150 mL with TSE. Following the pH adjustment

to 4.8 and sterilization, about 75 µL of each amylase (α-amylase and glucoamylase) and 2.0 mL of cellulase were added in order to start the SSF of TSE. SSF was done in triplicates (n=3) according to the same conditions as the fermentation described earlier.

Sample collection and *Candida glabrata* cell count

About 1.5 mL of culture broth was pipetted out at 0, 6, 12, 24, 36, 48, 72, 96 and 120 h during the fermentation. Prior to HPLC analyses, cell counting was performed using hemocytometer (Optik Labor, United Kingdom) and the viable cells were observed under a light microscope after methylene blue staining. The remaining supernatant was centrifuged (WiseSpin CF-10 High-Performance Microcentrifuge Set, Daihan Scientific, Korea) at 13,500 rpm for 3 min and was filtered through a 0.45 µm nylon membrane filter (Whatman, NJ, USA) to eliminate any solid residues. The filtrate was used for phenol-sulfuric acid assay (PSA) and High-Performance Liquid Chromatography (HPLC) analyses.

Phenol-sulfuric acid assay (PSA)

Total carbohydrate content of the samples was determined based on PSA assay. The assay was performed by measuring the optical density of the samples at 490 nm using a spectrophotometer (SP-880 Metertech, Taiwan). The amount of total carbohydrate present in the samples was determined based on a standard curve, which was plotted prior to the assay.

Analytical analysis

Quantification of ethanol, sugars, lactic acid and acetic acid was performed using High-Performance Liquid Chromatography (HPLC) (Waters 2695 Separations Module, Alliance HPLC System, United States) The HPLC system was equipped with a column heater, refractive index detector (2414 RI Detector) and computer controller. The protocols for separation and analyses of ethanol and other fermentation constituents were according to Vincent et al. (2015). Theoretical Ethanol Yield (TEY) of each substrate was determined based on ethanol produced and was calculated as follows:

TEY = (Ethanol produced (g/L) / conversion factor x initial concentration of sugar) x 100

Where,
Conversion factor for sugar to ethanol = 0.511
Conversion factor for biomass to sugar =1.1

Statistical analysis

Error bars were determined based on the standard deviation from the mean value of triplicate experiments. The data was statistically analyzed using One-way Analysis of Variance (ANOVA) and Tukey's *Post Hoc* test (SPSS Statistics Software version 22), and the differences were considered significant if $p < 0.05$.

RESULTS AND DISCUSSION

***Candida glabrata* cell growth using different carbohydrate feedstock**

In the current study, the ability of *C. glabrata*, isolated from local wine starter (ragi), in utilizing and fermenting different carbohydrates (mono-, di-, polysaccharides and complex mixture), represented by glucose, sucrose, maltose, starch, cellulose, and total sago effluent (TSE), to bioethanol and other byproducts were examined. Similar to other common yeasts, *C. glabrata* utilizes these different classes of carbohydrates differently, either for primary cell growth or for conversion to other secondary metabolites, alcohols, or organic acids.

A rapid and good specific growth rate is crucial for efficient fermentation and high yield of ethanol (Chang et al. 2018). For this reason, the cell population growth of *C. glabrata* in all substrates was monitored in order to gauge its growth profile. Based on the results from Figure 3, *C. glabrata* cells were propagating rapidly in the first 12 h in all fermentations. However, a notable increase in the first 6 h was only observed in fermentations employing glucose, starch with enzyme and TSE while the growth in other fermentations was relatively slower. After 48 h, a gradual decline in cell concentration was observed in all fermentations, due to substrate limitation and/or ethanol toxicity. The same observations were reported in studies elsewhere (Jönsson et al. 2013). Throughout the fermentation period, the trend of change of sugar uptake and alcohol production profiles was found to coincide with the logarithmic phase of the growth profile.

Ethanol production from glucose, maltose and sucrose

Figure 4 shows the time course of ethanol production during the fermentation by *C. glabrata* using different carbon sources. The ethanol production in fermentations using glucose increased sharply from 0 to 48 h, with the maximum production of 20.40 g/L (79.84% TEY) achieved at 72 h. After 72 h, the production of ethanol decreased slowly over time. At the later stage of fermentation, the ethanol concentration decreased in most of the fermentation because the yeast required energy to maintain growth. According to Li et al. (2017), the yeast consumed some ethanol to obtain energy due to the small amount of residual sugars in the media. From Figure 4, low ethanol yield was observed in the fermentation using sucrose, with the peak production of only 1.64 g/L at 6 h. On the other hand, no ethanol was detected in fermentations using maltose throughout the 5 days of incubation. This showed that *C. glabrata* does not produce particular enzyme to break down maltose. In the meantime, only low ethanol produced in fermentations using sucrose, which was probably caused by certain conditions such as concentration of starting materials, pH, or temperature during the fermentation.

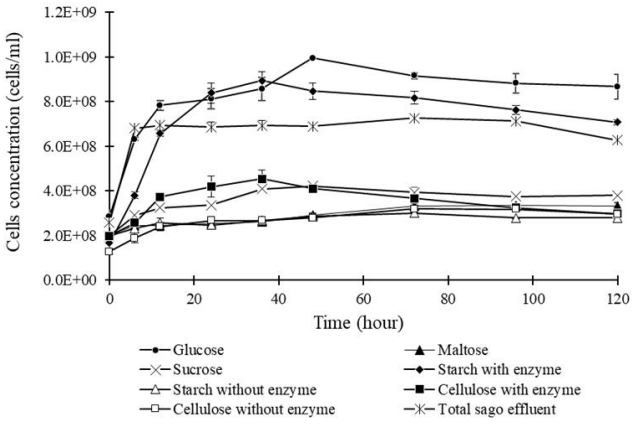


Figure 3. Time course of cell concentration during bioethanol fermentation by *C. glabrata* using different carbon sources. The data points represent the average of three independent experiments (n=3)

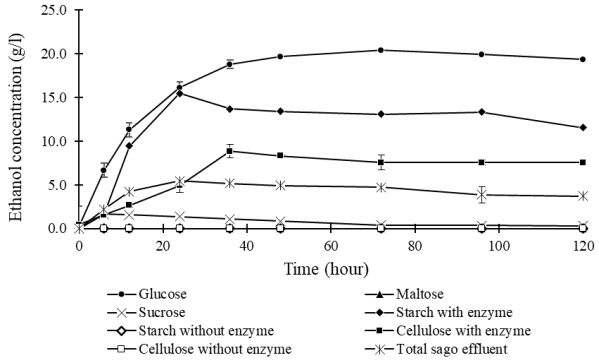


Figure 4. Time course of ethanol concentration during bioethanol fermentation by *C. glabrata* using different carbon sources. The data points represent the average of three independent experiments (n=3)

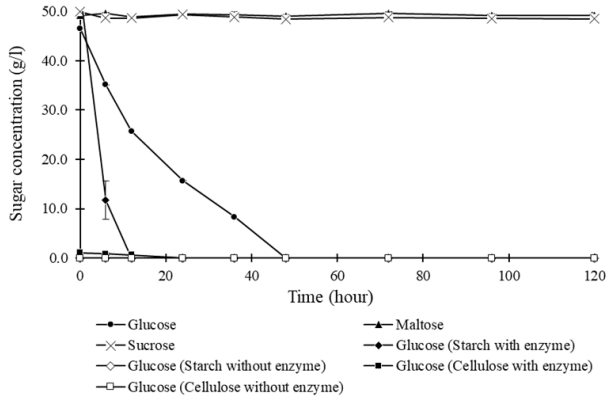


Figure 5. Time course of sugar concentration during bioethanol fermentation by *C. glabrata* using different carbon sources (simple sugars and complex sugars). The data points represent the average of three independent experiments (n=3)

The high efficiency of conversion of glucose to ethanol by *C. glabrata* was also documented previously by Barnett et al. (2000). Our study has shown that *C. glabrata* was capable of fermenting glucose efficiently. According to Mosier (2005), hexoses such as glucose can be fermented by most microorganisms species, especially yeasts. Glucose is also considered the best substrate for growing yeast cells and also ethanol fermentation (Ruriani et al. 2012). Conversion of glucose to ethanol at 79.84% of the theoretical value obtained in this work is considered high yield. The maximum yield is usually lower than 90% because it is usually limited by nutrients or substrates used to synthesize biomass and maintain the reactions. Another similar study conducted by Techaparin et al. (2017) on ethanol production from glucose by *C. glabrata* reported only a 42% conversion. By comparison, the *C. glabrata* strain used in this work showed higher efficiency of ethanol production, which renders its practicality for industrial applications.

Figure 5 shows the profile of sugar consumption during fermentation by *C. glabrata* using different substrates. The profile depicts a steady decrease of glucose concentration during the 48-hour incubation. However, the concentration of maltose and sucrose remained unchanged at approximately 48.0 g/L throughout the fermentation period. This observation is in agreement with the reports on the extremely narrow carbon utilization range of *C. glabrata* when compared to other yeasts (Turner and Butler 2014).

Simultaneous saccharification and fermentation (SSF) of starch, cellulose, and total sago effluent (TSE)

The ability of *C. glabrata* to saccharify starch, cellulose, and TSE for ethanol production was also evaluated in this work. Both starch and cellulose are classified as glucose-based polymers, with the latter more abundant in nature. However, these two carbohydrates differ in the orientation of the linkages between the glucose subunits (Jacques et al. 2003). As shown in Figure 5, the initial glucose concentration in fermentation medium containing starch was completely depleted after 12 h of fermentation, which was in line with the increase of ethanol starting at 6 h. The maximum concentration of ethanol (15.23 g/L) was achieved at 24 h. On the other hand, no glucose was detected in the fermentation medium containing starch when the supplementary enzymes were not added.

The results indicated that supplementary amylases are important in the alcoholic fermentation of *C. glabrata* as no ethanol was produced in the absence of the enzymes. These results can also be correlated with the sugar consumption profile (Figure 5) where glucose was only detected in media that was dosed with amylases. The conversion of starch to glucose occurred most efficiently on the first day of SSF as the maximum production of ethanol was recorded at 24 h, along with the depletion of glucose in the fermentation media. Our findings suggest that *C. glabrata* does not produce amylolytic enzymes to breakdown starch in the fermentation broth.

When cellulose powder was used as a carbon source, ethanol was only detected in the medium containing

supplementary cellulases. The ethanol concentration increased gradually over the 36 h of fermentation, peaking at about 8.83 g/L, as shown in Figure 4. After that, the ethanol yield started to remain constant until the end of the SSF period. On the contrary, in the absence of supplementary enzymes, no ethanol yield was recorded throughout the SSF period, as shown in Figure 4. This outcome indicates that cellulases are crucial for the conversion of cellulose to ethanol, as supported by the findings by Fox et al. (2012) where the cellulase action on the insoluble cellulosic substrates is modeled as a heterogenous biocatalytic reaction. The reaction involves endo- and exocellulases that either interact directly with soluble cello-oligosaccharides or form complexes with insoluble cellulose chains. Cello-oligosaccharides are saccharides that consist of 2 to 6 glucose by β-1-4-linkages, containing mainly cellobiose (Zhao et al. 2009). This study further confirms the inability of *C. glabrata* to breakdown cellulose and requires extracellular cellulases to convert cellulose into fermentable sugar.

SSF was also performed on TSE. The compositional analyses of TSE prior to SSF are presented in Table 1 and Table 2. The TSE used in the current study consisted of sago effluent hydrolysate (water component) and sago hampas (solid component), with 97.14% was the water component as shown in Table 1. Approximately 2.23% of the hydrolysate was starch, which can be converted to fermentable sugar through enzymatic hydrolysis. Further analysis of sago hampas was carried out to determine the exact amount of lignocellulosic contents. As outlined in Table 2, dried sago hampas used in this study contained 52.03% starch, 27.82% cellulose, 5.32% hemicellulose and 3% lignin. The results obtained in this work are comparable to a previous report by Hung et al. (2018), where sago hampas were reported to contain 55.4% starch, 23.6% cellulose, 9.1% hemicellulose, and 4.0% lignin. Furthermore, the starch content recorded in this study, which was in the range of 30% - 50% was comparable with that reported by Awg-Adeni et al. (2010). The variation of lignocellulosic composition can be associated with the difference in the sago species, growth conditions and maturity (Kim et al. 2010; Tye et al. 2011). The amount of cellulose and hemicellulose in biomass is the key in biofuel production, as they are convertible into bioethanol (Malherbe and Cloete 2003).

Figure 6 shows the profile of total soluble carbohydrates and sugars released during SSF of TSE. The initial total carbohydrate concentration was recorded at 20.57 g/L. At 6 h, the total carbohydrate content declined steeply to 4.98 g/L, giving a total reduction of approximately 75%. At 12 h, the concentration decreased further to 4.02 g/L and remained relatively constant until the end of the SSF period. The initial glucose concentration was 16.47 g/L at 0 h, before it decreased to 0.56 g/L at 6 h and became completely depleted after 12 h. For cellobiose, the initial concentration was 4.32 g/L and it decreased to 1.62 g/L and became plateau until the end of the incubation. Another wood sugar, arabinose was also detected in the TSE samples with the concentrations fluctuated slightly between 0.8 and 0.9 g/L, before peaking at 1.10 g/L at 120 h. There was no xylose detected at the initial stage of SSF until at 12 h when a concentration of 0.19 g/L was detected. The xylose concentration remained between 0.25 and 0.27 g/L throughout the remaining SSF period. A trace amount of arabinose with a concentration of 0.40 g/L was detected and the amount was found to remain constant throughout the incubation. Xylose and arabinose are the most abundant pentose sugars in hemicellulose (Bettiga et al. 2009). These sugars are released from xyloglucan, xylan, arabinan and arabinogalactan (substructures of pectin) from polysaccharides in plant cell (Battaglia et al. 2011).

Table 1. Compositional analysis of total sago effluent TSE comprising of sago hydrolysate and dried sago hampas.

Compositions	Amount (%)	
Sago hydrolysate (water component)	Water	94.91 ± 0.09
	Free Starch	2.23 ± 0.86
Dried sago hampas (solid component)	Starch	1.49 ± 1.11
	Cellulose	0.79 ± 1.23
	Hemicellulose	0.15 ± 2.00
	Lignin	0.09 ± 0.09
	Ash	0.06 ± 0.06
	Others	0.28 ± 0.00

Table 2. Compositional analysis of dried sago hampas The percentages was based on dry weight, n=3.

Compositions	% w/w
Starch	52.03 ± 1.11
Cellulose	27.82 ± 0.62
Hemicellulose	5.32 ± 0.96
Lignin	3.00 ± 0.05
Ash	1.95 ± 0.03
Others	9.88 ± 0.07

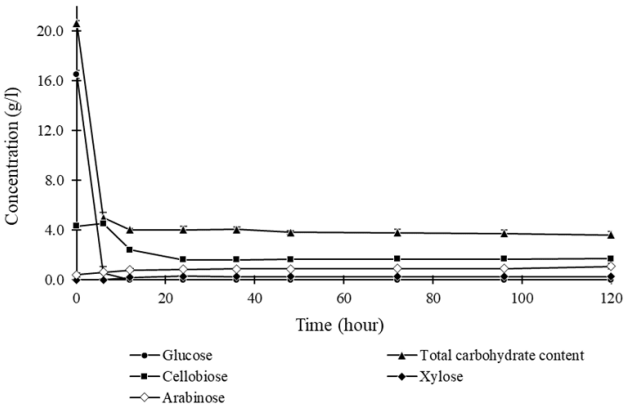


Figure 6. Time course of total carbohydrate content and sugar concentration (glucose, cellobiose, xylose, arabinose) during SSF of TSE. The data points represent the average of three independent experiments (n=3).

As shown in Figure 6, xylose was only detected at 12 h and the peak was found at 24 h. This showed that the breakdown of hemicellulose only started to occur at 12 h and onwards. In contrast, a trace amount of arabinose was detected from the beginning until the end of SSF. The degradation of pectin during the autoclaving process of media preparation may have resulted in the accumulation of arabinose at this stage. The concentration of arabinose was found to increase during the first 12 h, indicating the active breakdown of hemicellulose by xylanase into xylose and arabinose. After 24 h, the concentration of xylose and arabinose remained constant until the end of SSF implying the inability of *C. glabrata* to utilize both sugars. This is in parallel with a study by Barnett et al. (2000) and Ruriani et al. (2012) that reported the selective fermentation of *C. glabrata*. The same studies further reported that yeast cells basically use monosaccharides for their growth, but only a few of the monosaccharide compounds such as glucose, galactose and mannose can be converted into ethanol. Pentose sugars (five-carbon sugars) such as xylose and arabinose, can only be fermented by a small number of wild microorganisms, often with a low ethanol yield (Mosier 2005).

A complete breakdown of cellulose into glucose involves extracellular cellulases. Cellobiose, a disaccharide, is usually the intermediate product. Based on our results, the highest concentration of cellobiose was found in the beginning of SSF indicating the enzymatic hydrolysis of cellulose into cellobiose. Subsequently, β-glucosidases, a part of cellulase enzymes will cleave cellobiose into 2 glucose units (Lynd et al. 2002). The depletion of cellobiose denotes the effective enzymatic activity in the fermentation broth. However, cellobiose was only detected in low concentrations, and this in turn resulted in a low yield of glucose.

Based on Figure 6, a steep decrease of total sugar content during the first 6 h indicated that the substrates present in the fermentation broth were rapidly fermented into ethanol by *C. glabrata*. The same observations were also reported by Hung et al. (2018) who stated that not all sugars can be converted to ethanol. In addition, the results obtained in this study showed the presence of other unknown hexose sugars in the fermentation broth besides glucose.

Ethanol was produced at the early stage of SSF, with a concentration of 2.22 g/L recorded at 6 h, as shown in Figure 4. The increase in ethanol yield is highly correlated with the steep decrease in glucose concentration. After the depletion of glucose at 6 h, the ethanol concentration continued to increase until its maximum production at 24 h. The highest ethanol concentration was 5.44 g/L. This corresponds to an estimated 21.25% TEY, which is significantly lower than that reported by Hung et al. (2018) (79.65% TEY). The author reported a higher TEY because they used only sago hampas as substrate, while in this study, the mixture of sago effluent and sago hampas was used. Compared to sago hampas, TSE contains less lignocellulosic materials and thus produced less ethanol.

Analyses of fermentation by-products – Lactic acid and acetic acid

Figure 7 depicts the profile of lactic acid during fermentation by *C. glabrata* using different carbon sources. Lactic acid commonly comes from lactic acid bacteria (LAB). A sharp increase of lactic acid was observed in fermentations using glucose as a substrate with a peak of 3.71 g/L at 24 h, which was caused by the presence of LAB in the media throughout the fermentation. On the other hand, the lactic acid concentration was relatively low in fermentations that use other carbon sources.

Figure 8 illustrates the concentration of acetic acid during the fermentation by *C. glabrata* using different carbon sources. Overall, the level of acetic acid in all fermentations was recorded to be less than 0.5 g/L. Similar to lactic acid, acetic acid also comes from bacteria, acetic acid bacteria (AAB). A previous study stated that acetic acid also can be found in hydrolysates and it basically comes from acetyl side-chains in hemicellulose. According to Vincent et al. (2015), acetic acid is an important indicator of the efficiency of biomass hydrolysis where an increase in acetic acid concentration in the fermentation broth denotes a continuous degradation of the lignocellulosic components of TSE throughout the SSF period.

Although the presence of organic acids is common in typical fermentation systems, the concentrations of lactic acid and acetic acid must, however, be closely monitored to avoid a reduction in the ethanol production due to the changes in the pH of the fermentation media (Lin et al. 2012). According to Narendranath (2003), the presence of these acids in high concentrations can also be indicator of contamination, which could be caused by Lactobacilli. In this study, the overall concentrations of both lactic acid and acetic acid in the cultures were relatively low (<0.5 g/L). This indicated that the fermentations were free from contaminations caused by bacteria, except for the media containing glucose.

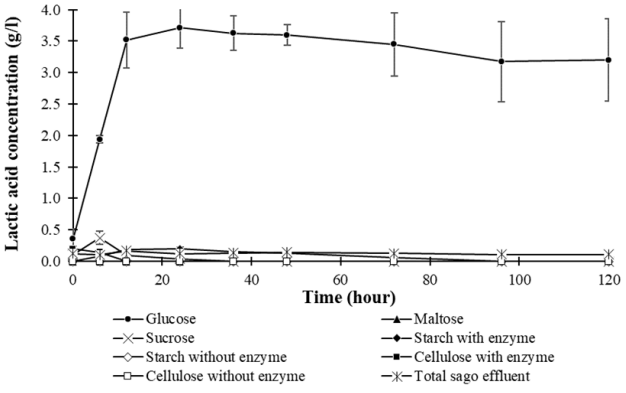


Figure 7. Time course of lactic acid concentration during bioethanol fermentation by *C. glabrata* using different carbon sources. The data points represent the average values of three independent experiments (n=3).

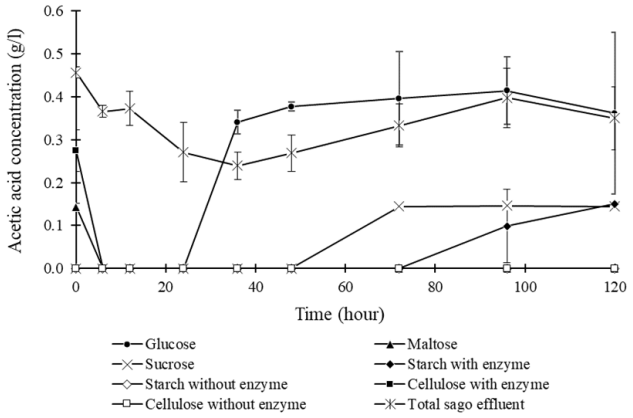


Figure 8. Time course of acetic acid concentration during bioethanol fermentation by *C. glabrata* using different carbon sources. The data points represent the average of three independent experiments (n=3)

Table 3. Maximum ethanol production and yield achieved in fermentation by *C. glabrata* using different substrates.

Substrate	Maximum production of ethanol (g/L)	Experimental theoretical yield (%)
Glucose	20.40 ± 0.30	79.84 ± 1.18a
Maltose	0.00 ± 0.00	0.00 ± 0.00b
Sucrose	1.64 ± 0.08	6.44 ± 0.30b
Starch with enzyme	15.23 ± 2.11	55.08 ± 7.50d
Starch without enzyme	0.00 ± 0.00	0.00 ± 0.00b
Cellulose with enzyme	8.83 ± 0.76	31.41 ± 3.31c
Cellulose without enzyme	0.00 ± 0.09	0.00 ± 0.00b
Total sago effluent	5.54 ± 0.09	21.25 ± 1.35e

Note: *Data are mean of triplicates ± S.D (Different superscript letters in the same column show significant differences, *p*-value < 0.05)

Table 3 outlines the overall maximum ethanol production and yield achieved in fermentation by *C. glabrata* using different substrates. The highest ethanol production was recorded in fermentations using glucose as a substrate. A maximum ethanol concentration of 20.4 g/L was produced, which corresponded to a theoretical yield of 79.84%. This clearly showed that in comparison to other carbon sources examined in this study, glucose is the best substrate for ethanol production by *C. glabrata*. The second best substrate was found to be starch when it was supplemented with enzymes. The maximum ethanol concentration achieved was 15.23 g/L and with a theoretical yield of 55.08%. In contrast, there was no ethanol yielded in fermentations using maltose, starch, and cellulose. Among all substrates tested in this work, only glucose and sucrose were consumed by *C. glabrata* to produce ethanol. Meanwhile, it is observed that soluble starch and cellulose were consumed only with the aids of the supplementary enzymes, with the former gave a higher yield. As for the SSF of TSE, the maximum ethanol production was 5.44 g/L (21.25% TEY). Although the ethanol concentration was relatively low compared to that achieved using other carbon sources, the results do reflect the ability of *C. glabrata* to grow on TSE. It is expected that higher yield of ethanol can be achieved upon further optimization of the SSF process.

In conclusion, the results from this study indicated that *C. glabrata* was capable to ferment glucose efficiently (79.84% of theoretical yield), making it as compared to other common fermenting yeasts such as *S. cerevisiae*, which was reported to yield TEY of 40 to 49% when fermenting 4 to 5% (w/v) glucose (Govindaswamy and Sane 2010; Kumar et al. 2011). Although *C. glabrata* is incapable of fermenting other sugars such as xylose and arabinose as well as polysaccharides like starch and cellulose without the aid of supplementary amylases and cellulases, the strain does show a promising potential to be used for fermenting starchy industrial waste such as TSE.

ACKNOWLEDGEMENTS

This work was supported by the Tun Openg Sago Chair Grant [F07/TOC/1518/2016].

REFERENCES

Ahorsu R, Medina F, Constantí M. 2018. Significance and challenges of biomass as a suitable feedstock for bioenergy and biochemical production: a review. Energies 11 (12): 3366. DOI: 10.3390/en11123366.

Ali SS, Nugent B, Mullins E, Doohan FM. 2016. Fungal-mediated consolidated bioprocessing: The potential of *Fusarium oxysporum* for the lignocellulosic ethanol industry. AMB Express 6: 13. DOI: 10.1186/s13568-016-0185-0.

Awg-Adeni DS, Abd-Aziz S, Bujang K, Hasaan MA. 2010. Bioconversion of sago residues into value-added products. Afr J Biotechnol 9 (4): 2016-2021.

Barnett JA, Payne RW, Yaroww D. 2000. Yeasts: Characteristics and identification. Cambridge University Press, USA.

Battaglia E, Hansen SF, Leendertse A, Madrid S, Mulder H, Nikolaev I, de Vries RP. 2011. Regulation of pentose utilisation by AraR, but not XlnR, differs in *Aspergillus nidulans* and *Aspergillus niger*. Appl Microbiol Biotechnol 91 (2): 387-397.

Bettiga M, Bengtsson O, Hahn-Hagerdal B, Gorwa-Grauslund MF. 2009. Arabinose and xylose fermentation by recombinant *Saccharomyces cerevisiae* expressing a fungal pentose utilization pathway. Microb Cell Fact 8: 40-52.

Carrasco C, Baudel H, Roslander C, Galbe M, Lidén G. 2013. Fermentation of the straw material paja brava by the yeast *Pichia stipitis* in a simultaneous saccharification and fermentation process. J Sustain Bioenergy Syst 3: 99-106.

Chang YH, Chang KS, Chen CY, Hsu CL, Chang TC, Jang HD. 2018. Enhancement of the Efficiency of Bioethanol Production by *Saccharomyces cerevisiae* via Gradually Batch-Wise and Fed-Batch Increasing the Glucose Concentration. Ferment 4 (2): 45-57.

Fidel PL, Vazquez JA, Sobel JD. 1999. *Candida glabrata*: Review of Epidemiology, Pathogenesis, and Clinical Disease with Comparison to *C. albicans*. Clin Microbiol Rev 12 (1): 80-96.

Fox JM, Levine SE, Clark DS, Blanch HW. 2012. Initial- and processive-cut products reveal cellobiohydrolase rate limitations and the role of companion enzymes. Biochem 51: 442-452.

Goering HK, van Soest PJ. 1970. Forage fiber analyses (apparatus, reagents, procedures, and some applications). Agriculture Handbook No. 379. United States Department of Agriculture, Washington, DC.

Govindaswamy S, Vane LM. 2010. Multi-stage continuous culture fermentation of glucose-xylose mixtures to fuel ethanol using genetically engineered *Saccharomyces cerevisiae* 424S. Bioresour Technol 101: 1277-1284.

Hajar S, Noorhisham T, Nurina A. 2012. Short Technical Communication Yeast Identification from domestic ragi for food fermentation by PCR method. Int Food Res J 19: 775-777.

Hung CH, Adeni DSA, Johnny Q, Vincent M. 2018. Production of bioethanol from sago hampas via Simultaneous Saccharification and Fermentation (SSF). Nusantara Biosci 10 (4): 240-245.

Ingledew WM. 1999. Alcohol production by *Saccharomyces cerevisiae*: A yeast primer. In: Lyons TP, Kelsall DR (eds) Nottingham University Press, United Kingdom.

Jacques KA, Lyons TP, Kelsall DR. 2003. The Alcohol Textbook (4thed.). Nottingham University Press, United Kingdom.

Jönsson LJ, Alriksson B, Nilvebrant NO. 2013. Bioconversion of lignocellulose: Inhibitors and detoxification. Biotechnol Biofuels 6 (16): 1-10.

Kim JH, Block DE, Mills DA. 2010. Simultaneous consumption of pentose and hexose sugars: An optimal microbial phenotype for efficient fermentation of lignocellulosic biomass. Appl Microbiol Biotechnol 88 (5): 1077-1085.

Kofli NT, Dayaon, SHM. 2010. Identification of Microorganism from *Ragi* for Bioethanol Production by API Kit. J Appl Sci 10 (21): 2751-2753.

Kumar RS, Shankar T, Anandapandian KTK. 2011. Characterization of alcohol-resistant yeast *Saccharomyces cerevisiae* isolated from Toddy. Int Res J Microbiol 2: 339-405.

Li X, Deng Y, Yang Y, Wei Z, Cheng J, Cao L, Mu D, Luo S, Zheng Z, Jiang S, Wu X. 2017. Fermentation Process and Metabolic Flux of Ethanol Production from the Detoxified Hydrolyzate of Cassava Residue. Front Microbiol 8: 1-12.

Lin Y, Zhang W, Li CJ, Sakakibara K, Tanaka S, Kang HN. 2012. Factors affecting ethanol fermentation using *Saccharomyces cerevisiae* BY4742. Biomass Bioenergy 47: 395-401.

Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. 2002. Microbial cellulose utilization: Fundamentals and biotechnology. Microbiol Mol Biol Rev 66: 506-577.

Malherbe S, Cloete TE. 2002. Lignocellulose biodegradation: Fundamentals and applications. Rev Environ Sci Biotechnol 1: 105-114.

Merico A, Sulo P, Piskur J, Compagno C. 2007. Fermentative lifestyle in yeasts belonging to the Saccharomyces complex. FEBS J 274: 976-989.

Mohammad S, Awg-Adeni DS, Bujang KB, Vincent M, Baidurah S. 2020. Potentials of sago fibre hydrolysate (SFH) as a sole fermentation media for bioethanol production. IOP Conf Ser: Mater Sci Eng 716 (1): 012001. DOI: 10.1088/1757-899x/716/1/012001.

Mosier N. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 96 (6): 673-686.

Narendranath, N. 2003. Bacterial contamination and control in ethanol production. In: Jacques KA, Lyons TP, Kelsall DR (eds) Nottingham University Press, United Kingdom.

Pagliardini J, Hubmann G, Alfenore S, Nevoigt E, Bideaux C, Guillouet SE. 2013. The metabolic costs of improving ethanol yield by reducing glycerol formation capacity under anaerobic conditions in *Saccharomyces cerevisiae*. Microb Cell Fact 12 (1): 29. DOI: 10.1186/1475-2859-12-29.

Ruriani E, Sunarti TC, Meryandini A. 2012. Yeast isolation for bioethanol production. Hayati 19 (3): 145-149.

Ştefănescu-Mihăilă RO. 2016. Rural economy and bioethanol production. Sustainability 8: 1148. DOI: 10.3390/su8111148.

Techaparin A, Thanonkeo P, Klanrit P. 2017. High-temperature ethanol production using thermotolerant yeast newly isolated from Greater Mekong Subregion. Braz J Microbiol 48 (3): 461-475.

Tsuyoshi N, Fudou R, Yamanaka S, Kozaki M, Tamang N, Thapa S, Tamang JP. 2005. Identification of yeast strains isolated from marcha in Sikkim, a microbial starter for amylolytic fermentation. Int J Food Microbiol 99: 135-146.

Turner SA, Butler G. 2014. The Candida Pathogenic Species Complex. Cold Spring Harb Perspect Med 4 (9). DOI: 10.1101/cshperspect.a019778.

Tye YY, Lee KT, Abdullah WNW, Leh CP. 2011. Second-generation bioethanol as a sustainable energy source in Malaysia transportation sector: Status, potential and future prospects. Renew Sustain Energy Rev 15 (9): 4521-4536.

Vincent M, Senawi BR, Esut E, Muhammad-Nor N, Adeni DS. 2015. Sequential saccharification and simultaneous fermentation (SSSF) of sago hampas for the production of bioethanol. Sains Malays 44 (6): 899-904.

Vincent M, Hung HC, Baran PRM, Azahari AS, Adeni DSA. 2018. Isolation, identification and diversity of oleaginous yeasts from Kuching, Sarawak, Malaysia. Biodiversitas 19 (4): 1266-1272.

Vincent M, Junaidi F, Bilung LM, Suhaili N, Husaini AA, Kanakaraju D. 2020. Simultaneous reclamation of sago starch processing effluent water and *Rhizopus oligosporus* cultivation at different pH conditions. J Water Environ Technol 18 (4): 254-263.

Watanabe I, Nakamura T, Shima J. 2010. Strategy for simultaneous saccharification and fermentation using a respiratory-deficient mutant of *Candida glabrata* for bioethanol production. J Biosci Bioeng 110 (2): 176-179.

Wei CCS, Seng L, Hussaini AASA, Zulkarnain A, Apun K, Bilung L, Vincent M. 2015. Molecular technique identification of the microbial population in palm oil mill effluent (POME). J Oil Palm Res 27 (3): 293-298.

Wong SH, Vincent M. 2019. Development of *Rhodotorula mucilaginosa* strain via random mutagenesis for improved lipid production. Malays J Microbiol 15 (7): 566-576.

Zhang X, Lei H, Chen S, Wu J. 2016. Catalytic co-pyrolysis of lignocellulosic biomass with polymer: A critical review. Green Chem 18 (15): 4145-4169.

Zhao Y, Lu W, Wang H. 2009. Supercritical hydrolysis of cellulose for oligosaccharide production in combined technology. Chem Eng J 150 (2-3): 411-417.

NUSANTARA BIOSCIENCE
Vol. 13, No. 1, pp. 11-15
May 2021

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n130102

Effectiveness of maggot extractions and secretion (E/S) of *Lucilia sericata* in reducing wound surface in experimental scalding burn injury

ZAHRA SADAT AMIRI[1], KAMRAN AKBARZADEH[1], MASOUMEH DOURAGHI[2], KHOSROU M. ABDI[3], ABBAS AGHAEI AFSHAR[4], JASEM GHAFFARI[1], RAZIEH SHABANI KORDSHOULI[1], MORTEZA AKBARI[5], JAVAD RAFINEJAD[1,♥]

[1]Department of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Sciences. Tehran, Iran.
♥email jrafinejad@yahoo.com
[2]Division of Microbiology, Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences. Tehran, Iran
[3]Department of Medicinal Chemistry, Faculty of Pharmacy and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences. Tehran, Iran
[4]Department of Medical Entomology and Vector Control, School of Public Health, Kerman University of Medical Sciences. Kerman, Iran
[5]Department of Public Health, School of Health, Ilam University of Medical Sciences. Ilam, Iran

Manuscript received: 18 October 2020. Revision accepted: 23 December 2020.

Abstract. *Amiri ZS, Akbarzadeh K, Douraghi M, Abdi KM, Afshar AA, Ghaffari J, Kordshouli RS, Akbari M, Rafinejad J. 2021. Effectiveness of maggot extractions and secretion (E/S) of* Lucilia sericata *in reducing wound surface in an experimental scalding burn injury. Nusantara Bioscience 13: 11-15. Lucilia sericata* larvae have been successfully used as medicinal larvae for wound healing. This study was performed to evaluate the therapeutic effects of the primary ointment made from excretory-secretory substances of *L.sericata* larvae on experimental burn wounds on rabbits under laboratory conditions. Extraction of ES from the third stage of *L.sericata* larvae and antibiogram testing was done. Four rabbits were randomly divided into four groups; three standard third-grade burn wounds were created on the back of each rabbit for intervention groups (B, D) of wounds were used respectively from the original ointment made from ES and ES without accompanying material. Nitrofurazone ointment was used for the positive (C) and placebo ointment was used for the negative (A). On the 21st day, all scars of wounds in groups B and D were separated and the wound was free of infectious tissue, whereas in groups A and C that the scars were clinging to the base. The level of the ulcer was significantly lower in the E/S intervention group and the infectious tissue was not observed during the treatment of group B and D wounds. Methicillin-resistant *Staphylococcus aureus* (MRSA) did not show resistance to excretory-secretory substances of *L. sericata* larvae. The debridement and recovery process was faster in the ES-treated group and the initial ointment than in the control group. The results showed that maggot extractions and secretion (E/S) of *L. sericata* in reducing wound healing is appropriate for treatment.

Keywords: Burn, *Lucilia sericata*, ointment, secretion-excretion, wounds

INTRODUCTION

A Necrophagous maggot is the larva of some flies (Order Diptera) that have a beneficial property. They have increasingly being applied to clean wounds by destroying, liquefying, and ingesting only devitalize / necrotic tissue for centuries which is known as maggot therapy (Yan et al. 2018). In this method, larvae of flies Calliphoridae family, including *Lucilia sericata* (Greenbottle fly) and *Calliphora vicina* using for wound healing (Yan et al. 2018).

Many people around the world suffer from burn injuries (Mogoșanu and Grumezescu 2014). Burn ulcer is a type of skin injury that often long-term treatment with high economic costs. Moreover, may become infected during treatment and become more painful, instead of gradually improving (Sterling et al. 2010). Burn ulcers are caused mainly by exposure to extra heat, electricity, light, radiation as well as chemical elements. It mostly occurs only on the skin (Murphy and Evans 2012; Sterling et al. 2010). In addition to the local effect on the skin, heat has many general effects on the body. These complications are specific to burn injuries and are not generally seen in wounds caused by other injuries (Tiwari 2012).

Maggots were first used to treat wounds during the American Civil War. However, in 2004 the Food and Drug Administration (FDA) authorized the use of maggots for medical use (Sherman 2005). *L. sericata* larvae are successfully used as medicinal larvae in wound healing and help the healing process by proteolytic digestion, disinfection of necrotic tissue, and stimulation of active promotion of granulation tissue formation (Steenvoorde et al. 2007). Regardless of the preference of the mode of action, larvae of *L. sericata* initiate their activities in the wound by chemical secretions and excretions (Nigam et al. 2006; Sherman 2014). Maggot extractions and secretion (ES) treatment have been shown to aid the healing process by stimulates the regeneration of blood vessels and also has a proliferative effect on the growth of human endothelial cells (Elshehaby et al. 2017). Many studies have shown evidence that larvae have antimicrobial activity in two ways, including antimicrobial action by ingestion larvae of wound bacteria, which are killed when they enter the larval

digestive tract also through the antimicrobial activity of secretions and larval excretions, including salivary gland secretions and fecal wastes (Valachova et al. 2013). However, the main mechanisms in wound healing with ES treatment are not yet completely understood, This method became popular around the world for the treatment of chronic and infectious wounds (Hou et al. 2007).

Today, despite advances in technology and healthcare, chronic wound management remains a major challenge and therefore requires alternative methods. This study has been conducted to study excretion/secretions (E/S) of larvae of *L. sericata* on the healing of experimental burn wounds in rabbits.

MATERIALS AND METHODS

Rearing of flies

Lucilia sericata was rearing continuously in the fly insectary of Tehran University of Medical Sciences from 2013. The temperature and relative humidity in the insectary were 27±2°C and 50±10% respectively. The light/dark regimen was 16/8 hours. The adult flies were fed by sugar, milk powder, palm dates, and water but their larvae fed on the chicken liver for rearing.

Ethical approval

The ethical committee of Tehran University of Medical Sciences, Iran performed ethical approval using rabbits. (Ethics code: IR.TUMS.SPH.REC.1397.4977)

Extracting of E/S

As much as 300 (in three equal replicates) of third instar larvae of *L. sericata* with 12-14 millimeters in length were selected to find the average weight of 100 larvae. This weight was used as a standard for selecting larvae for extraction. For extracting 3.7 grams of larvae were selected and washed with normal detergent as the first step. Washing with ddH2O, submerging in EtOH 70% for 30 seconds, and again washing with ddH2O were the next steps for cleaning the larvae before the extraction process. Extracting of E/S was started with the put of these larvae into a disinfected laboratory beaker and adding 1000 microliter of PBS (Phosphate Buffered Saline). The beaker was covered with a sterile mosquito net and put in the dark area at room temperature for 5 hours. After this time duration, the liquid in the beaker was centrifuged at 10000 g for 10 minutes. The supernatant, which has been mentioned as pure E/S, was stored at-20°C for future use.

Protein assay

Three replicates of extracted E/S of the larvae were assayed with a BCA kit (cat.T9300A) according to manufacturer construction.

Microbial examination of E/S

The Antimicrobial activity of E/S was tested in vitro using the agar well diffusion method (Scott 1989) and Mueller Hinton Agar medium. Bacterial inoculums that were used for antimicrobial activity were a sensitive strain of *Staphylococcus aureus* and MRSA (methicillin-resistant *S. aureus*). A dilution of 0.5 McFarland of this bacteria was spread on Mueller Hinton agar using a sterile swab, which has moistened in the solution. Wells of 6 mm were punched in the inoculated plate, which was filled with six dilutions of E/S (1, ½, ¼, 1/8, and 1/16). Also, PBS and Cefoxitin Antibiotic were used for negative and positive control, respectively. PBS was used for making dilutions of E/S as well as filling of at least one of the wells in each plate as the negative control. As much as 80 microliters of each dilution were pipetted in the wells as treatment. Three replicates of each dilution were tested. The growth inhibition zone around each well was measured after 24 hours of incubation at 37°C. This test was performed out in triplicate and zones of inhibition were measured in mm. scale.

Experimental ointment

For better stability and permeability of E/S, an experimental ointment has prepared by mixing 1gr of urea and 10gr of Eucerine with the E/S extract.

Producing experimental burn injury

Male rabbits (n=4; age, 16-18 months; weight, 2.5±2 kg) were selected in the Laboratory of Experimental Animals in the School of Public Health of Tehran University of Medical Sciences. The lab had controlled conditions at 25±2°C and a 12/12 dark/light regimen. The animal models had free access to food and water. The Ethical Committee of the School of Public Health of Tehran University of Medical Sciences approved all steps of the project. Ketamine and Xylazine were used for anesthetizing rabbits via intraperitoneal injection with doses of 100 and 50 mg/kg respectively. Three wounds with about 20 mm in diameter were produced on the back of the rabbits using hot water after complete anesthetizing, disinfecting by EtOH70%, and local shaving. Ten seconds were enough to produce each of the 3rd degrees of burn wounds. Treating was started after 24 hours from the time of burning. Each of four rabbits was selected randomly to allocate to one of four testing groups including A, B, C, and D. Groups D and B were treatment groups for treating with pure E/S and experimental ointment respectively. Groups C and A were control groups for treating with Nitrfurazone ointment (as positive control) and placebo (as negative control) respectively. The wounds were left uncovered after each treatment.

Data collecting

The inspection was done at 24 hours intervals to photographing the wound surfaces and repeating of treatments. Each photo was used for the measurement of the necrotic and granulated tissues by Matlab software. Observations were recorded due to the appearance of the wounds, bad odors as well as any infectious discharge. On the last day of the study untreated rabbit's unconsciousness and disappeared.

RESULTS AND DISCUSSION

The average weight of 300 individuals of third larvae with 12-14 mm in body length was 3.7±0.2 g. This weight was used for future tests instead of counting 100 larvae for each replicate.

Results of protein assay showed that the average of whole proteins in E/S of 100 individuals of third larvae was 1159.7±44.7 µg/mL.

Results of agar well diffusion tests showed that the pure E/S is the most effective for removing both sensitive and resistant strains of *S. aureus*. Mean zone inhibition of pure E/S were 19.3±0.58 mm and 17.6 ±0.58mm in diameter for sensitive and resistant strains of *S. aureus* respectively (Table 1).

Repeated measure analysis was used for evaluating the trend of reducing the wound surface over time, which can be mentioned as a trend of improvement in all wounds. The analysis showed that the reduction of the wound surface in each group was significant from the first day of starting (P≤0.001). Due to the LSD analysis and comparison of mean wound size in groups over time, wound size changes in the E/S group with the negative control group have a significant difference (P≤0.011). (Figure 1).

Pure E/S (purple line), experimental ointment (green line), positive control with nitrofurazone (yellowish line), negative control (blue line).

The covariance Test showed a significant difference in reducing wound surfaces between negative control and each of pure E/S and experimental ointment as well as between positive control and pure E/S (Table 2, Figure 2).

Numerous research has been focused on the antibacterial activity of maggots ES against gram-positive and gram-negative bacteria but the results have been different. The most important effect of burning is the loss of protective function of skin against dangerous pathogenic microorganisms (Murphy and Evans 2012) such as *S. aureus*, *Pseudomonas aeruginosa*, and some other microorganisms (Van Duin et al. 2016). Therefore the risk of infection is high for burn patients (Coban 2012). The results of this study showed that pure E/S is the most effective for removing both sensitive and resistant strains of *S. aureus* (MRSA). Regarding the antibacterial effect of maggots, it was found that the maggot ingests and kill *E. coli* when it passes through the midgut (Mumcuoglu et al. 2001). Thomas et al. (1999) confirmed the antibacterial activity of secretions against a range of bacteria, including *S. aureus* and methicillin-resistant *S. aureus* (MRSA) in a preliminary laboratory study (Thomas et al. 1999).

Long-time hospitalization will increase the risk of infection (Shupp et al. 2010) with special attention to drug-resistant infections (Branski et al. 2009). However, in maggot therapy, there is no need for surgery and hospitalization, and compared to other treatment methods has a lower cost.

The results of this study showed that the reduction of the wound surface in each group was significant from the first day (P≤0.001) and wound size changes in the E/S group with the negative control group have a significant difference (P≤0.011). In another study, It was found *L.sericata* larval secretions could modulate the mRNA expression of some wound healing-related genes and contains components that are effective in wound healing (Akbas et al. 2020).

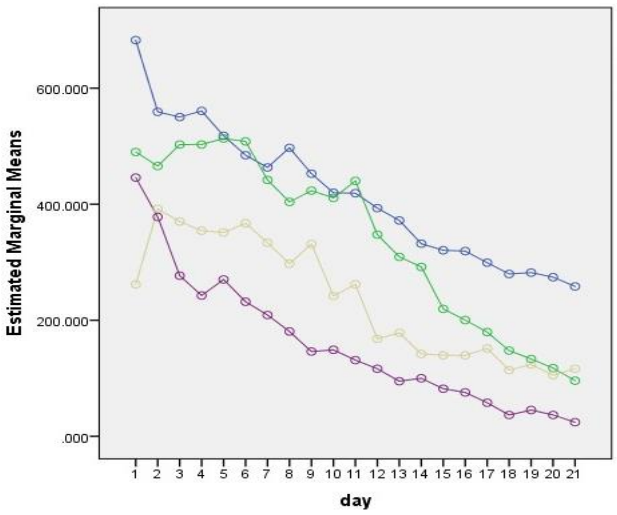


Figure 1. Comparing of average reducing trends in wound surfaces among control groups

Table 1. Zone inhibition of various dilutions of E/S of 100 larvae of *L. sericata* in agar well diffusion test**.**

Type of *Staphylococcus aureus*	Replicates	Dilutions of E/S				
		1	**½**	**¼**	**1/8**	**1/16**
Sensitive *S. aureus*	1	19	17	13	9	7
	2	19	14	13	10	-
	3	20	14	10	10	-
	Average	19.33	15	12	9.66	
	SD	0.58	1.73	1.73	0.58	
Resistant *S. aureus*	1	17	15	11	9	-
	2	18	12	12	8	-
	3	18	14	10	8	-
	Average	17.67	13.67	11.00	8.33	
	SD	0.58	1.53	1.00	0.58	

The most important aid for healing burn wounds is wound bed preparation, which has different methods .The standard method for dealing with such wounds is fast and wide debridement with a graft (Madihally et al. 2003). Whatever the method, debridement is an integral part of it; this is possible in both mechanical and chemical (enzymatic) forms. The history of wound enzymatic treatments using plant or bacterial-derived enzymes dates back to about 60 years before World War II (Hafezi et al. 2009). The enzymes trypsin, leucine, aminopeptidase, and carboxypeptidases A and B have been isolated from the secretions of *L. sericata* larvae (Vistnes et al. 1981). Schmidtchen et al. (2003) used animal models to show the secretory properties of maggot E/S in resolving necrotic tissue, they also identified several proteases (Schmidtchen et al. 2003).

Debridement should be done in almost all of the burn ulcers for treatment (Mann and Heimbach 1996). Non-selective debridement, which is usual for the treatment of wounds, may damage basement tissues other than necrotic. One of the best selective methods for debridement is maggot therapy (Colin et al. 1996) which has a good background in the healing ulcers caused by burning, chronic infection, diabetes complications, and post-surgical wounds (Wu et al. 2017). In addition to healing wounds, maggot disinfects wounds and stimulates the regenerative process by proteolytic digestion (Nigam et al. 2006).

Managing pain in the treatment of burn wounds is crucial (Mann and Heimbach 1996) which may limit the use of free larvae in maggot therapy by *L.sericata* (Mumcuoglu et al. 2012) especially for use in burn wounds. Elimination of physical contact can be a solution for solving the painful use of larvae in burn wounds without reducing their effects in debridement and subsequent wound healing.

Larval secretions using deoxyribonuclease (DNAse) can destroy microbial DNA as well as human DNA in necrotic tissues (Brown et al. 2012). Biofilms and biological materials in wounds have become increasingly known as a problem. Therefore, focus on new strategies for the eradication of severe infections associated with biofilm is very important. The presence of small antibacterial agents (1 kDa) in E/S showed that against a range of bacteria including *S. aureus* is active (Elshehaby et al. 2017). E/S enzymes can dissolve biofilms and inhibit biofilm growth (Elshehaby et al. 2017; Harris et al. 2009).

The E/S extract from *L. sericata* larvae with its proteolytic properties can be effective in the chemical debridement of burn wounds, the process of debridement, preparation of the wound bed, and treatment. According to the results of this study, we can see a significant change in the treatment of such wounds. The application of the results of this research, due to its experimental and fundamental nature, can be a basis for further research on volunteers or drug manufacturing. The use of proteolytic enzymes is very extensive and can be used in all wounds that have necrotic tissue and require debridement, including compression, diabetic, and other chronic wounds. It is recommended that further research be continued in this area.

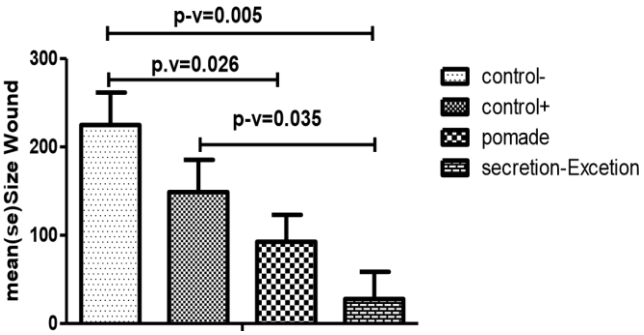


Figure 2. Comparisons of average reduction in wound size at last day of treatment (day 21). : pure E/S, : experimental ointment, : positive control, : negative control

Table 2. Comparisons of average reduction in wound size at last day of treatment (Day 21)

Name groups (A)	Name group contrastable (B)	Dispute mean	Sig.b
Placebo	Ointment	132.2*	.026
	Ointment Nitrofurazone	75.9	.238
	Excretion-Secretion	196.9*	.005
Pomade	Placebo	-132.2*	.026
	Ointment Nitrofurazone	-56.2	.282
	Excretion-Secretion	64.6	.179
Pomade Nitrofurazone	Placebo	-75.9	.238
	Ointment	56.2	.282
	Excretion-Secretion	120.9*	.035
Excretion-Secretion	Placebo	-196.9*	.005
	Ointment	-64.6	.179
	Ointment Nitrofurazone	-120.9*	.035

ACKNOWLEDGEMENTS

We would like to thank the department of microbiology of the school of public health at the Tehran University of Medical Sciences. This study was part of an M.Sc. dissertation funded and supported by Tehran University of Medical Sciences, Iran (Grant no. 9511263002).

REFERENCES

Akbas F, Ozaydin A, Polat E, Onaran I. 2020. *Lucilia sericata* larval secretions stimulating wound healing effects on rat dermal fibroblast cells. Rec Nat Prod 14: 340-354.

Branski LK, Al-Mousawi A, Rivero H, Jeschke MG, Sanford AP, Herndon DN. 2009. Emerging infections in burns. Surg Infect 10: 389-397.

Brown A, Horobin A, Blount D, Hill P, English J, Rich A, Williams PM, Pritchard D. 2012. Blow fly *Lucilia sericata* nuclease digests DNA associated with wound slough/eschar and with P*seudomonas aeruginosa* biofilm. Med Vet Entomol 26: 432-439.

Coban YK. 2012. Infection control in severely burned patients. World J Crit Care Med 1: 94-101

Colin D, Kurring P, Quinlan D, Yvon C. 1996. Managing sloughy pressure sores. J Wound Care 5: 444-446.

Elshehaby M, Tony M, Sultan AR, Reheem A, Baset A. 2017. Antibacterial properties of larval secretions of the blowfly, *Lucilia sericata* (Diptera: Calliphoridae). Egypt Acad J Biol Sci 9: 1-12.

Hafezi F, Elmirad H, Pedram MS. 2009. Determination of the macroscopic effect of kiwi fruit on wound healing in rats (a new effective drug for the treatment of deep chronic wounds). Iran J Surg 17: 1388-1397.

Harris LG, Bexfield A, Nigam Y, Rohde H, Ratcliffe NA, Mack D. 2009. Disruption of *Staphylococcus epidermidis* biofilms by medicinal maggot *Lucilia sericata* excretions/secretions. Intl J Artif Organs 32: 555-564.

Hou L, Shi Y, Zhai P, Le G. 2007. Antibacterial activity and in vitro anti-tumor activity of the extract of the larvae of the housefly (*Musca domestica*). J Ethnopharmacol 111: 227-231.

Wu JC, Lu RR, Huo R, Fu HB, Song SM. 2012. Maggot therapy for repairing serious infective wound in a severely burned patient. Chin J Traumatol 15: 124-125.

Madihally S, Solomon V, Mitchell R, Van De Water L, Yarmush M, Toner M. 2003. Influence of insulin therapy on burn wound healing in rats. J Surg Res 109: 92-100.

Mann R, Heimbach D. 1996. Prognosis and treatment of burns. West J Med 165: 215-220.

Mogoşanu GD, Grumezescu AM. 2014. Natural and synthetic polymers for wounds and burns dressing. Int J Pharm 463: 127-136.

Mumcuoglu K, Davidson E, Avidan A, Gilead L. 2012. Pain related to maggot debridement therapy. J Wound Care 21: 400-405.

Mumcuoglu KY, Miller J, Mumcuoglu M, Friger M, Tarshis M. 2001. Destruction of bacteria in the digestive tract of the maggot of *Lucilia sericata* (Diptera: Calliphoridae). J Med Entomol 38: 161-166.

Murphy PS, Evans GR. 2012. Advances in wound healing: A review of current wound healing products. Plast Surg Intl 2012: 1-8.

Nigam Y, Bexfield A, Thomas S, Ratcliffe NA. 2006. Maggot therapy: The science and implication for CAM Part I—history and bacterial resistance. Evid. Based Compl Altern Med 3: 223-227.

Schmidtchen A, Wolff H, Rydengard V, Hansson C. 2003. Detection of serine proteases secreted by *Lucilia sericata* in vitro and during treatment of a chronic leg ulcer. Acta Derm Venereol 83: 310-311.

Sherman RA. 2005. Age-old therapy gets new approval. Adv Skin Wound Care 18: 12-15.

Sherman RA. 2014. Mechanisms of maggot-induced wound healing: What do we know, and where do we go from here?. Evid Based Compl Altern Med 2014: 592419. DOI: 10.1155/2014/592419.

Shupp JW, Pavlovich AR, Jeng JC, Pezzullo JC, Oetgen WJ, Jaskille AD, Jordan MH, Shoham S. 2010. Epidemiology of bloodstream infections in burn-injured patients: A review of the national burn repository. J Burn Care Res 31: 521-528.

Steenvoorde P, Jacobi CE, Van Doorn L, Oskam J. 2007. Maggot debridement therapy of infected ulcers: Patient and wound factors influencing outcome-a study on 101 patients with 117 wounds. Ann R Coll Surg Engl 89: 596-602.

Sterling J, Heimbach D, Gibran N. 2010. Management of the Burn Wound. ACS Surgery: Principles and Practice. Marcell Decker, Hamilton, ON.

Thomas S, Andrews AM, Hay NP, Bourgoise S. 1999. The anti-microbial activity of maggot secretions: results of a preliminary study. J Tissue Viability 9: 127-132.

Tiwari V. 2012. Burn wound: How it differs from other wounds?. Indian J Plast Surg 45: 364-372.

Valachova I, Bohova J, Kozanek M, Takac P, Majtan J. 2013. *Lucilia sericata* medicinal maggots: A new source of antimicrobial compounds. Formatex 2013: 1745-1753.

van Duin D, Strassle PD, DiBiase LM, Lachiewicz AM, Rutala WA, Eitas T, Maile R, Kanamori H, Weber DJ, Cairns BA, Napravnik S, Jones SW. 2016. Timeline of healthcare-associated infections and pathogens after burn injuries. Am J Infect Control 44: 1511-1516.

Vistnes L, Lee R, Ksander G. 1981. Proteolytic activity of blowfly larvae secretions in experimental burns. Surgery 90: 835-841.

Yan L, Chu J, Li M, Wang X, Zong J, Zhang X, Song M, Wang S. 2018. Pharmacological properties of the medical maggot: A novel therapy overview. Evid Based Compl Altern Med 2018: 4934890. DOI: 10.1155/2018/4934890.

NUSANTARA BIOSCIENCE
Vol. 13, No. 1, pp. 16-23
May 2021

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n130103

Histological aberrations and mode of damage of cowpea (*Vigna unguiculata*) by *Colletotrichum destructivum*

DAVID NWAZUO ENYIUKWU♥, ANDERSON CHIDI AMADIOHA, CHARLES CHIMEZIE ONONUJU
Department of Plant Health Management, Michael Okpara University of Agriculture. Umudike Km 10, Umuahia-Ikot-ekene Road, PMB 7267 Umuahia, Abia State, Nigeria. Tel.: +234-9092309790, ♥email: enyidave2003@gmail.com

Manuscript received: 11 October 2020. Revision accepted: 28 December 2020.

Abstract. *Enyiukwu DN, Amadioha AC, Ononuju CC. 2021. Histological aberrations and mode of damage of cowpea (*Vigna unguiculata*) by* Colletotrichum destructivum. *Nusantara Bioscience 13: 16-23.* Leaf and stem of healthy 2-week old cowpea (*Vigna unguiculata* L. Walp.) seedlings were inoculated with spore suspension of the *Colletotrichum destructivum* O'Gara. Sections of the infection courts were prepared and examined under digital microscope. The results showed that the infection process began 12 hours after inoculation (hai) with the germination of spores of the fungus. Large multi-lobed primary hypha (somewhat spherical in shape) developed from the infection peg and remained inside a single epithelial cell for about 3 days. Towards the end of this time, the primary hyphae developed thin filamentous tentacles that punctured and branched into adjoining cells, initiating necrotrophic phase of the disease. During this period, typical anthracnose symptoms began to develop on the infected organs of the crop. The entire infection process lasted a maximum of 96 h, at the end of which acervulus that bore a single seta emerged on infected crop lesions. The mechanism of damage of the pathogen involved intra-cellular and inter-cellular colonization of the host tissues early and late in the disease cycle. The integrity of the affected tissues' cells was compromised by passage and colonization of *Colletotrichum destructivum* O'Gara evidenced by lack of clear-cut middle lamella and cell boundaries. Anthracnose affected cells dispossessed of vital nutrients became turbid, devoid of turgidity and vigor. Thus, these results have strong implications for plant health management in that effective environment-compliant control of the fungus should be initiated on or before the third day following arrival of the conidia of the pathogen on the crop. Seeds are major agents of introduction of *Colletotrichum* spp. to disease-free locations. Findings from this study also support that strong trans-border control of seeds of the crop should be maintained since the pathogen is known to be seed-borne and demonstrates sufficient cross-infection of crop plants.

Keywords: Anthracnose, *Colletotrichum destructivum*, cowpea, hemibiotrophy, hyphal colonization

INTRODUCTION

The place of legumes especially cowpea (sometimes called southern pea) (*Vigna unguiculata* L. Walp.) (Figure 1.A) in the nutrition of the third world cannot be overemphasized. The crop serves as both vegetable and pulse crop. It remains a major provider of proteins, amino acids, essential vitamins, and minerals in many meat-scarce locations of tropical third worlds (Enyiukwu et al. 2018; Falade et al. 2018). Though the crop is important as bio-fertilizer in farming systems and a source of valuable amino acids in human nutrition, however, economic production of this important legume is deeply constrained by arthropod pests and pathogenic diseases; amongst which anthracnose (Figure 1.B) is major (Adegbite and Amusa 2008; Akinbode and Ikotun 2008a,b).

Colletotrichum species are underscored as causal agents of anthracnose which are commonly associated with various tropical and sub-tropical legumes in both field and storage (Begum et al. 2007; 2013). *Colletotrichum* spp. are ranked amongst the top 10 most damaging plant pathogens on a world scale; causing large scale economic losses in the production, shelf life and marketing chains of agro-produce including solanum and legumes (Sharma et al. 2017; Coates et al. 2019; Guarnaccia et al. 2019; Xie et al. 2019) In China, India, Brazil, Uganda, South Africa, Ethiopia, Nigeria and various parts of Asia *C. gloeosporioides*, *C. fragerie*, *C. dermatium*, *C. lindemuthianum* and *C. destructivum* have been reported to be associated with anthracnose in cowpea (Enyiukwu 2017). In Nigeria, *C. lindemuthianum* and *C. destructivum* have been advanced as cause of anthracnose of the crop in the country; however, extensive molecular evaluations of many accessions of both pathogens have conclusively upheld the later as solely responsible for the disease in the crop (Latunde-Dada et al. 1996; 1997; Adegbite and Amusa 2008; Akinbode and Ikotun 2008a,b; ASHC 2015). Besides cowpea, curly dock, angula (*Eruca sativa*), tobacco, lucerne, alfalfa, *Arabidopsis thaliana*, and dodder are affected by anthracnose due to members of *Colletotrichum destructivum sensu lato* (Lee and Kim 2001; Patel et al. 2014; Enyiukwu et al. 2014; Da Silva 2017). Though *Colletotrichum* spp are host-specific, cross-infection of hosts has been reported in the genera (Coates et al. 2019).

During no crop seasons, *C. destructivum* overwinters in the seed coats, cotyledons, crop thrash, or related crop debris until the next cropping season (Enyiukwu and Awurum 2013a; Enyiukwu et al. 2014; Guarnaccia et al. 2019). Under warm temperatures (27-28^{o}C especially at night), prolonged moist conditions or free moisture on susceptible host surfaces or soils (lasting 24-48 h); spores of the fungus germinate to initiate the disease on the crop

(Miles et al. 2013; Enyiukwu and Awurum 2013b; Falade et al. 2018). Dark, depressed, irregularly shaped, water-soaked lesions develop on affected host stems, branches, petioles, and lamina (Figure 1.B) which coalesce rapidly to girdle plant (Coates et al. 2019). In Nigeria, incidence and yield losses as high as 83 % and 50-100 % respectively have been ascribed to moderate to severe attacks of anthracnose disease on cowpea accessions grown in different agro-ecological zones of the country (Awurum 2013; Enyiukwu et al. 2014).

In their relationships with host crops, *Colletotrichum* species exhibit endophytic, latent, necrotrophic, and hemibiotrophic lifestyles; however, hemibiotrophy is the most common life-strategy (Smith et al. 1999; Da Silva et al. 2017). Hemibiotrophy is sub-categorized into sub-cuticular intramural strategy which is common in *C. capsici* and amongst some pathogens in *C. gloeosporioides* clade; and intracellular hemibiotrophic lifestyle which characterizes *C. destructivum sensu lato* (Enyiukwu et al. 2014; Da Silva et al. 2017). *C. destructivum* and related species live intracellular hemibiotrophic lifestyle (Latunde-Dada et al. 1996; 1997; 1999). The successes of *Colletotrichum* spp. in the colonization of susceptible hosts and initiating anthracnose disease is reported to depend among other variables on their ability to elaborate specialized infection structures from their conidia such as appressoria and infection pegs on target tissues (Amadioha 1994; Tucker and Talbot 2001; Damm et al. 2009; 2014). In the case of *Colletotrichum destructivum sensu lato*; the infection peg penetrates the host cuticle mechanically, forms the primary hypha by which it establishes short-lived biotrophic lifestyle in a single epidermal cell which lasts between 48-72 h post-inoculation (Shen et al. 2001; Da Silva et al. 2017). After this time, the pathogen graduates to a damaging enzymes-assisted necrotrophic relationship characterized by extensive development of thin secondary h\yphae into adjoining cells, and emergence of anthracnose symptoms (depressed dark tan lesion with black acervuli) on the crop (Latunde-Dada et al. 1996; 1999). The lesions of the disease on the crop (Figure 1.B) stems from anatomical aberrations as the fungus wades through and breaks down host's tissues; causing reduction in photosynthetic area, lowering photosynthetic rate; reduction of total chlorophyll occasioned by toxic metabolites from the fungus; and depletion of essential nutrients and vital electrolytes (Meyer et al. 2001; Lobato et al. 2014; Markson et al. 2014). Besides, in stem infections, the fungus compromises interlocking cross walls responsible for strength and rigidity of stems and branches leading to tipping over and collapse of seedlings (Amadioha and Enyiukwu 2019a,b).

In affected cowpeas, substantial leakage of electrolytes, loss of vital nutrients such as proteins, carbohydrates lipids, crude fiber, calcium, and phosphorus from the crop; and a general reduction of quality of their products have been reported (Begum et al. 2008; 2013). In several field and storage evaluations, average loss of proximate and elemental nutrients ranging between 20 to 30 % attend the interaction of susceptible varieties of cowpea with the pathogen within 1 month period (Amadioha and Enyiukwu 2019a,b). Parallel evaluations of the effects of related fungi on yams, sweet and Irish potatoes revealed a nutrient loss profile of 30-60 % during the 3-6 months storage period (Markson et al. 2014). *C. destructivum* and other crop decimating fungi utilize host-derived proteins, electrolytes, and metabolites to support their own growth, accumulate biomass and generate energy (Strivastava et al. 2013; Amadioha and Enyiukwu 2019a, b). Smith et al. (1999) and Latunde-Dada et al. (1999) noted that infection process of *Colletotrichum* species is attended by distortions in the anatomy and physiology of the susceptible crop cultivar.

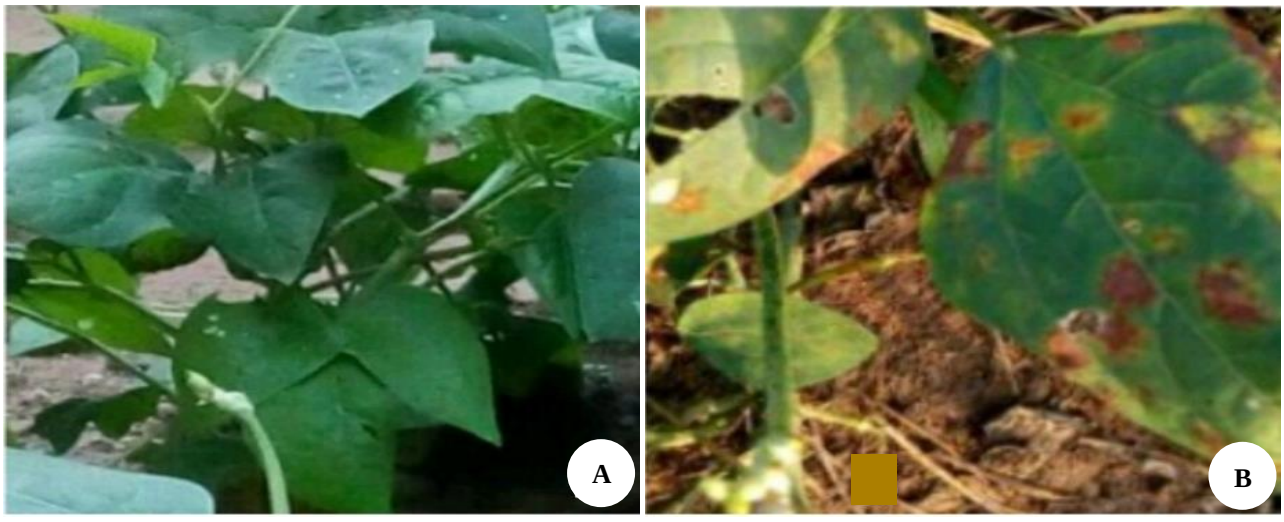


Figure 1. Cowpea (Var. IAR-48) growing in the field. A. Healthy (uninfected) cowpea. B. Anthracnose infected cowpea showing dark-tan irregular lesions on leaf of the crop

Though the identity and infection process of cowpea anthracnose pathogens have been studied in South Africa on one hand and Southwestern Nigeria on the other; however, no similar studies have been conducted on the crop in Southeast region of Nigeria. So far, therefore, information on the infection process and extent of damage to cowpea tissues by *C. destructivum* and its synonyms during infection and colonization in the area is to the best of our knowledge non-existent. Against the backdrop of the huge loss profile on the biological and economic yields of the crop due to the disease; therefore, this work was undertaken to study the infection process and determine the extent of anatomical damage done to stem and leaf tissues of cowpea by *C. destructivum* during infection and colonization of the crop in the humid Umudike, Southeast, Nigeria.

MATERIALS AND METHODS

Experimental site

The experiment was conducted at the Crop Science Laboratory and greenhouse of the Department of Plant Health Management, College of Crop and Soil Sciences as well as the Microbiology Laboratory of the College of Veterinary Medicine; all of the Michael Okpara University of Agriculture, Umudike, Abia State. The University is located at latitude $5^{o}29^{1}$N, longitude $7^{o}33^{1}$E, and an altitude of 121.08 meters above sea level (GPS Coordinates 2017).

In vitro experiment

Isolation and identification of the causal agent

Pods of infected cowpea (*Vigna unguiculata* (L.) Walp) with typical symptoms of anthracnose were collected from the University Research Farm. The pods were cut in bits using a surgical blade, sterilized in 70 % ethanol and finally washed in several changes of 200 ml of sterile distilled water. The tissues were placed in Petri dishes containing moistened Whatman No 1 filter paper, and incubated for 7 days at 27^{0}C. Then 39.50 g of dehydrated potato dextrose agar (PDA) (Oxoid® ThermoScientific Product, England, UK) was dissolved in 1000 ml of sterile distilled water in a 2L flask, stirred thoroughly with a glass stirrer, then stoppered and autoclaved at 15 Psi for 30 minutes. The mycelial growth from the plated tissues was repeatedly sub-cultured to obtain the pure culture of the organism maintained on PDA as prepared above. The isolate was subjected to pathogenicity tests by re-inoculating it into 2-week old cowpea seedlings. About 4 days after inoculation of the seedlings, typical anthracnose symptoms resembling those observed on the diseased pods were observed on the leaf blades and petioles of the seedlings (Ogu and Owoeye 2013; Markson et al. 2014). Thus, confirming that the organism is pathogenic.

Slides of the organism were then prepared, mounted and examined under a microscope. The organism's identity was confirmed to be *C. destructivum* by the aid of fungi identification manual by Barnett and Hunter (1995), annotated genera of *Colletotrichum* species by Damn et al. (2009; 2014) and monographs of the International Mycological Institute IMI (1995).

Preparation of spore suspension

The spores of the pathogen *C. destructivum* were collected from 8-day old culture-agar stock in Petri dish by lifting 60 cm^2 piece into a beaker containing 200ml of sterile distilled water. This was sieved through 4-folds of sterile cheesecloth to remove agar and mycelial mesh; and the filtrate was then centrifuged for 10 minutes. Thereafter the spores suspension was standardized using a hemocytometer counting slide to 10^5 spores/ml (Awurum and Enyiukwu 2013b; Alberto 2019).

Preparation of seedlings for the study

Cowpea (Var. IAR: 48) seeds obtained from the Research and Training Unit of the College of Crop and Soil Sciences, were surface-sterilized in a 0.5 % sodium hypochlorite solution for 1 minute, and rinsed in 3 changes of 200 ml of sterile water. The seeds were sown 3 per 20 cm diameter plastic pot containing 4 kg heat-sterilized topsoil; and kept moist by light watering twice daily. At 8 days after sprouting, the seedlings were thinned to two per pot and remained so till 2 weeks after planting (WAP) when they were used for histological studies (Enyiukwu et al. 2018).

Histological studies

The method according to Latunde-Dada and Lucas (2007) was adopted for this study. Fresh uninfected leaves and stems were excised from the 2-week-old seedlings of cowpea (Var. IAR-48) as raised above; and inoculated on the surface (abaxial surface for the leaves) with four 3 µl droplets of conidial suspension (1.0 x 10^5 spores/ml of distilled water) (Vasic et al. 2014; Xie et al. 2019) of *C. destructivum*. The inoculated leaves and stems were incubated at 25°C in the dark chamber under humid conditions (Latunde-Dada et al. 1997). In the control experiment leaves and stems were treated with sterile de-ionized water in place of the conidial suspension of the fungus.

Pieces of the infected and healthy cowpea leaves and stems were cut after 72 h (1-4 day) intervals from the infection courts and de-colorized in a 0·15% (w/v) solution of trichloroacetic acid in a 3:1 (v/v) mixture of ethanol and chloroform for 12 h. They were then stained in a 0·025% (w/v) solution of aniline blue in lactophenol for 2 h (Xie et al 2019). Light microscopic examinations were made on sections of the stained tissues with Olympus digital compound microscopic fitted with the software Scopevision (Version 9.0) camera and photographed. The photograph was taken of both healthy (uninfected) and diseased leaves and stems of cowpea infected with *C. destructivum.*

RESULTS AND DISCUSSION

Penetration and anatomical damage of cowpea leaf tissues by *Colletotrichum destructivum*

The process of colonization and damage of cowpea leaf tissues by the *C. destructivum* is captured in Figures 2-5. The conidium of the test fungus which is hyaline, non-septate, smooth-walled, ovoid, straight or slightly curved with more or less tapered ends (Figure 3), came in contact with and stuck on the cuticle of the leaf and germinated at 12 hai to produce germ tube, at the end of which it developed appressorium (Figure 3). Under this structure, it produced penetration peg which penetrated through the cuticle into a single epithelial cell; to form a multi-lobed somewhat spherical vesicle at about 48 hai (Figure 4.A-B). From this large primary structure thin multilateral hyphae developed at about 72 hai and initiated the tissue colonization (Figure 4.C-D) ramifying within the host tissue and subsequent production of the lesions ensued which was seen as the symptom of anthracnose disease of cowpea from 96 hai (Figure 4.E-F). The colonization of the tissues by the hyphae of the test fungus was both inter-cellular (Figure 5.A) and intra-cellular (Figure 5.B-C).

Anatomical aberrations of cowpea stem tissues due to the test fungus

The photomicrograph presented in Figure 6 shows comparative anatomical structures of healthy (uninfected) and infected cowpea stem with *C. destructivum*. The histological studies results indicated that the fungus strongly affected the integrity of stem tissues of the crop. All the cells of the healthy stem of the test cowpea (epithelial, cortical, vascular, and pith) were intact and well defined; they remained turgid and full of vitality, with the cementing middle lamella at the boundaries of the cells being clearly visible (Figure 6.A). In contrast, however, the activity of *C. destructivum* damaged the stem tissues of infected cowpea (Figure 6.B). The infected tissue cells were turbid and dispossessed of their vital nutrients during colonization of the pathogen, resulting in their distorted cell boundaries and lack of turgidity and vigor (Figure 6.B).

Discussion

Results of this study showed that conidia of the test fungus were hyaline, non-septate, smooth-walled, ovoid-shaped, straight structures (Figure 3.A). This description tallies with those given by other workers who reported the same features for the fungus conidia with average dimension of 15 x 4 µm isolated from lucerne, alfalfa, and cowpea (Latunde-Dada et al. 1996; 1997; 1999. O'Connell et al. 2004). The results also showed that conidia upon contact with the host began to germinate at 12 hai and developed septum (Figure 3 B.a). This strongly agrees with the report of Latunde-Dada et al. (1996; 1997) who noted that the conidia of the fungus conidia took 12 h to germinate and develop septum on cowpea and lucerne inoculated with the disease in Southwest, Nigeria. It also agrees with Shen et al. (2001) who reported that the conidia of the organism germinated on tobacco at about the same time. This, however, was earlier than 16 and 18 hai reported for *C. capsici* and *C. lindemuthianum* on pea and common bean respectively; but later than 4 and 6 hai for *C. truncatum* and *C. dermatium* on cowpea leaf and stem specimens (Smith et al. 1999). Similarly, that the germination time recorded for the conidia of the test pathogen in this study is at par with 12 hai reported for *C. lindemuthianum* on cowpea by Bailey et al. (1990) may suggest that both organisms might be the same fungus since *C. lindemuthianum* has been proven incapable of infecting cowpea (Latunde Dada et al. 1996; Adegbite and Amusa 2008; Akinbode and Amusa 2008 a,b; Enyiukwu et al. 2014; ASHC 2015).

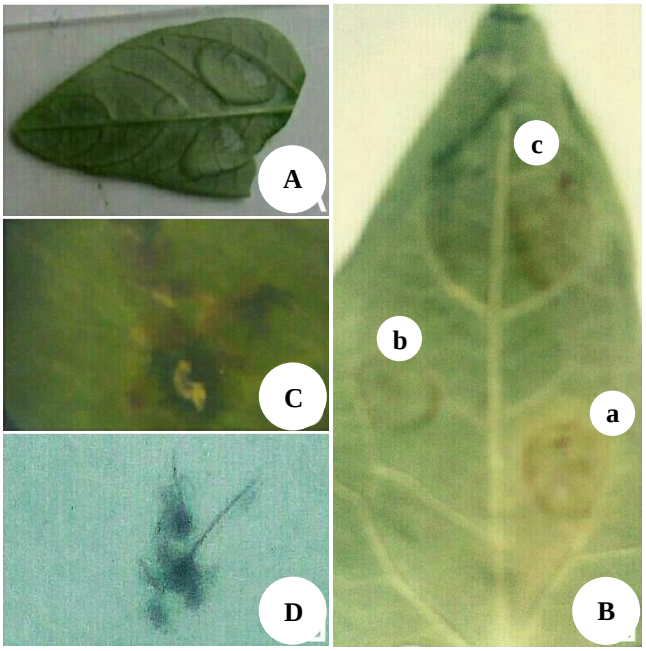


Figure 2. Photomicrograph of healthy (uninfected) and infected cowpea leaf tissues. (A) Healthy (uninfected) leaf inoculated with a drop (0.05 ml) of inoculum at 0 h, (B) Appearance of necrotic spots on leaf 72 hai, (C) Mature lesion on leaf 96 hai, (D) Acervuli on lesion at ≥ 96 hai. Magnification: 1x

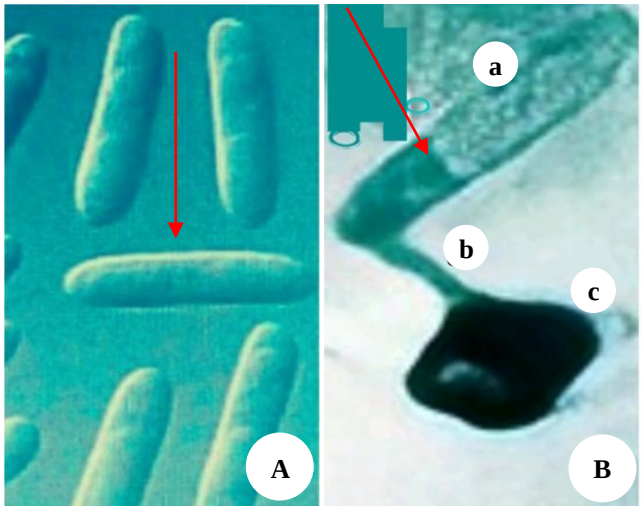


Figure 3. Conidium of *Colletotrichum destructivum* O'Gara, (A) Conidium at 0 h after inoculation on leaves (arrow = no septum observed), (B) Conidium at 12 Hai on cowpea leaf showing

formation of germ tube and appressorium (arrow = septum observed). Magnification: 400x

At the pre-penetration stage, conidia (Figure 3A) and other propagules of plant pathogenic fungi secrete surface acting proteins (hydrophobins and integrins) with which they attach to the host and resist agitation (Tucker and Talbot 2001). The successful attachment of the test conidium in this study may have been due to these compounds. The germination of *C. destructivum* spores observed in this study was followed by the formation of germ tube, development of appressorium at the tip of the germ tube (Figure 3.Bc) from where penetration peg developed. This structure compromised and penetrated the host cuticle-epidermal layer directly within 24 h. This is consistent with the report of many workers who reported direct penetration of the target hosts by *Colletotrichum* species (Da Silva et al. 2017; Xie et al. 2019). For instance, *C. dermatium* has been reported to penetrate cowpea (Smith et al. 1999) and *C. destructivum* also penetrated tobacco, lucerne and cowpea directly (Latunde-Dada et al. 1996; 1997; Shen et al. 2001). Many workers believe that this direct penetration was achieved through melanin-generated turgor pressure (Liao et al. 2012) assisted by hydrolyzing fungal cutinases (Pandey et al. 2012; Adelene et al. 2015) mustered to compromise certain phytoalexins (kievetone and phaseolidin of the plant defense system (Latunde-Dada and Lucas 2001). The tip of the infection peg remained and gradually enlarged inside the lumen of a single epidermal cell, forming large somewhat spherical vesicle which became multi-lobed and multi-septate at about 48 hai and gradually elongated to form the primary hyphae (Figure 4.A-B). The organism remained in this state till mid or late in the third day (about 72 hai). That the large primary hyphae remained inside a single epidermal cell for this length of time (72 hai) agrees with the characteristic transient localized biotrophy characteristic commonly demonstrated by members of *C. destructivum sensu lato* such as *C. higginsianum, C. linicola, C. lentis, C. tabacum,* and *C. taneceti* and *C. horri* on a variety of crops as reported by other workers (Shen et al. 2001; Barimani et al. 2013; O'Connell et al. 2014; Da Silva et al. 2017; Xie et al. 2019). However, it sharply contrasted with non-localized infection strategy of *C. graminicola, C. falcatum* and *C. lindemuthianum* on susceptible crop varieties where in the initial biotrophic phase many epidermal cells were invaded at once by their primary hyphae (Da Silva et al. 2017). Throughout this stage of biotrophy, the crop remained symptomless.

The primary hyphae that had formed and successfully established inside the epidermal cell at penetration, expanded to develop narrow filamentous secondary hyphae in all directions (Figure 4.C). The pathogen invaded adjacent epidermal and mesophyll cells late into the third day after inoculation. In a parallel study of C. obiculare causal agent of cucumber anthracnose; Qi et al. (2013) also noted formation of secondary hyphae from large constricted primary hyphae at this time on leaves of the crop. This leads to the appearance of chlorotic water-soaked necrotic spots on the test leaf surface at about 96 hai (Figure 4.C-D). Shen et al observed that the organism incited typical symptoms of the disease on tobacco at 96 hai. This is congruent with our observation in this study where the pathogen caused appearance of anthracnose lesions on the leaves of cowpea (Var. IAR-48) at 4 days after inoculation. However, our observation slightly deviated from the report of Latunde-Dada et al. (1996; 1997) who in a related study on lucerne and cowpea in Southwest Nigeria found typical symptoms of anthracnose to begin to emerge on the crops at 120 hai. The differences in the time of appearance of the symptoms of the disease may have been occasioned by differences in inoculum potentials of the strains of the pathogen in the regions, or differences in variety, stage of maturity of host plant, or prevailing environmental factors during the experiments.

The ability of the fungus to switch nutrition strategy from latent symptomless biotrophy to a damaging necrotrophy is triggered (or influenced) by changes in the synthesis and activity of cell wall degrading enzymes and secondary metabolites at the host plant-pathogen interface (Smith et al. 1999; Da Silva et al. 2017). The secondary hyphae is reported to secrete a diversity of cell-wall-macerating enzymes [polygalacturonase (PG), pectin lyase (PL), pectin methylesterase (PME), pectin methyl galacturonase (PMG), pectin transeliminase (PTE), carboxymethyl cellulose (CMC), etc.] which inactivate host anti-stressor peroxidases [superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), gluthatione peroxidase (GPX) in advance of their spread (Kumaran et al. 2013; Oliveira et al. 2014). With this army of enzymes, the fungal secondary hyphae rapidly attack, de-cement neighboring cell walls, and create expanding necrotic lesions (Figure 4.D) as they digest the tissues (Figure 4.E) (Qi et al. 2012; Plant Symbiosis 2015). Acervulus having a single-celled seta (a characteristic diagnostic feature of *C. destructivum* as against *C. lindemuthianum* that has multiple setae) developed on the surface of leaf lesion at 96 hai (Figure 2.C-D; Figure 4.F), thus completing the asexual cycle of the fungus. The production of only one seta on its acervulus by the test fungus in this study strongly conforms to submissions of production of only a single seta on the lesion of anthracnose-affected tobacco, lucerne and cowpea by *C. destructivum* (Latunde-Dada et al. 1996; 1997; Shen et al. 2001). Thus, the result of this study clearly showed a two-stage infection process − symptomless biotrophic nutrition (12-72 hai) and voraciously damaging necrotrophic nutrition (72-≥96 hai) – and as such the pathogen demonstrated a hemibiotrophic lifestyle. Inemibiotrophy is reported as a common lifestyle with some other members of the genera *Colletotrichum* such as *C. trifolii, C. lindemuthianum* as well as *C. destructivum* (Damm et al. 2009; 2014; Da Silva et al. 2017).

Results of the study presented in Figure 5 showed that the hyphae of *C. destructivum* macerated the affected cells both intra-cellularly and inter-cellularly; beginning first with degradation of the lumen of the initial single epidermal cell (Figure 5.A-B). Late in the infection process, the pathogenic hyphae compromised the cell walls and the cementing middle lamella as they enlongated and infected other epidermal and mesophyll tissues (Figure 5.C).

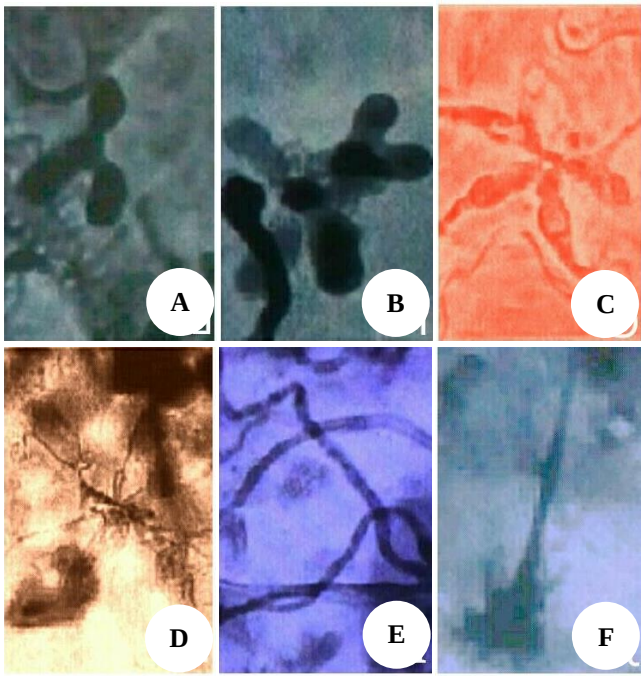


Figure 4. Infection process of *Colletotrichum destructivum* on cowpea. A-B. Fungal vesicle develops into multi-lobed structure 48 hai. C-D. Multi-lobed primary structure elongates into thinner secondary hyphae and ramifies neighboring epidermal cells with appearance of necrotic lesion on leaf 72 hai. E. Intercellular network of hyphae inside the cell at 96 hai. F. Acervulus on leaf lesion 96 hai. Magnification: 400x

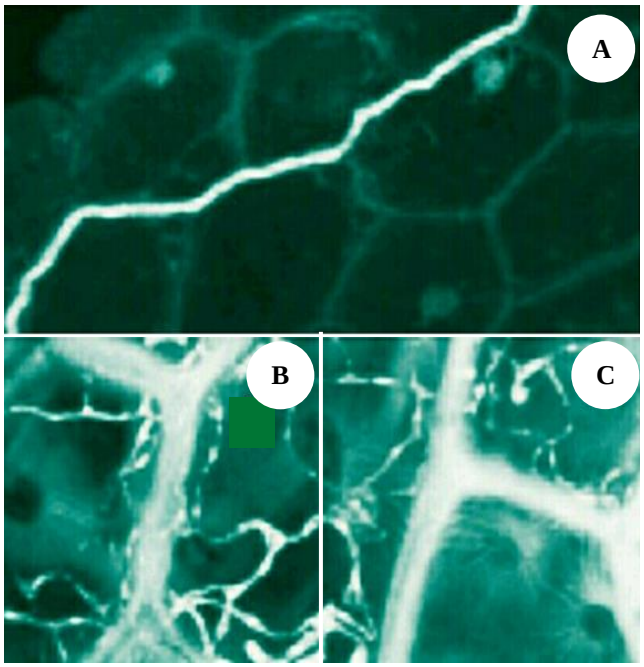


Figure 5. Photomicrograph of infected cowpea leaf tissue showing Intra-cellular and inter-cellular colonization by *Colletotrichum destructivum.* A. Intra-cellular hyphae in a cell during early infection by the pathogen. B. Infected and colonized cell with mass of intracellular hyphae of *C. Destructivum*. C. Intercellular hypha during late infection degrading along middle lamella of leaf tissues by the pathogen. Magnification: 400x

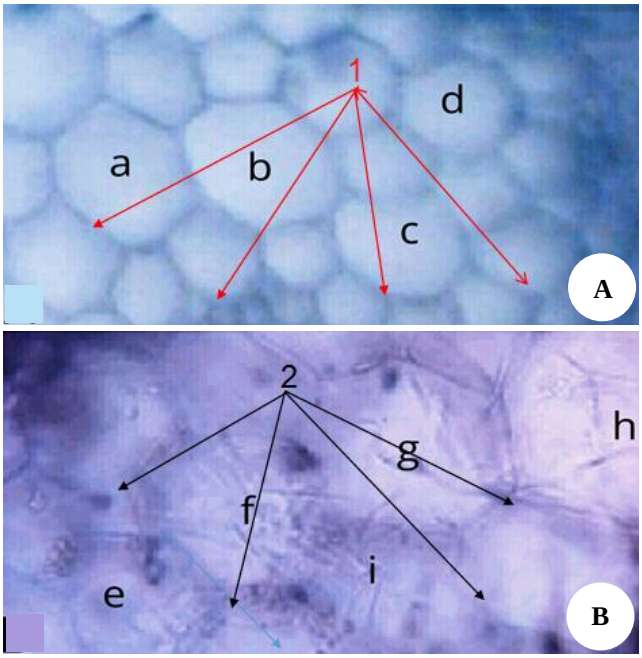


Figure 6. Photomicrograph of healthy (uninfected) and infected cowpea stem. A. Healthy (uninfected) cowpea stem tissue, a-d intact (undisrupted) cells, and 1 uncompromised cell boundaries. B. Infected cowpea stem tissue, e-i disrupted cells and 2 compromised cell boundaries of infected cells due to activities of *C. Destructivum.* Magnification: 400x

This feature is consistent with the report of other workers such as Latunde-Dada et al. (1996; 1997), Shen et al. (2001) and Da Silva et al. (2017) who noted localized hemibiotrophy characterized by intra- and intercellular colonization as a prominent attribute of *C. higginsianum, C. linicola, C. fuscum C. tabacum* and *C. destructivum* which distinguishes them from *C. graminicola, C. Sublineola, C. lindemuthianum* and *C. falcatum* whose biotrophic strategy is non-localized. It was also non-congruent with the reported biotrophic phase of *C. truncatum* causal agent of anthracnose and brown blotch diseases of chilli pepper and cowpea respectively whose lifestyles are characterized by sub-cuticular intramural hemibiotrophy in both ferns and higher plants (Reboledo et al. 2015).

The study showed that the fungus as it ramified and colonized cowpea, depleted the crop of valuable growth factors of the crop as observed in the disparity in growth between the uninfected (control) stem (Figure 6.A) and the infected stem tissues (Figure 6.B). Unlike the uninfected control experiment (Figure 6.A), the infected tissues were turbid, and devoid of vitality, turgidity and vigor; and had weak and loose middle lamella compromised by fungal passage; and cleared, open spaces representing points of nutrient extraction, depletion, and degradation by the test fungus (*C. destructivum*) (Figure 6.B). This observation is in agreement with the findings of Amadioha (1994; 2012) and Markson et al. (2014) who also noted such points of nutrient degradation between tomato and potato tissues infected and colonized both inter- and intra-cellularly by

Rhizopus stolonifer, *Botrydiplodia theobromae*, and *Penicillium expansium* than the control experiment.

In conclusion, this study clearly showed that the pathogen's infection process lasted ≥96 hours hai; and demonstrated that the organism lives intra-cellular hemibiotrophic lifestyle. The first biotrophic phase lasted about 72 hai whereas it switched to a damaging necrotrophy in the later phase (72-≥96 hai). This later phase was attended by massive loss of tissue integrity and vitality. This complex lifestyle of *Colletotrichum destructivum* amongst other variables contributes to make managing cowpea anthracnose difficult. It is generally known that seeds remain a popular means of propagating cowpea. As such, they remain also a viable means of distributing seed-associated pathogen far and wide into disease-free locations. And given that *Colletotrichum destructivum* can potentially cross-infect hosts, all these, therefore, have far reaching implications for bio-security since *C. destructivum* can survive saprophytically on crop thrash and as spores on seed coats or endophytically as dormant mycelia on cotyledons. Again, being that the pathogen is very highly variable, available resistant varieties against the disease are not long-lasting. Understanding the lifestyle of the pathogen could contribute enormously towards developing proper diagnostics and tailoring appropriate control measures for the disease. Quarantining and strict control of trans-border trade in bean seeds is thus necessary and strongly recommended. Also, since the pathogen takes 3 days after infection to initiate and maintain biotrophic nutrition strategy before switching to a damaging necrotrophic phase; this implies that appropriate bio-pesticide or conventional fungicide must be applied at this point to achieve maximum control of the pathogen, increase yield and quality of cowpea, and lessen toxic chemical load on the environment.

REFERENCES

Adegbite AA, Amusa AN. 2008. The major economic field diseases of cowpea in the humid agro-ecologies of South-Western Nigeria. *Afr J Biotechnol* 7 (25): 4705-4712.

Adelene SMA, Ford R, Taylor PWJ. 2015. The role of cutinase and its impact on pathogenecity of *Colletotrichum truncatum*. *Plant Pathol Microbiol 6* (3): 259-264.

ASHC (African Soil Health Consortium). 2015. Anthracnose of cowpea *Colletotrichum destructivum*. http://african soilhealth.cabi.org/wpcms/wp-content/uploads/2015/02/7-legumes-anthracnose-cowpea.pdf&ved+2aU… Accessed November 30, 2020.

Akinbode AO, Ikotun T. 2008a. Evaluation of some bioagents and botanicals in *in vitro* control of *Colletotrichum destructivum*. Afr J Biotechnol 7 (7): 868-872.

Akinbode AO, Ikotun T. 2008b. Efficacy of certain plant extracts against seed-borne infection of *Colletotrichum destructivum* on cowpea (*Vigna unguiculata*). *Afr J Biotechnol* 7 (20): 3683-3685.

Alberto E. 2013. How to calculate the concentration of fungal spores suspension. www.researchgate.net/post/How_to-calculate_the_concentration_of_spores_fungal_... Accessed June 13, 2020.

Amadioha AC, Enyiukwu DN. 2019a**.** Biochemical composition of seed and husk of cowpea (*Vigna unguiculata* L. Walp.) infected by *Colletotrichum destructivum* O'Gara in storage. *Annu Res Rev Biol* 31 (1): 001-007**.**

Amadioha AC, Enyiukwu DN. 2019b. Alterations of biochemical composition of leaf and stem of cowpea (*Vigna unguiculata* L. Walp.) by *Colletotrichum destructivum* O'Gara in Nigeria. *J Exp Agric Int 32(*2): 001-007.

Amadioha AC. 1994. Pectolytic enzymes of *Rhizopus arrhizus* produced in culture and infected potato tubers. *Fitopatol Brasilera* 19: 139-143.

Awurum AN. 2013. Varietal screening and response of cowpea to some foliar diseases iand cropping systems n Southeast Nigeria. *J. Sust. Agric. Environ*. 14(1&2): 59-78.

Bailey JA, Nash C, O'Connell RJ, Skipp RA 1990. Infection process and host specificity of a *Colletotrichum* species causing anthracnose disease of cowpea, Vigna unguiculata. *Mycol. Res*. 94: 800-804.

Barimani M, Pethybridge SJ, Vaghefi N, Hay FS, Taylor PWJ. 2013. A new anthracnose disease of pyrethrum caused by *Colletotrichum tanaceti* sp. nov. Plant Pathol 62 (6): 1248-1257.

Barnett HL, Hunter BB, 1995. Illustrated Genera of Imperfect Fungi (3rd Ed). Burgress Publishing Co., Minneapolis.

Begum JMAJ, Venudevan B, Jayanthi M. 2013. Storage fungi in groundnut and the associated seed quality deterioration – A review. *Plant Pathol J* 12 (3): 127-134.

Begum MM, Sariah M, Puteh AB, Zainal-Adidin MA. 2008. Pathogenicity of *Colletotrichum truncatum* and its influence on soybean seed quality. *Int J Agric Biol* 10 (4): 393-398.

Begum MM, Saviah M, Puteh AB, Abidim MAZ. 2007. Detection of seed-borne fungi and site of infection by *Colletotrichum truncatum* in naturally infected soybean. *J Agric Res* 2 (9): 812-819.

Coates L, Cooke T, Forsberg l. 2019. The biology and management of *Colletotrichum* diseases in production nurseries. Agric-Sci Queensland, Department of Agriculture and Forestry Ecosciences Precinct. In: Plant Health, Biosciences, Risk Management and Capacity Building. www.greenhousemag.com/gm/011-colletotrighum-common-plant-disease...Accessed November 23, 2020.

Da Silva dos Crous PW, Ades PK, Hyde KD, Taylor PWJ. 2017. Life styles of *Colletotrichum* species and implication for plant biosecurity. *Fungal Biol Rev* 155-168. DOI: 10.1016/jfbn2017.05.001.

Damm U, Wonderberg JHC, Cannon PF, Crous PW. 2009. *Colletotrichum* species with curved conidia from herbaceous plants. Fungal Divers 45-88.

Damm U, O'Cornell RJ, Groenevald JR, Crous PW. 2014. *Colletotrichum* species complex, hemibiotrophic pathogens of forage and field crops. Stud Mycol 79: 31-84.

Enyiukwu DN, Amadioha AC, Ononuju CC. 2018. Significance of cowpea leaves for human consumption. *Greener Trends Food Sci Nutr* 1 (1): 001-010.

Enyiukwu DN, Awurum AN. 2013b. Comparative fungitoxicity of benomyl and extracts of *Carica papaya* roots and seeds and piper guineense seeds on *Colletotrichum destructivum* O"Gara. *Continental J Biol Sci* 6 (1): 38-42.

Enyiukwu DN, Awurum AN, Ononuju CC, Nwaneri JA. 2014. Biology and management strategies of cowpea anthracnose disease caused by *Colletotrichum* species. *Greener J Biotechnol Biochem* 1 (2): 052-065.

Enyiukwu DN, Awurum AN. 2013a. Fungitoxic effects of *Carica papaya* and *Piper guineense* extracts against *Colletotrichum destructivum* in the glasshouse. *Continental J Agric Sci* 7 (1): 23-28.

Enyiukwu DN. 2017. Effects of extracts of some medicinal plants on *Colletotrichum destructivum* O'Gara causing anthracnose disease of cowpea (*Vigna unguiculata* L. Walp.) in Nigeria. [Doctoral dissertation, Dissertation]. Department of Plant Health Management, MOUAU, Nigeria.

Falade MJ, Enikuomehin OA, Borisade OA, Aluko M. 2018. Control of cowpea (*Vigna unguiculata* L. Walp.) diseases with intercropping of maize (*Zea mays* L.) and spray of plant extracts. *J Adv Microbiol* 7 (4): 001-010.

GPS (Global Positioning Systems) Coordinate. 2017. Google Play Store. www.google.com/gps...Accessed June 2018.

Guarnaccia L, Gilardi G, Martino I, GaribaldiA, Gullino L. 2019. Species diversity in *Colletotrichum* causing anthracnose of aromatic and ornamental *Lamiaceae* in Italy. *Agronomy* 9: 613. DOI: 10.103390/agronomy9100613.

IMI (International Mycological Institute), Kew, UK. 1995. Description of Pathogenic fungi and Bacteria Nos 316-317.

Kumaran RS, Yong-Keun C, Jeon HJ, Jung H, Kyung-Guen S, Kim HJ. 2013. *In vitro* antagonistic efficacy of plant extracts on the enzyme

activity of *Colletotrichum gloeosporioides*. *Afr J Microbiol Res* 7 (12): 1069-1076.

Latunde-Dada AO, Bailey JA, Lucas JA. 1997. Infection process of *Colletotrichum destructivum* O'Gara from lucerne (*Medicago sativa* L.). Eur J Plant Pathol 103 (1): 35-41.

Latunde-Dada AO, Bailey JA, Lucas JA. 2001. The plant defense activator acibenzolar-S-methyl primes cowpea (*Vigna unguiculata* L. Walp.) seedlings for rapid induction of resistance. *Physiol Mol Plant Pathol* 58 (5): 199-208. DOI: 10.1016/ppp.2001.0327.

Latunde-Dada AO, Lucas JA 1999. Stomatal penetration of cowpea (*Vigna unguiculata*) leaves by a *Colletotrichum* species causing latent anthracnose. *Plant Pathol* 48: 777-785.

Latunde-Dada AO, Lucas JO. 2007. Localized hemibiotrophy in *Colletotrichum*: Cytological and molecular taxonomic similarities among *C. destructivum, C. linicola and C. truncatum*. *Plant Pathol* 56 (3): 437-447.

Latunde-Dada AO, O'Cornell RJ, Nash C, Pring RJ, Lucas JA, Bailey JA. 1996. Infection process and the identity of the hemibiotrophic anthracnose fungus (*Colletotrichum destructivum* O'Gara) from cowpea (*Vigna unguiculata* L Walp.). *Mycol Res* 100: 1133-1141.

Lee HB, Kim CJ. 2002. First report of *Colletotrichum destructivum* on curly dock. *Plant Dis* 86: 1271. DOI: 1094/PDIS.2002.86.11.1271A.

Liao CY, Chen MY, Chen YK, Kuo KC, Ghung KR, Lee MH. 2012. Formation of highly branched hyphae by *Colletotrichum acutatum* within the fruit cuticle of *Capsicum spp*. *Plant Pathol* 61: 262-270.

Lobato AKS, Goncalves-Vidigal MC, Vidigal-Filho PS, Andrade CAB, Kvitschal MV, Bonato CM. 2010. Relationships between leaf pigments and photosynthesis in common bean plants infected by anthracnose. *New Zealand J Crop Hortic Sci* 38 (1): 29-37.

Markson AA, Omosun G, Umana EJ, Madonagu RE, Amadioha AC, Udo SE. 2014. Differential response of *Solanum tuberosum* L. and *Ipomea batatas* L. to three pathogens. *Int J NatSci* 2(1): 40-51.

Meyer S, Saccardy-Adji K, Rizza E, Gevity B. 2001. Inhibition of photosynthesis by *Colletotrichum lindemuthianium* in bean leaves determined by chlorophyll flourescence imaging. *Plant Cell Environ* 24: 947-985.

Miles TD, Gillett JM, Jarosz AM, Schilder AMC. 2013. The effect of environmental factors on infection of fruit by *Colletotrichum acutatum*. *Plant Pathol* 2013: 1-10. DOI: 10.1111/ppa.12061.

O'Connell R, Herbert C, Screenivasaprasad S, Khatib M, Esqurre-Tugaye M-T, Dumas B. 2004. A novel *Arabidopsis-Colletotrichum* pathosystem for molecular dissection of plant-fungal interactions. *MPMI* 17 (3): 272-282.

Ogu DE, Owoeye AE, 2013. *In vitro* control of anthracnose disease of cowpea (*Vigna unguiculata* L. Walp.) caused by *Colletotrichum destructivum* O'Gara with *Cyathula prostata* and *Diodia scandens* Sw leaf extracts. *J Intercultural Ethnopharm* 2 (1): 29-36

Oliveira JTA, Barreto ALM, Vasconcelos IM, Eloy YRG, Gondim DMF, Fernandes CF, Frier-Filho FR. 2014. Role of antioxidant enzymes, hydrogen peroxide and PR-proteins in the compatible and incompatible interactions of cowpea (*Vigna unguiculata* L. Walp.) genotypes with the fungus *Colletotrichum gloeosporioides*. *J Plant Physiol Pathol* 002-008.

Pandy A, Pandy BK, Muthukumar M, Yadava LP, Chauham UK 2012. Histological studies of infection process of *Colletotrichum gloeosporioides* Penz and Sacc. on *Mangifera indica* L. Plant Pathol. J. 11: 18-24.

Patel JS, Costa de Novaes MI, Zhang. 2014. First report of *Colletotrichum higginsianum* causing anthracnose of arugula (E*ruca sativa*) in Florida. *Plant Dis* 98 (9): 1269. DOI: 10.1094/PDIS-09-13-0926-PDN.

Plant Symbiosis Anonymous. 2015. *Colletotrichum* species – Medicinal plant archives. www.medicinal_plant_archive_us/plant-symbiosis/colletotrichum…, Retieved November 19, 2016.

Qi T, Hao C, Ma Q. 2013. Histological studies of *Colletotrichum orbiculare* on susceptible and resistant cucumber cultivars. *Cucurbit Genet Cooperative Rep* 25-26: 004-006.

Reboledo G, De Campos, R, Alvarez A, Montesano M, Mara H, De Leon IP. 2015. *PhyscomitreIla patens* activate defense responses against the pathogen *Colletotrichum gloeosporioides*. *Int J Mol Sci* 16: 22280-222298.

Sharma G, Maymon M, Freeman S. 2017. Epidemiology, pathology and identification of *Colletotrichum* including a novel species associated with avocado (*Persea americana*) anthracnose in Israel. *Sci Rep* 7: 15839. DOI: 10.1038/s41598-017-11596-w.

Shen S, Goodwin P, Hsiang T. 2001. Hemibiotrophic infection and identity of the fungus *Colletotrichum*, causing anthracnose of tobacco. *Mycol Res* 109 (11): 1340-1347.

Smith JE, Korsten L, Aveling TAS. 1999a. Infection process of *Colletotrichum* dematium on cowpea stems. *Mycol Res* 103 (2): 230-234.

Smith JE, Korsten L, Aveling TAS. 1999b. Evaluation of seed treatments for reducing *Colletotrichum dematium* on cowpea seed. S*eed Sci Technol* 27 (2): 591-598.

Strivastava S, Strivastava M, Kumar R, Sinha A. 2013. Effect of seed-borne mycoflora on the protein and amino acid contents of *Jatropha carcus* L. seeds during storage. *Int J Plant Res* 26 (2): 271-279.

Tucker SL, Talbot NJ. 2001. Surface attachment and pre-penetration stage development by plant pathogenic fungi. *Annu Rev Phytopahol* 39 (1): 385-417.

Vasic T, Bulajic A, Krnjaja VS, Jevremovic D, Zivkovic S, Andjelkovic B. 2014. First report of anthracnose on alfalfa caused by *Colletotrichum linicola* in Serbia. *Plant Dis* 98 (9): 1276. DOI: 10.1094/PDIS-03-14-0258-PDN.

Xie L, Zhang JZ, Cai L, Hyde KD, 2010. Biology of *Colletotrichum horrri*, causal agent of Persimmon anthracnose. *Mycology* 1 (4): 242-253.

NUSANTARA BIOSCIENCE
Vol. 13, No. 1, pp. 24-28
May 2021

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n130104

Isolation and enumeration of bacteria responsible for nosocomial infections from houseflies and determining their susceptibility to poison bait

AZAM HAEIDARI[1], DAVOOD KESHAVARZI[2], PARVIZ OWLIA[3], HASSAN VATANDOOST[1], ATIYEH RAFINEJAD[1], JAVAD RAFINEJAD[1,♥]

[1]Department of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Sciences. Tehran, Iran.
♥email: jrafinejad@gmail.com
[2]Research Center for Health Sciences, Institute of Health, Department of Medical Entomology and Vector Control, School of Health, Shiraz University of Medical Sciences. Shiraz, Iran
[3]Molecular Microbiology Research Center, Faculty of Medicine, Shahed University. Tehran, Iran

Manuscript received: 8 September 2020. Revision accepted: 30 December 2020.

Abstract. *Haeidari A, Keshavarzi D, Owlia P, Vatandoost H, Rafinejad A, Rafinejad J. 2021. Isolation and enumeration of bacteria responsible for nosocomial infections from houseflies and determining their susceptibility to poison bait. Nusantara Bioscience 13: 24-28.* Nosocomial infections represent a serious public health concern in developing countries. Houseflies are one of the most common household pests carrying different pathogenic organisms. The purpose of this study was to isolate and enumerate bacteria species from house flies and to determine their susceptibility to Agita® fly bait. Flies were collected from two hospital environments between July to December 2014, in Yazd Province of Iran. Bacterial species were isolated from the outer surfaces of flies, and Agita® efficacy was evaluated based on lethal time (LT50) after 1, 2, 4, 8, 16, and 32 minutes. Three species of bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) responsible for nosocomial infections have been isolated and enumerated from flies. Among the 30 flies collected, 17, 24, and 3 flies were contaminated with *E. coli*, *S. aureus*, and *Ps. aeruginosa*, respectively. The minimum number of isolated bacteria was 3×10^2 CFU/mL, whereas the highest number was 2.4×10^5 CFU/mL. The susceptibility results showed that, despite the existence of a significant effect for heterogeneity in both field and laboratory strains (p-value < 0/05) and an increase in the mortality of houseflies during the time, there was no significant difference between two strains regarding the efficacy of Agita® against houseflies. The findings of the present study revealed and confirmed that houseflies have an important role in the spread of nosocomial infections in hospital environments, and they are susceptible to Agita® fly bait.

Keywords: Agita, housefly, nosocomial infections, susceptibility

INTRODUCTION

The housefly (*Musca domestica*) is a common insect of the family Muscidae, order Diptera (Rassi et al. 2020). They are diurnal insects that live in the human environment and its surrounding, in terms of behavior, feeding, flying power, and quick handling, transmit mechanically many different pathogens to human's food and living environment (Zahn and Gerry 2020; Elyasigomari et al. 2020). There are more than 100 species that can transmit pathogens, but the two most common household pests are flies and cockroaches that transmit mechanically by oral supplements, hairs of body, legs, wings, feces, and vomit (Shiravand et al. 2018; Kobayashi et al. 2020). House flies and cockroaches can transmit various pathogens, including bacteria, protozoa viruses, and parasitic eggs that can cause internal disorders, diarrhea, and typhoid (Sahi 2019; Nwankwo et al. 2020). The hospital setting is one of the places that flies can cause serious problems for human health. Contamination in hospitals and medical centers to bacterial pathogenic agents (nosocomial infections), is the most complicated problem worldwide in both developed and developing countries (Nazari et al. 2017; Park et al. 2019). A previous study by Shiravand et al. (2018) indicated that nosocomial infection is a leading cause of death in all countries. Nosocomial infections have a significant impact on the length of hospital stay, mortality in hospitalized patients and medical care cost. *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* are the most important pathogenic bacteria causing nosocomial infections**.** The increasing antibiotic multidrug resistance of these bacteria has created many problems (Nwankwo et al. 2020). Therefore, the study of the number of pathogens carried by house fly is necessary to be done.

Chemical control is one of the most frequently used interventions to control house flies (Levchenko et al. 2019). Poison bait formulations such as Agita are one of the current control strategies, as it is cost-effective and reduces insecticide exposure to non-target organisms. Agita bait is a contact and stomach insecticide with a mixture of two active ingredients; thiamethoxam (a neonicotinoid insecticide) and tricosene (a housefly pheromone) (Nurita et al. 2008; Ong et al. 2015). The toxicity mechanism of the neonicotinoids is an agonist to the nicotinic acetylcholine receptor. Tricosene is used in this formulation to attract

male and female flies (Nurita et al. 2008). There is no information on the house fly susceptibility to the Agita fly bait in Iran. Therefore, the objective of this study was to determine the efficacy of house fly bait formulations against *M. domestica* under laboratory conditions.

MATERIAL AND METHODS:

Study area and sampling

Two public hospitals were randomly selected from two areas of Yazd between June to December 2014 for this descriptive-analytic study. There were 30 adult houseflies trapped from various parts of the hospitals. Collected houseflies were put into sterile test tubes and then transported to the microbiology laboratory within two hours. Yazd is the driest city in Iran, with a yearly precipitation amount of 49 millimeters (1.9 in) and only 23 days of precipitation, with summer temperatures very frequently above 40°C (104°F) in blazing sunshine without humidity.

Microbiological analysis

Each fly was placed in 5 mL physiologic serum and was vortexed for 30 seconds. Then, 100 µl of physiologic serum was inoculated in the Brain Heart Infusion Agar culture medium. Inoculated Petri dishes were incubated for 24 hours at 37°C. After incubation, the appearance of the colonies was observed, and the number of colonies was counted.

One hundred µl physiologic serum was culture on the various culture media, i.e., Nutrient Agar, Brain Heart Infusion Agar, Cetrimide Agar, Brad Parker Agar (BPA) and MacConkey Agar (production of IBRESCO Company, Iran) and Mannitol Salt Agar (MSA) and Eosin Methylene Blue (EMB) (production of MERCK Company, Germany) to specify cultivation of *P. aeruginosa*, *E. coli*, and *S. aureus*. The various inoculated culture media were incubated for 24 hours at 37°C. After incubation, the number of colonies based on the specific characteristics of the bacteria was determined.

Chemicals and Preparation

Agita® 10 WG (Basel, Switzerland) contains thiamethoxam (10.0% w/w) and Tricosene (0.5g/kg). The required concentration was prepared according to the manufacturer's instruction (10g/13mL water) and was rubbed onto three paper sheets (10 cm x 10 cm) as bait targets and placed in separate cages. Three replicates were conducted for each bait test (20 flies for each replicate. In the control group, the paper was soaked with water.

Bait evaluation

The first generation (F1) of adult flies were used for testing. Flies were starved for 12 h before the tests. Efficacy was evaluated based on lethal time (LT50) after 1, 2, 4, 8, 16 and 32 minutes of contact time. After contact times, flies were transferred into a disposable cup and the mortality was scored 24 hours later. The mortality of the flies was assessed daily at a certain time for 2 weeks. Data were analyzed using Probit analysis. The 95% confidence interval overlap of LT50 was compared for statistical significance.

RESULTS AND DISCUSSION

Isolation and enumeration of bacteria species

Thirty flies were used to isolate the bacteria responsible for nosocomial infections. There were 25 flies (83.3%) were found to carry one or more species of bacteria. Seventeen flies were carrying *E. coli*, 24 flies were carrying *S. aureus*, and 3 flies were contaminated with *P. aeruginosa* respectively. There were 2 flies (6.7%) carrying 3 bacteria species, and 16 flies (53.4%) carrying *E. coli*, and *S. aureus* species. *E. coli* contamination was observed by the appearance of metallic luster colony from 17 flies samples (Figure 1). The minimum number of isolated bacteria was found to be 3×10^2 CFU/mL, while the highest number was 2.4×10^5 CFU/mL (Table 1.). The housefly (*Musca domestica*) has a wide distribution in all parts of the world and a close association with humans and their environment, therefore housefly can be one of the most important mechanical vectors of bacteria, viruses, fungi, protozoa, and worm eggs (Zahn and Gerry 2020; Elyasigomari et al. 2020). The results of bacterial isolation from the outer surface of the fly body show that flies can be carriers of various species of bacteria and vector of several diseases because they are common around households, garbage, human, and animal excreta (Pace et al. 2017; Kavran et al. 2019). This study showed that house flies can carry dangerous bacteria such as *P. aeruginosa*, *E. coli*, and *S. aureus* that are medically important. *P. aeruginosa* is associated with a progressive loss of lung function in cystic fibrosis patients (Mogayzel et al. 2014).

Staphylococcus aureus contamination in houseflies has been reported in several previous studies (Nazari et al. 2017; Nwankwo et al. 2020). One of the most common human pathogens and the most important cause of hospital-acquired infections is *S. aureus*. *S. aureus* can cause a wide range of diseases, including serious infections such as septicemia, endocarditis, wound infections, bacteremia, and osteomyelitis in hospitalized patients and is one of the common causes of mortality in hemodialysis patients (Salgado-Pabón et al. 2013; Tong et al. 2015).

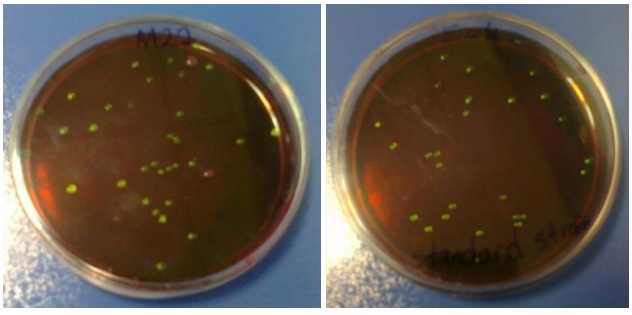


Figure 1. Green metallic luster colonies belong to the standard strains of *Escherichia coli* (right) and M22 fly (left) on the EMB growth medium

Table 1. Total bacteria count and bacteria species isolated from the body surfaces of the collected flies

Sample No.	Count			Sample No.	Count		
	E. coli	*S. aureus*	*Ps. aeruginosa*		*E. coli*	*S. aureus*	*Ps. aeruginosa*
M1	9×10^2	10^2	----	M16	----	9×10^2	----
M2	4×10^2	2×10^2	----	M17	10^2	3×10^2	----
M3	8×10^3	6×10^3	----	M18	----	6×10^2	----
M4	2×10^2	10^2	----	M19	6×10^2	4×10^2	----
M5	3×10^3	10^2	----	M20	----	----	----
M6	5×10^3	3×10^3	3×10^2	M21	----	----	----
M7	4×10^3	4×10^2	----	M22	2×10^3	----	
M8	8×10^2	1.1×10^4	----	M23	----	10^2	----
M9	1.6×10^3	2×10^3	----	M24	----	3.4×10^3	----
M10	4.6×10^3	7×10^3	----	M25	----	3.8×10^2	----
M11	----	----	----	M26	----	----	----
M12	----	----	----	M27	10^2	4.3×10^3	----
M13	9×10^2	5×10^2	----	M28	----	1.9×10^2	----
M14	5×10^2	4×10^2	----	M29	----	2×10^2	----
M15	8×10^2	8×10^2	4×10^2	M30	----	1.2×10^2	----

Table 2. Probit regression line parameters related to the results of experiments of Ajita insecticide on two field and laboratory strains.

Tests	Y-intercept	Slope ± SE	LT_{50} , 95% C.I.	LT_{90}, 95% C.I.	X^2 (df)	p value
Field strain	-1/41	1/92 ±/338	5/47	25/47	45/3 (4)	</05
Lab strain	1/48	1/77±/237	6/85	36/42	24/3 (4)	</05

Escherichia coli contamination had the highest frequency of occurrence which is in line with previous studies (Salgado-Pabón et al. 2013; Nwankwo et al. 2020; Sobur et al. 2019). *E. coli* and *S. aureus* are the two most important pathogens causing urinary tract infections in humans (Zahn and Gerry 2020). A study by Chaiwong et al. (2014) in Thailand showed that bacterial contamination (*P. aeruginosa, E. coli*, and *S.* aureus) in *C. megacephala* was 11-12 times greater than *M. domestica*.

Sasaki et al. (2000) reported a similar result on *E. coli* contamination in house flies. *E. coli* reproduce in the oral parts of the housefly, and three days after swallowing the bacteria was disposed of by the fly, and survive for up to four days in the gut of the housefly. House flies transmit bacteria both mechanically, via contaminated mouthparts and legs, and biologically, via ingestion of microbes and excretion in vomit or feces. (Sasaki et al. 2000). We assume that the bacteria are multiply in the digestive tract of houseflies. The digestive tract of house fly may provide a favorable environment for the horizontal transfer of antibiotic resistance genes among bacteria (Zahn and Gerry 2020). Therefore, flies may be involved in the spread of drug-resistant bacteria in different places.

In the present study, bacterial contamination in houseflies was very high including *E. coli, Ps. aeruginosa*, and *S. aureus*. These bacterial isolates were medically very important because they can result in serious infections. These bacteria were collected from the outer surface of the housefly's body, therefore, bacterial contamination on houseflies collected from the hospital environment may reflect the contamination status of the hospital environment.

Bait evaluation

The mortality rate showed that the death occurred immediately after the flies feed the feeding from Agita. One minute after feeding, there were 22 flies from the laboratory and 26 flies from the field were dead. Over time, the mortality rate of flies in both strains increased (Figure 2).

The results of probit analysis of the susceptibility tests revealed that with increasing time (after one minute), the probability of death of house fly collected from the field was increased to 1.928 ± 0.38, while that of the house fly from the laboratory was 1.77 ± 0.128. The median lethal time (LT50) and LT90 for houseflies from the field were 5.47 minutes and 25.47 minutes, respectively. LT50 and LT90 values for houseflies from the laboratory were 6.85 and 36.42 minutes, respectively (Table 2.). The regression lines of the susceptibility for houseflies from the field and laboratory were presented in Figure 3. The susceptibility results showed that, despite the existence of a significant effect for heterogeneity in both species of field and laboratory (p-value < 0/05) and an increase in the mortality of houseflies during the time, there was no significant difference between two strains regarding the efficacy of Agita® against houseflies.

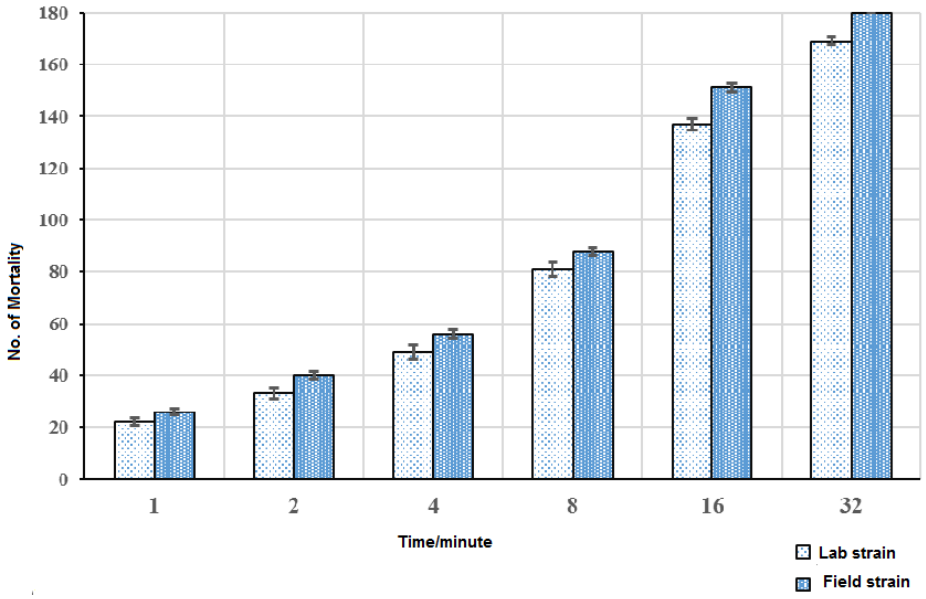


Figure 2. Effect of Agita on the number of mortality in adult flies during specific times.

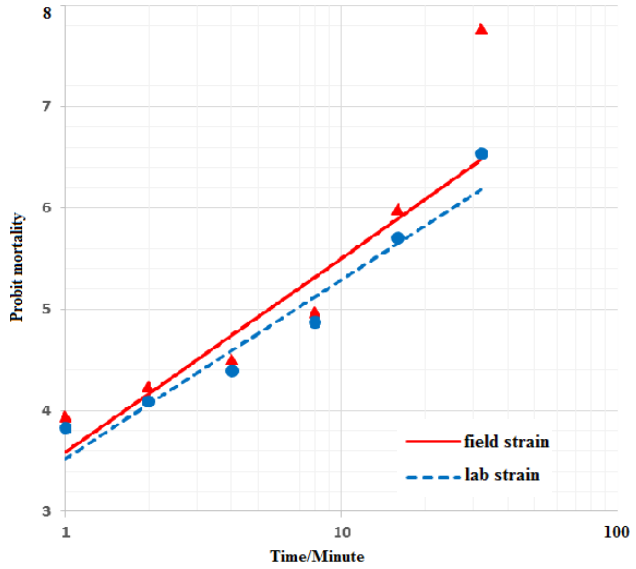


Figure 3. Probit regression lines of susceptibility test for adult flies to Agita fly bait

The results of the susceptibility tests showed that the resistance ratio was 0.79. Resistance ratio (RR) was obtained according to the following formula :

RR = LT50 of field population/LT50 of susceptible laboratory population

Based on the RR value, houseflies collected from the field were more susceptible to Agita. A previous study by Nurita et al. (2008) on synanthropic flies showed that the Agita fly bait was effective for 4 weeks but its effectiveness was significantly reduced on week 6th. A study by Ong et al. (2015) indicated a minor resistance to Agita, hence there is a possibility of increasing Agita resistance in houseflies. Agita irritates before the knockdown effect and consequently, the houseflies fly away and die away from the insecticide source (Msangi et al. 2005). These results indicate that Agita fly bait performs well against houseflies. Evaluation of the residual efficacy of insecticides or chemical baits is important in formulating effective fly control programs. There is a need to extend the effectiveness of fly baits for a longer period and economically feasible to use in fly control programs (Nurita et al. 2008). It has been reported that the effectiveness of Quick Bayt® is longer compared to Agita®. The difference in effectiveness could be due to pellets or granules of Quick Bayt® are homogenous pellets (Nurita et al. 2008).

The results of this study are expected to motivate the relevant hospital authorities regarding insect control so that it can prevent infections caused by bacteria and stem hotspots of their release in the hospitals. Agita fly bait can be used as part of an integrated pest management program for house flies and also for application as a spot spray insecticidal bait. The presence of houseflies indicates a lack of sanitation and unhygienic conditions. The findings of this study suggest that houseflies can be efficient vectors for the mechanical transmission of multidrug-resistant diseases causing organisms, especially from a health-care environment. Considering the importance of this issue: hospital officials should pay attention to healthcare waste management, hospital inputs particularly windows covered with a net to prevent the entry of flies, if necessary. The Agita fly bait formulation can be used for outdoor fly control in the hospital.

REFERENCES

Chaiwong T, Srivoramas T, Sueabsamran P, Sukontason K, Sanford M, Sukontason K. 2014. The blow fly, *Chrysomya megacephala*, and the house fly, *Musca domestica*, as mechanical vectors of pathogenic bacteria in Northeast Thailand. Trop Biomed 31 (2): 336-346.

Elyasigomari A, Keshavarzi D, Ahmed Yusuf M, Hassanzadeh A, Marvi N, Shahriari-Namadi M, Nassiri Z. 2017. Isolation of bacteria from the digestive tract of *Periplaneta americana* and *Polyphaga aegyptiaca* (Blattodea: Blattidae) in Khuzestan Province, Southwestern Iran. Orient Insects 51 (4): 345-52.

Kavran M, Petrić D, Ignjatović-Ćupina A, Zgomba M. 2019. Evaluation of Different Monitoring Methods for *Musca domestica* L. 1758 (Diptera: Muscidae) Indoor Population. Contemp Agric 68 (3-4): 103-12.

Kobayashi M, Ooi HK, Taira K. 2020. Effects of anthelmintics on the pinworm *Blatticola blattae* in laboratory-reared German cockroaches Blattella germanica. Parasitol Res 119 (9): 3093-3097.

Levchenko MA, Silivanova EA, Balabanova GF, Bikinyaeva RH. 2019. Insecticide susceptibility of house flies (*Musca domestica*) from a livestock farm in Tyumen region, Russia. Bulgarian J Vet Med 22 (2): 213-9.

Mogayzel Jr PJ, Naureckas ET, Robinson KA, et al. 2014. Cystic Fibrosis Foundation pulmonary guideline. Pharmacologic approaches to prevention and eradication of initial *Pseudomonas aeruginosa* infection. Ann Am Thorac Soc 11 (10): 1640-1650.

Msangi S, Lawrence B, Masenga C. 2006. Comparative effectiveness of neonicotinoid (Thiamethoxam) and organophosphate (Azamethiphos) against synanthropic house flies. Eur J Res 15 (4): 493-497.

Nazari M, Mehrabi T, Hosseini SM, Alikhani MY. 2017. Bacterial contamination of adult house flies (*Musca domestica*) and sensitivity of these bacteria to various antibiotics, captured from Hamadan City, Iran. J Clin Diagn Res 11 (4): DC04.

Nwankwo EO, Ekemezie CL, Adeyemo S. 2020. Evaluation of microbial flora of the external surface of housefly (*Musca domestica*) in Umuahia Metropolis, Abia State, Southeast Nigeria. Calabar J Health Sci 3 (1): 9-15.

Nurita AT, Abu Hassan A, Nur Aida H, Norasmah B. 2014. Field evaluations of the granular fly bait, Quick Bayt® and the paint-on fly bait, Agita® against synanthropic flies. Trop Biomed 25 (2): 126-33.

Ong SQ, Ahmad H, Jaal Z, Rus AC. 2015. Comparative effectiveness of insecticides for use against the house fly (Diptera: Muscidae): determination of resistance levels on a Malaysian poultry farm. J Econ Entomol 109 (1): 352-9.

Pace RC, Talley JL, Crippen TL, Wayadande AC. 2017. Filth fly transmission of *Escherichia coli* O157: H7 and *Salmonella enterica* to lettuce, Lactuca sativa. Ann Entomol Soc Am 110 (1): 83-89.

Park R, Dzialo MC, Spaepen S, Nsabimana D, Gielens K, Devriese H, Crauwels S, Tito RY, Raes J, Lievens B, Verstrepen KJ. 2019. Microbial communities of the house fly *Musca domestica* vary with geographical location and habitat. Microbiol 7 (1): 147-153.

Rassi Y, Oshaghi M, Azizi K, Rafizadeh S. 2020. Dipteran diversity and succession pattern on carcass of rabbits treated with opium dross: Implication in forensic medicine. Biodiversitas 21 (7): 3135-3141.

Sobur A, Haque ZF, Sabuj AA, Ievy S, Rahman AT, El Zowalaty ME, Rahman T. 2019. Molecular detection of multidrug and colistin-resistant *Escherichia coli* isolated from house flies in various environmental settings. Future Microb 14 (10): 847-58.

Sasaki T, Kobayashi M, Agui N. 2000. Epidemiological potential of excretion and regurgitation by *Musca domestica* (Diptera: Muscidae) in the dissemination of *Escherichia coli* O157: H7 to food. J Med Entomol 37 (6): 945-949.

Shiravand B, Rafinejad J, Enayati A, Bani-Ardalani M, Vatandoost H, Keshavarzi D, Rafinejad A, Saneidehkordi A. 2018. Assessing the susceptibility status of cypermethrin resistance in German cockroaches (Blattella germanica: Blattellidae) to hydramethylnon gel bait. J Kerman Univ Med Sci 25 (5): 396-404.

Sahi NM. 2019. Study Impact of the Negative Relationship between Isolated Bacteria from the Left and Right Wing of the *Musca Domestica* Fly. Indian J Med Forensic Med Toxicol 13 (3): 208-12.

Salgado-Pabón W, Breshears L, Spaulding AR, Merriman JA, Stach CS, Horswill AR, Peterson ML, Schlievert PM. 2013. Superantigens are critical for *Staphylococcus aureus* Infective endocarditis, sepsis, and acute kidney injury. MBio 4 (4): e00494-00413.

Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler VG. 2015. Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management. Clin Microbiol Rev 28 (3): 603-661.

Zahn LK, Gerry AC. 2020. Diurnal flight activity of house flies (*Musca domestica*) is influenced by sex, time of day, and environmental conditions. Insect 11 (6): 391-396.

NUSANTARA BIOSCIENCE
Vol. 13, No. 1, pp. 29-40
May 2021

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n130105

Ethnoveterinary practice of medicinal plants in Chhatradev Rural Municipality, Arghakhanchi District of Western Nepal

ALOK DHAKAL♥, SUSHANK KHANAL, MEENA PANDEY
Paklihawa Campus, Institute of Agriculture and Animal Science, Tribhuvan University, Rupandehi, Lumbini, Nepal.
♥email: dhakalalok06@gmail.com

Manuscript received: 26 November 2020. Revision accepted: 16 January 2021.

Abstract. *Dhakal A, Khanal S, Pandey M. 2021. Ethnoveterinary practice of medicinal plants in Chhatradev Rural Municipality, Arghakhanchi District of Western Nepal. Nusantara Bioscience 13: 29-40.* The use of medicinal plants is a traditional system in treating domestic animals in Nepal. This study was done to investigate and document knowledge about using different plants with medicinal value to cure different animal ailments in Chhatradev Rural Municipality, Arghakhanchi district of western Nepal. A total of 100 elderly people rearing domestic animals and having traditional knowledge of ethnoveterinary plants were selected for interview. A semi-structured questionnaire was employed, and interviews were conducted for addressing detailed ethnoveterinary information. The uses of 103 plant species belonging to 56 families were documented for the treatment of 21 animal ailments. Fabaceae was found to be a dominant family with 10 plant species followed by Poaceae (8 species). The most commonly used plant parts were leaf, seed, and fruit. Herbs were dominant with 44 plant species followed by trees (32 species), shrubs (14 species), and climbers (13 species). The wide application of the medicinal plants as the form of paste formulation was observed for 41 plant species, while 25 plant species were used as powder formulation followed by Juice (21 species), raw (20 species), decoction (19 species), infusion (5 species), and roasted formulation (3 species). Oral route was the most common route followed by dermal and ocular. It was found that the informant consensus factor (ICF) values varied from 0.858 to 0.96. A high informant consensus factor was found for ailment of reproductive category (0.96) followed by respiratory (0.957), gastrointestinal (0.949) while the least was in urinary problem category (0.858). The highest citation frequency was found for *Trachyspermum ammi* (L.) Sprague (247) followed by *Myristica fragrans* Houtt. (111), *Sesamum indicum* L. (109), *Saccharum officinarum* L. (107) and *Zea mays* L. (97). The wide use and rich knowledge of ethnoveterinary practice were found in the study area. This study might be handy to discover useful ethnopharmaceutical agents applicable in the livestock industry. Conservation programs should be done from the government level and study on the use of medicinal plants for treating animal diseases is recommended.

Keywords: Animal care, indigenous knowledge, livestock, Nepal, traditional veterinary healers

INTRODUCTION

Medicinal plants have been used by humans for human as well as animal ailments, and disease treatment from the earliest history of human beings (Bartha et al. 2015; Petrovska 2012). Ethnoveterinary medicine is the traditional approach relating to the treatment and maintenance of animal health care. It is following folklore beliefs, skills, and practices of people by using locally available plant species (Hassan et al. 2017; Katerere and Luseba 2010; Ryan 2014). These medicinal plants discovered by traditional societies are an important source of new potential therapeutic drugs (Pan et al. 2014).

The current studies have the equal concern of improving human medication as well as animal health by the use of locally available medicinal plants. In contrast to modern veterinary medicine, ethnoveterinary practices have been established through trial and error methods with deliberate experimentation by farmers in the fields (Baskota and Doj Raj 2013). The increase in the cost of caring for and maintaining animal health in modern health facilities has made researchers increase their enthusiasm towards the study of ethnoveterinary medicinal plants (Phondani et al. 2010). Nepal is predominantly an agricultural county with about 90% of people in rural areas own livestock as a part of their livelihood (MOAD 2017). For a developing country like Nepal with a huge number of poor farmers, ethnoveterinary practice for animal treatment is a sustainable way of veterinary medicine practice in the new era (NAVS 2015). The indigenous use of ethnoveterinary medicinal plants is of great boon to the developing countries because of its quick accessibility and affordability where there is no easy access to the modern veterinary care facilities (Aziz et al. 2018). Although ethnoveterinary medicinal plants carry great potential in the treatment of the animals, proper documentation of the plants has not been done in the majority of the rural areas of Nepal. The knowledge has been transferred from one to the next generation only verbally or orally (Raut and Shrestha 2012) which is not a dependable way to preserve the knowledge of ethnoveterinary practices. The ease of access to modern health facilities, increase in development of socio-economic aspects, failure to transfer indigenous knowledge regarding medicinal plants, change in technology, and unplanned urbanization have led the use of

ethnoveterinary medicinal plants in a great threat (Subedi 2017; Weckmüller et al. 2019).

As ancient practice of herbal treatment of livestock diseases are still significant today, and are still used by many farmers, veterinarian, Ayurvedic physicians, and *Vaidyas*, so that this study was conducted to investigate, and document local knowledge about the use of different plants with medicinal value in Chhatradev Rural Municipality of Arghakhanchi district in Nepal. The objectives to carry this study were (i) to document the plants with medicinal values used in the treatment of various ailment category of the animals, (ii) to disseminate the formulation technique and the plant part used, (iii) to determine the informant consensus factor and (iv) to record the plant species with the highest citation frequency. This study will be a useful resource for the conservationist, veterinarians, phytochemists, and Ayurvedic physicians to conduct pharmacological studies in the coming days, and will be a valuable asset for the farmers for the treatment of animals health.

MATERIALS AND METHODS

Study area

Chhatradev Rural Municipality is a rural municipality of Arghakhanchi district located in Lumbini province of Nepal. It has an area of 87.62 square kilometres. The climate is tropical to subtropical type with cool and humid. The majority of people are Brahman, Chhetri, Magar, and other ethnic groups like Kumal, Gurung, Kami, Damai, Sarki, Thakuri, Sanyasi, Newar, Badi, and others (CBS 2012). The rural people are not only dependent on forests and natural vegetation to fulfill their daily needs of fuelwood, fodder, grasses, leaf litter, etc., but also for the treatment of ailments and diseases using medicinal plants. The map of the study area is shown in Figure 1. People here are acquainted more with the traditional ethnobotanical knowledge, and are well practitioner of folk beliefs.

Data collection

The data were collected from July to September 2020. A total of 100 elderly, knowledgeable, and experienced persons rearing domestic animals who had traditional knowledge of ethnoveterinary plants were selected. A questionnaire was prepared, and interviews were taken from them addressing detailed ethnoveterinary information. The interview process followed international codes of ethics (ISE 2006).

Plant specimen collection and identification

Some of the collected specimens were identified in the field, whereas others were identified with the help of standard botanical literature. Nomenclature of the identified species follows standard kinds of literature (Aryal et al. 2016; DPR 2015; IUCN Nepal 2000; POWO 2017).

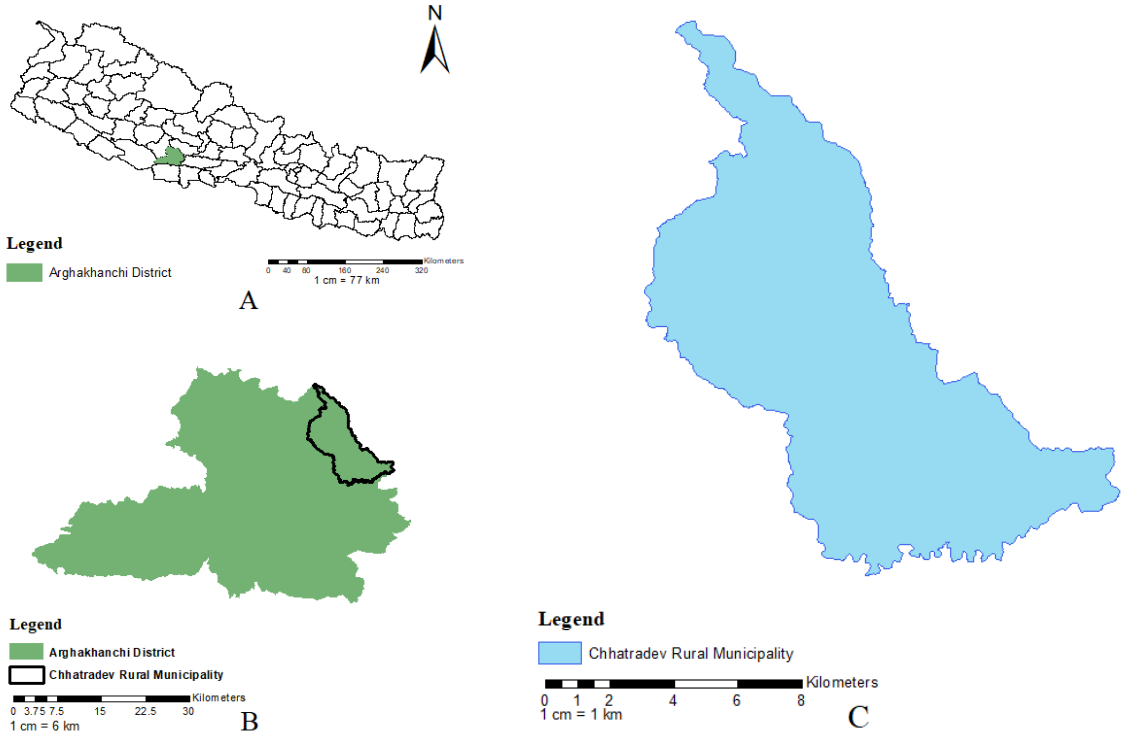


Figure 1. A. Map of Nepal showing Arghakhanchi districts with green color and other hollow ones are other districts. B. Map of Arghakhanchi district showing Chhatradev Rural Municipality. C. Map of Chhatradev Rural Municipality

Data organization

After completion of fieldwork, data entry was done in Microsoft Excel 2016. The life form of the medicinal plants was classified into herbs, shrubs, trees, and climbers. The plant part utilized was classified into bark, bulb, flower, fruit, latex, leaf, rhizome, root, seed, stem, tuber, and whole plant. The preparation technique was classified into different groups, i.e., powder, paste, juice, infusion, raw, decoction, and roast. Route of administration was categorized into oral, dermal, and ocular. The uses of medicinal plants were categorized into ten major categories: reproductive, respiratory, gastrointestinal, antipyretic, parasitic, general weakness, eye, dermatological, osteological, and urinary problems.

Data analysis

For analysis of socio-demographic profile, Microsoft Excel 2016 was used.

Informant consensus factor (ICF)

To determine the agreement among informants in terms of the use of plants, the Informant Consensus Factor (ICF) was calculated (Heinrich et al. 1998) . It was followed in the number of Ethnoveterinary studies of Medicinal Plants (Adeniran et al. 2020; Tariq et al. 2014).

$$ICF = \frac{Nur - Nt}{Nur - 1}$$

Where; ICF = Informants consensus factor, Nur = number of use reports from informants for a particular plant-use category; Nt = number of taxa or species that are used for that plant use category for all informants.

The result of this index ranges from 0 to 1. ICF values are low if plants are chosen randomly or if there is no exchange of information about their use among informants, and approach one (1) when there is a well-defined selection criterion in the community and/or if information is exchanged between informants.

Citation frequency (Cf%)

Cf of medicinal plants is useful to determine the most commonly used medicinal plants in the study area.

$$Cf\ \% = \frac{n}{N} \times 100$$

Where; n refers to the number of times a particular species was mentioned, N refers to the total number of times that all species were mentioned.

RESULTS AND DISCUSSION

Socio-demographic profile of the informants and their domestic animal composition

Out of 100 informants, more than half of the respondents were male (58) while there were 42 female respondents. Most of the interviewees (72) were older than 50 years and 28 interviewees were between 35-50 years old. Agriculture was the main source of household income to 70 respondents while 19 people had government services, and 11 had remittance as the way to make a living. Among the informants, there were not any illiterate while the majority (70) of the informants had gained primary education, 19 informants had gained secondary education, and the rest (11 informants) had got other higher forms of education (Table 1). Livestock (Buffalo, Goat, Cow, Poultry) were important economic sources of informants. The majority of the respondents (98) kept buffalo, 81 respondents kept goats, 28 respondents kept poultry and 4 respondents kept cow with their total number 191, 237, 191, and 7 respectively (Figure 2).

Source of ethnoveterinary knowledge

The majority of the respondents received the knowledge of ethnoveterinary use of plants from their ancestors whereas some came from neighbors, newspapers, and radio. Elderly people, mainly male, were rich in ethnoveterinary knowledge, and more concerned about the conservation of plants than younger people. This observation has also been reported by Khan et al. (2015).

Table 1. Socio-demographic characteristics of respondents

Description	Frequency
Age	
35- 50	28
>50	72
Gender	
Female	42
Male	58
Educational level	
Primary	70
Secondary	19
Others	11
Primary occupation	
Agriculture	70
Government services	19
Remittance	11

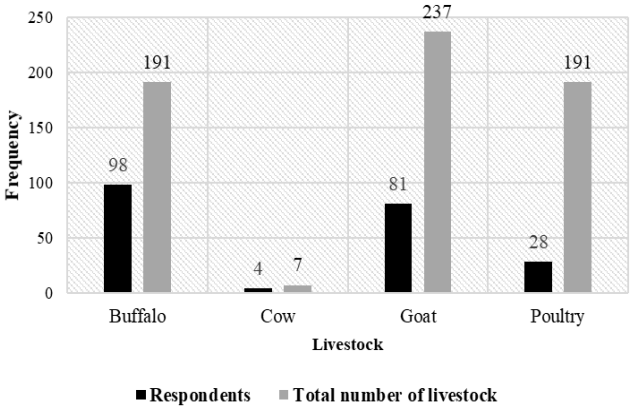


Figure 2. Respondents with the number of their livestock in the study area

Table 2. List of the ethnoveterinary medicinal plants identified in the study area

Botanical name	Family	Local name	Life form	Part used	Form of medication	Route	Method of use and application
Justicia adhatoda L.	Acanthaceae	Asuro	Shrub	Whole plant	Decoction	Oral	Decoction prepared from the whole plant is given twice a day orally until cure of common cold
				Leaf	Paste	Dermal	Leaves are ground to make a paste and mixed with turmeric powder to cure ectoparasite
Achyranthes aspera L.	Amaranthaceae	Datyun	Herb	Whole plant	Raw	Oral	Fed as a feed to cure endoparasite
Chenopodium album L.	Amaranthaceae	Bethe	Herb	Whole plant	Juice	Oral	The whole plant is crushed and obtained juice is given orally to treat dysentery
Allium sativum L.	Amaryllidaceae	Lasun	Herb	Bulb	Paste	Oral	*Allium sativum* L. and *Mentha arvensis* L. are mixed in a ratio of 1: 2. They are crushed and given to animal to cure diarrhea
						Dermal	Bulb is crushed with camphor to make paste and applied to the wound.
Mangifera indica L.	Anacardiaceae	Aap	Tree	Bark	Powder	Dermal	The bark is dried then ground with camphor and applied to the burn area
Rhus javanica L.	Anacardiaceae	Bhakkimlo	Tree	Fruit	Infusion	Oral	Ripe fruits are soaked in water, and water is given twice a day to animal by mixing with curd to treat diarrhea and dysentery
Semecarpus anacardium L.	Anacardiaceae	Bhela	Tree	Fruits	Juice	Dermal	Fruit is ground with mustard oil and applied to wound
Centella asiatica (L.) Urb.	Apiaceae	Ghodtapre	Herb	Whole plant	Raw	Oral	The whole plant is fed to treat urinary disorders.
Trachyspermum ammi (L.) Sprague	Apiaceae	Juwano	Shrub	Seed	Powder, Decoction	Oral	The required amount of dry seed is crushed and fed with water, cornflour meal. It can also be boiled in water and fed twice a day to cure cough, diarrhea, and fever. Whereas dried seeds of *Trachyspermum ammi* (L.) Sprague, *Sesamum indicum* L. and *Myristica fragrans* Houtt. are crushed to make powder, and given to animals by mixing with cornflour to cure constipation and increase milk production.
Calotropis gigantea (L.) Dryand.	Apocynaceae	Aank	Tree	Leaf	Paste	Dermal	Lightly crushed leaves are warmed on fire and kept on swelling joints
Holarrhena pubescens Wall. ex G.Don	Apocynaceae	Indrajau	Tree	Bark	Decoction	Oral	Decoction obtained from bark is given to the livestock to treat constipation, diarrhea, and dysentery
Marsdenia tenacissima (Roxb.) Moon	Apocynaceae	Bilajor	Climber	Root	Paste	Dermal	Root paste is applied on swelling bone area
Acorus calamus L.	Araceae	Bojho	Herb	Rhizome	Paste	Dermal	Paste prepared from the rhizomes is applied to the body of animals to remove lice
Colocasia esculenta (L.) Schott	Araceae	Karkalo	Herb	Leaf, Stem	Decoction	Oral	Small pieces are made by cutting them and given to animal by cooking with cornflour to cure endoparasite and diarrhea
Agave cantula Roxb.	Asparagaceae	Ketuki	Herb	Leaf	Decoction	Oral	Juice extracted from boiled leaf is given orally to cure fever
					Juice		Leaves are ground to obtain juice and fed for any urinary related problem
Aloe vera (L.) Burm.f.	Asphodelaceae	Ghyu kumari	Herb	Leaf	Raw	Dermal	Jelly portion of leaf is rubbed in burn area and cure mastitis
Begonia picta Smith	Begoniaceae	Magarkanche	Herb	Root	Infusion	Ocular	Water obtained after infusion of the root is applied to treat conjunctivitis of buffalos
Oroxylum indicum (L.) Kurz	Bignoniaceae	Tatahalo	Tree	Bark	Powder	Oral	Bark of *Oroxylum indicum* (L.) Kurz and *Schima wallichii* (DC.) Korth. are dried, then ground to obtain powder, and fed to cure diarrhea
				Leaf, seed	Paste		Fresh leaves and seeds are ground to make a paste, and fed to cure urinary problem
Cynoglossum zeylanicum Thumb. ex. Lehm.	Boraginaceae	Bhere kuro	Herb	Whole plant	Powder	Ocular	Powder obtained from dried plant is blown to eye problem
					Paste	Dermal	Paste of plant mixed with water and applied to cure skin scabies and wound
Brassica nigra L.	Brassicaceae	Tori	Herb	Seed	Juice	Oral	The mustard oil is directly fed to cure endoparasite and uterus prolapse
					Paste	Dermal	Mustard cake is mixed with maize flour, and fed to increase milk production
							Mustard cake is applied to cure skin scabies

Cannabis sativa L.	Cannabaceae	Ganja	Herb	Leaf, seed	Powder	Oral	The mixture of leaf and seed is ground then drenched with water to cure diarrhea, fever, and urinary related problems
Crateva unilocularis Buch.-Ham	Capparaceae	Simlikan	Tree	Leaf, stem	Raw	Oral	The grass and edible stem are directly fed to cure endoparasite
Carica papaya L.	Caricaceae	Mewa	Tree	Seed	Powder	Oral	Matured dry seeds are crushed to make powder, and given orally to cattle once a day for up to six days against internal parasites
Terminalia bellirica (Gaertn.) Roxb.	Combretaceae	Barro	Tree	Fruit	Powder	Oral	Fresh fruit is given orally to treat diarrhea
Commelina benghalensis L.	Commelinaceae	Khane jhar	Herb	Leaf	Paste	Dermal	Paste obtained from leaf is made and applied locally on fresh wounds in animals as antiseptic
Artemisia indica L.	Compositae	Titepati	Herb	Leaf	Juice	Dermal	Leaf and kerosene are crushed to make a juice and applied to cure ectoparasite
				Whole plant	Decoction	Oral	*Artemisia indica* L., *Azadirachta indica* A.Juss. and *Melia azedarach* L. (Leaf) are taken and cut into pieces, mixed with cornflour, cooked, and fed to cure endoparasite
				Leaf	Paste	Dermal	Leaf and camphor are crushed to make a paste and applied in a wounded area twice a day
Tagetes patula L.	Compositae	Sayapatri	Herb	Root	Paste	Oral	Fresh roots are ground and fed to treat urinary problem
Cuscuta reflexa Roxb.	Convolvulaceae	Aakash beli	Climber	Stem	Infusion	Oral	Stem along with local tomato, ghee, butter, and curd is placed in a copper vessel over a night. It is given orally to cattle against endoparasites
Cucumis sativus L.	Cucurbitaceae	Kakro	Climber	Seed	Powder	Oral	Dried seeds are crushed to make powder and fed by drenching with water to cure urinary problems
Cucurbita maxima Duchesne	Cucurbitaceae	Farsi	Herb	Fruit	Decoction	Oral	Ripen yellow pumpkin is cooked with cornflour and feed to cure mastitis
Solena heterophylla Lour.	Cucurbitaceae	Golkankri	Climber	Root	Paste	Dermal	The paste prepared from the root is applied to cure mastitis
				Whole plant	Raw	Oral	The whole plant is considered a nutritious feed, and also used to increase milk production
Trichosanthes cucumerina L.	Cucurbitaceae	Chichinno	Climber	Seed	Powder	Oral	Dried seeds are crushed to make powder and fed by mixing with turmeric to cure endoparasite
				Fruit	Raw		Fruit is fed raw to treat mastitis and removing the placenta
Cyperus difformis L.	Cyperaceae	Mothe Jhar	Herb	Root	Paste	Oral	The root nodules are crushed and fed to cure any urinary related problem
Dioscorea deltoidea Wall. ex Griseb.	Dioscoreaceae	Bhayakur	Climber	Tuber	Juice	Oral	Juice obtained from tuber is given twice a day is to treat constipation
Equisetum debile Roxb. ex Vaucher	Equisetaceae	Kurkure Jhar	Herb	Stem	Paste	Dermal	Paste obtained from stem is applied externally on fractured bone
Albizia procera (Roxb.) Benth.	Fabaceae	Jukret	Shrub	Bark	Powder	Oral	Dried bark is crushed to make powder, and drenched with water to cure bone related problems
Bauhinia purpurea L.	Fabaceae	Koiralo	Tree	Leaf	Raw	Oral	Oral administrations of leaves as feed to enhance lactation
				Bark	Decoction	Dermal	Slightly warm stem bark decoction is used to cure wounds
Cassia fistula L.	Fabaceae	Rajbrikshya	Tree	Fruit, seed	Powder	Oral	Dried seed and fruit are ground to make powder, and fed to cure urinary problem
Dalbergia sissoo Roxb.	Fabaceae	Sissoo	Tree	Leaf	Juice	Oral	Leaf is crushed to obtain juice, and then mixed with churning curd, and fed to cure diarrhea
Erythrina stricta Roxb.	Fabaceae	Phaledo	Tree	Bark	Infusion	Dermal	Bark is soaked in water and applied over the body to treat scabies
Lens culinaris Medikus	Fabaceae	Masuro	Herb	Seed	Decoction	Oral	Seeds are cooked with maize flour and given to animal twice a day to increase milk production
Mimosa pudica L.	Fabaceae	Lajjawati jhar	Herb	Whole plant	Raw	Oral	Fed whole plant to animal as feed to cure uterus prolapse
Phanera vahlii (Wight & Arn.) Benth.	Fabaceae	Bharla	Climber	Bark	Powder	Oral	Dried bark is ground to make powder and fed with honey to treat joint-related problem

Trigonella foenum-graecum L.	Fabaceae	Methi	Herb	Seed	Infusion	Oral	Soaked seeds are mixed with cornflour meal, and given orally to cure chronic cough and fever
					Decoction		*Trigonella foenum-graecum* L. and *Zingiber officinale* Roscoe are mixed and fed to cure joint swelling
Mentha arvensis L.	Lamiaceae	Pudina	Herb	Leaf	Paste	Oral	*Mentha arvensis* L. and *Allium sativum* L. are mixed in a ratio of 2: 1, and feed after crushing to cure diarrhea
						Dermal	The whole plant is rubbed into the body of the animal to cure ectoparasite
Ocimum tenuiflorum L.	Lamiaceae	Tulsi	Shrub	Leaf	Paste	Dermal	Leaf paste is applied externally to healing wounds
Pogostemon benghalensis (Burm. F.) O. Ktze	Lamiaceae	Rudilo	Herb	Leaf, flower	Powder	Oral	Leaf and flower are dried in sun. Powder is prepared by crushing them and fed to cure diarrhea
					Paste	Dermal	Leaf and flower are crushed to make a paste, and applied to cure a wound
Lindera neesiana (Wall. ex Nees) Kurz.	Lauraceae	Siltimur	Tree	Fruit	Juice	Oral	The fruit juice is given to cure any weakness
Asparagus racemosus Willd	Liliaceae	Kurilo	Shrub	Root	Paste	Oral	Tuberous roots are crushed and mixed with cattle feed for any bone related problem and mastitis
Linum usitatissimum L.	Linaceae	Aalus	Shrub	Seed	Powder	Oral	*Linum usitatissimum* L. and *Sesamum indicum* L. are fried and crushed to make powder. It is then fed orally to cure mastitis. Similarly, Powder obtained from dried seed of *Linum usitatissimum* L., *Sesamum indicum* L. and *Trachyspermum ammi* (L.) Sprague are mixed and fed with cornflour twice a day to increase milk production
Viscum album L.	Loranthaceae	Hardjor laharo	Climber	Stem	Paste	Dermal, Oral	The stem is crushed and paste is applied to the swelling area. It can also be fed by mixing with ghee and honey
Abelmoschus esculentus (L.) Moench	Malvaceae	Bhindi	Shrub	Root	Paste	Oral	Root is crushed and fed to cure urinary related problems
Bombax ceiba L.	Malvaceae	Simal	Tree	Flower	Juice	Oral	Flower is crushed and fed to cure constipation
				Bark	Powder		Powder obtained from bark is mixed with powder of pepper and ginger, and then given orally to cure dysentery
				Bark	Juice		Raw bark is crushed to obtain juice and given to remove the placenta
				Seed	Paste	Dermal	The seed is ground with mustard oil, and then applied to treat the wounds
Corchorus capsularis L.	Malvaceae	Jute Plant	Shrub	Stem	Roast	Oral	Stem is burned, and obtained ash is mixed with mustard, then fed to treat diarrhea
Osbeckia stellata Buch. Ham ex D. Don.	Melastomataceae	Aangaru	Shrub	Leaf, Fruit	Decoction	Oral	A decoction of the leaf and fruit twice a day is given to domestic animals for any kind of weakness
Azadirachta indica A.Juss.	Meliaceae	Neem	Tree	Leaf, seed	Paste	Dermal	Paste is made by crushing leaf and applied to cure ectoparasite, and fed to cure wound
					Decoction	Oral	*Azadirachta indica* A.Juss. , *Artemisia indica* L and *Melia azedarach* L. are taken and cut into pieces, then cooked with cornflour to cure endoparasite
Melia azedarach L.	Meliaceae	Bakaino	Tree	Leaf, seed	Juice	Dermal	Seed and leaf are crushed to make juice. It is applied by mixing with turmeric and salt to cure ectoparasite
					Decoction	Oral	*Melia azedarach* L., *Azadirachta indica* A.Juss., and *Artemisia indica* L. are taken and cut into pieces. It is cooked with cornflour then feed to treat endoparasite
					Paste	Dermal	Seed and leaf are crushed to make a paste. It is applied to cure wound by mixing with camphor
Tinospora cordifolia (Thunb.) Miers	Menispermaceae	Ganigurjo	Climber	Stem	Decoction	Oral	Stem is made into small pieces, then boiled with water and fed to cure chronic cough and constipation
					Powder		The powder is obtained from dried stem and given orally with water to treat diarrhea

Artocarpus heterophyllus Lam.	Moraceae	Rukh katar	Tree	Fruit	Raw	Oral, Dermal	Ripen jackfruit is fed directly, and the fruit core is applied in teats to cure mastitis
Ficus religiosa L.	Moraceae	Peepal	Tree	Bark	Powder	Dermal	Dry bark is ground to obtain powder and applied to the burn area
Ficus semicordata Buch.-Ham. ex Sm.	Moraceae	Khanyu	Tree	Leaf	Raw	Oral	Leaves as fodder are given to remove the placenta
Morus australis Poir.	Moraceae	Ban kimbu	Tree	Root	Paste	Oral	Fresh root is crushed to make a paste and fed to animal for treating endoparasite
Musa x paradisiaca L.	Musaceae	Malvoc Kera	Herb	Fruit	Raw	Oral	Ripen banana is given to treat mastitis
Myristica fragrans Houtt.	Myristicaceae	Jaifal	Tree	Seed	Powder	Oral	Powder is obtained from seeds of *Myristica fragrans* Houtt., *Trachyspermum ammi* (L.), and *Sesamum indicum* L., then fed to cure chronic cough, constipation. Solely it is given to increase milk production and urinary problems
Fraxinus floribunda Wall.	Oleaceae	Lankuri	Tree	Bark	Powder	Oral	Bark is ground to make powder to cure diarrhea
					Paste	Dermal	A paste of bark is applied to treat broken legs and arms of animal
Oxalis corniculata L.	Oxalidaceae	Chariamilo	Herb	Leaf	Juice	Ocular	Juice obtained by pounding fresh leaves is dropped into the eyes for any eye problems
Sesamum indicum L.	Pedaliaceae	Kalo Til	Herb	Seed	Powder	Oral	Powder is obtained from seeds of *Sesamum indicum* L., *Trachyspermum ammi* (L.) Sprague and *Myristica fragrans* Houtt, and then fed to cure chronic cough and constipation *Sesamum indicum* L., *Trachyspermum ammi* (L.) Sprague and *Linum usitatissimum* L. are fried and crushed to make powder and fed to cure mastitis and increase milk production
Phyllanthus emblica L.	Phyllanthaceae	Amala	Tree	Fruit, leaf	Paste	Dermal	Paste is made by crushing leaves and fruit. Then applied to the burn area
Pinus roxburghii Sarg.	Pinaceae	Sallo	Tree	Latex	Juice	Dermal	Latex obtained from stem is mixed with mustard oil and applied to wound skin
Piper nigrum L.	Piperaceae	Marich	Climber	Seed	Powder	Ocular	Powder is prepared and blown to the eye gently to cure eye-related problem
Scoparia dulcis L.	Plantaginaceae	Chini jahr	Herb	Stem	Paste	Dermal	The stem is crushed, and the paste is applied to the swelling area. Cloth is generally used to make it attach to the swelling area
Cynodon dactylon (L.) Pers.	Poaceae	Dubo	Herb	Whole plant	Raw	Oral	Whole plant is fed to livestock twice a day to enhance the lactation
					Paste	Dermal	Paste is made by crushing the whole plant and applied in a wounded area
Eleusine coracana Gaertn.	Poaceae	Kodho	Herb	Seed	Decoction	Oral	Seeds are boiled in water and mixed with cornflour meal to cure chronic cough and any weakness
Eulaliopsis binnata (Retz.) C.E. Hubb.	Poaceae	Babio khar	Herb	Whole plant	Raw	Oral	Grass is given raw to cure weakness
Hordeum vulgare L.	Poaceae	Jau	Herb	Leaf	Juice	Ocular	Newly born leaf is crushed to obtain juice and used to cure eye-related problems
				Seed	Decoction	Oral	Seed is boiled in water. The obtained Oily material is fed orally to treat mastitis
Imperata cylindrical (L.) P.Beauv.	Poaceae	Siru	Herb	Root	Paste	Oral	Root is crushed to make a paste and used to cure endoparasite
Saccharum officinarum L.	Poaceae	Ukhu	Herb	Stem	Juice	Oral	Stem is crushed to obtain juice, boiled, and fed by mixing with cornflour to increase milk production
				Leaf	Raw	Oral	Leaf is given as feed to remove placenta
Thysanolaena maxima (Roxb.) Kuntz	Poaceae	Amriso	Herb	Leaf	Raw	Oral	Leaves as a feed are given to remove placenta
Zea mays L.	Poaceae	Makai	Herb	Fruit	Paste	Oral	Head smut infected corn is crushed by mixing with lemon juice and fed to treat diarrhea
				Seed	Decoction		Maize flour is made from corn seed and boiled with salt and water to increase milk production
Prunus persica (L.) Batsch.	Rosaceae	Aaru	Tree	Leaf	Juice, Paste	Dermal	Newly born leaf buds are taken and crushed to make juice. Then it is mixed with camphor and applied when endoparasites are seen. It is also applied to treat wound
Rubus ellipticus Sm.	Rosaceae	Aiselu	Shrub	Leaf	Paste	Dermal	Leaves are crushed to make a paste and applied in the wound area

Aegle marmelos (L.) Corrêa	Rutaceae	Bel	Tree	Fruit	Paste	Oral	The fruit is crushed and made paste to treat fever
Boenninghausenia albiflora (Hook.) Rchb. ex Meissn	Rutaceae	Upiya jhar	Herb	Leaf	Juice	Dermal	Leaves juice is applied over the body to remove flea, bug, lice, and external parasites
					Paste		Fresh twigs leaves are ground with camphor and applied to the wound
Citrus limon (L.) Osbeck	Rutaceae	Kagati	Shrub	Fruit	Juice	Oral	Lemon juice is mixed with egg. If head smut infected corn is available then its powder is mixed and given to animal to cure fever
					Raw	Dermal	Juice is rubbed to swelling area
Citrus maxima Merr.	Rutaceae	Bhogate	Shrub	Leaf	Paste	Oral	Leaves are crushed and mixed with cornflour to cure endoparasite
					Juice	Ocular	Juice obtained is mixed with salt and applied to eye problem
Citrus medica L.	Rutaceae	Bimiro	Tree	Root	Juice	Oral	Juice obtained by pounding root and given orally to treat endoparasite
Osyris wightiana Wall. ex Wight	Santalaceae	Nun dhiki	Shrub	Bark	Powder	Oral	Dried bark is crushed to make powder and fed to cure joint swelling
Sapindus mukorossi Gaertn.	Sapindaceae	Ritho	Tree	Fruit	Paste	Oral	Fruit with its covering are cooked with cornflour and water to cure urinary problem
Diploknema butyracea (Roxb.) H.J.Lam	Sapotaceae	Chyuri	Tree	Root	Paste	Oral	Root is crushed to make a paste and fed to cure fever
				Fruit		Dermal	Fruit is crushed and paste obtained is applied to cure mastitis
				Bark			Bark is ground with water and applied by mixing with camphor to cure the wound
Capsicum annuum L.	Solanaceae	Khursani	Shrub	Fruit	Raw	Oral	Fruit is directly given to the animal to treat urinary related problem
Datura metal L.	Solanaceae	Dhatur	Herb	Fruit	Roast	Oral	Fruits are roasted on open fire, and fed to treat diarrhea, dysentery and fever
Solanum lycopersicum L.	Solanaceae	Tamatar	Herb	Fruit	Juice	Dermal	Fruit is crushed, and then the juice is applied to the burn area
					Raw	Oral	Fruit is directly fed to livestock to cure endoparasite
						Dermal	Fruit juice is applied to cure mastitis
Solanum melongena L.	Solanaceae	Bhenta	Herb	Fruit	Roast	Oral	The fruit is roasted and fed to animal to cure diarrhea
				Leaf	Paste		The newly born leaf is crushed and fed to remove the placenta
Solanum tuberossum L.	Solanaceae	Aalu	Herb	Tuber	Raw	Dermal	Tuber is cut and rubbed externally on burns twice a day
Schima wallichii (DC.) Korth.	Theaceae	Chilaune	Tree	Bark	Powder	Oral	Bark of *Schima wallichii* (DC.) Korth. and *Oroxylum indicum* (L.) Kurz are dried, then ground to obtain powder, and given orally to cure diarrhea. *Schima wallichii* (DC.) Korth. alone is given to cure endoparasite
Urtica dioica L.	Urticaceae	Sisno	Herb	Leaf	Decoction	Oral	Leaf is boiled, and water is fed to an animal to cure mastitis and urinary problem
Vitex negundo L.	Verbenaceae	Simali	Tree	Leaf	Decoction	Dermal	Boiled extract of the leaf is applied on scabies and other skin infections in animals
Ampelocissus divaricata (Wall. ex M.A.Lawson) Planch.	Vitaceae	Purreni	Climber	Stem	Raw	Ocular	One end of fresh-cut stem is brought near to eye and watery like substance oozing out from stem is let go into eye by blowing air
Cissus repens Lam.	Vitaceae	Charchare laharo	Climber	Root	Juice	Ocular	Stem is gently crushed to obtain juice and applied to any eye problem
Curcuma longa L.	Zingiberaceae	Besar	Herb	Rhizome	Powder, Paste	Dermal	A dried rhizome is crushed and mixed with mustard oil. Then, applied in burn area, wound and to treat ectoparasite.
					Powder	Ocular	Little powder is blown to the eye to treat eye-related problem
Elettaria cardamomum (L.) Maton	Zingiberaceae	Alaichi	Herb	Seed	Powder	Oral	Powder is made by crushing dry seeds and fed to animal to cure fever
Zingiber officinale Roscoe	Zingiberaceae	Aduwa	Herb	Rhizome	Paste	Oral,	Raw rhizome is crushed and given to cure diarrhea, mastitis. It is crushed with *Trigonella foenum-graecum* L. to treat joint swelling
						Dermal	Raw rhizome is ground with camphor to treat wounds

Plant diversity and uses

The present study revealed the ethnoveterinary use of 103 plant species belonging 56 families to cure 21 animal ailments. Mostly, plants were collected by local people from forests, marginal land, grassland, and cultivated fields. Out of 56 families, Fabaceae was found to be dominant with 10 plant species similar to the findings of Acharya et al. (2015). This might be due to its higher abundance in the study area and high bioactivity. Fabaceae was followed by Poaceae (8 species), Rutaceae and Solanaceae (5 species each), Cucurbitaceae and Moraceae (4 species each), Anacardiaceae, Apocynaceae, Lamiaceae, Malvaceae, and Zingiberaceae (3 species each). The remaining 45 families had less than 3 species each. In contrast to our study, Raut and Shrestha (2012) found Liliaceae as a dominating family. This difference in observation might be due to different vegetation of the study area or might be due to differences in traditional beliefs. The list of the ethnoveterinary medicinal plants identified in the study area is shown in Table 2. whereas Families with their number of plant species are shown in Table 3.

Plant parts used and their life forms

It was found that the informants used varieties of plant parts for treating different ailments of animals. However, the most commonly used plant part was leaf (of 32 plant species), followed by seed (of 21 plant species), fruit (of 20 plant species), and bark (of 13 plant species). A study by Malla and Chhetri (2012); Acharya et al. (2015) also found leaf as the most used plant part similar to this present study. A preferred use of leaf might be ease of collection as compared to other plant parts. The leaf is also the center for many physiological processes and contains different metabolites (Tariq et al. 2014). The collection of leaves over other parts have no threat to the survival of the plant (Poffenberger et al. 2010). In contrast to this study, Acharya and Acharya (2010) found maximum use of whole plant whereas maximum use of fruit and seed was found by Raut and Shrestha (2012). The plant parts and the number of plant used reports are provided in Figure 3. In addition to this, the data on the life form of plants indicate that most plant species were herbs (44 species), trees (32 species), shrubs (14 species), and climbers (13 species) (Figure 4). Herbs are available everywhere and easy to collect as compared to other life forms. Herbs were also found to be the prevailing life form in the study by Acharya et al. (2015). It might be due to ease of collection, storage, extraction, and transportation than other life forms. But, in contrast to this study Acharya and Acharya (2010) found climbers as mostly used life form.

Preparation technique and routes of administration

The application of the medicinal plants as the form of paste formulation was observed for 41 plant species while 25 plant species were used as powder formulation followed by Juice (21 species), raw (20 species), decoction (19 species), infusion (5 species), and roasted formulation (3 species). In contrast to this study, juice was the widely used preparation technique in previous studies in other parts of Nepal (Acharya and Acharya 2010; Raut and Shrestha 2012).

Table 3. Taxonomic diversity of medicinal plants

Family name	Number of plant species
Acanthaceae	1
Amaranthaceae	2
Amaryllidaceae	1
Anacardiaceae	3
Apiaceae	2
Apocynaceae	3
Araceae	2
Asparagaceae	1
Asphodelaceae	1
Begoniaceae	1
Bignoniaceae	1
Boraginaceae	1
Brassicaceae	1
Cannabaceae	1
Capparaceae	1
Caricaceae	1
Combretaceae	1
Commelinaceae	1
Compositae	2
Convolvulaceae	1
Cucurbitaceae	4
Cyperaceae	1
Dioscoreaceae	1
Equisetaceae	1
Fabaceae	10
Lamiaceae	3
Lauraceae	1
Liliaceae	1
Linaceae	1
Loranthaceae	1
Malvaceae	3
Melastomataceae	1
Meliaceae	2
Menispermaceae	1
Moraceae	4
Musaceae	1
Myristicaceae	1
Oleaceae	1
Oxalidaceae	1
Pedaliaceae	1
Phyllanthaceae	1
Pinaceae	1
Piperaceae	1
Plantaginaceae	1
Poaceae	8
Rosaceae	2
Rutaceae	5
Santalaceae	1
Sapindaceae	1
Sapotaceae	1
Solanaceae	5
Theaceae	1
Urticaceae	1
Verbenaceae	1
Vitaceae	2
Zingiberaceae	3

Similarly, different types of vehicles were found to be used for the administration of plant recipes like water, mustard oil, cornflour, curd, and water. Out of them cornflour and water were the most commonly used. The common route of administration was oral, followed by dermal, and ocular. Maximum use of oral route of medication was similar to the findings of Raut and Shrestha (2012). Nasal way of administration was not found in this study. Different preparation techniques and routes of administration with number of plants are shown in Table 4 and Table 5 respectively. Similar to our study, a single plant, as well as a combination of two or more plant species, was used to cure ailments (Acharya et al. 2015).

Informant consensus factor (ICF)

Informant consensus factors of different ailment categories are shown in Table 6. It was found that the ICF values vary from 0.858 to 0.96 with an average value of 0.926. High informant consensus factor was found for reproductive (0.96), followed by respiratory (0.957), gastrointestinal (0.949) while the least was for urinary problems (0.858). The high ICF value for the reproductive category indicated that reproductive ailments are common in the area. Furthermore, three plant species were used for four ailments categories, eight plant species for three ailments categories, twenty-nine plant species for two ailments categories, and sixty-three plant species were used for single ailment category (Figure 5).

Citation frequency (Cf%)

The total number of times that all species mentioned was 2450 times (N). The highest citation frequency was found for *Trachyspermum ammi* (L.) Sprague 247 (10.08%) followed by *Myristica fragrans* Houtt. 111 (4.53%), *Sesamum indicum* L. 109 (4.44%), *Saccharum officinarum* L. 107 (4.36%), and *Zea mays* L. 97 (3.95%). The top 20 plant species with the highest citation frequency are provided in Table 7. n refers to the number of times a particular species was mentioned.

Table 4. Preparation technique with a number of ethnoveterinary medicinal plants identified in the study area

Preparation technique	Number of plants
Paste	41
Powder	25
Juice	21
Raw	20
Decoction	19
Infusion	5
Roast	3

Table 5. Routes of administration of the ethnoveterinary medicinal plants identified in the study area

Route	Number of plants
Oral	76
Dermal	41
Ocular	9

Table 6. Informant consensus factor by categories of ailments in the study area

Ailment category	Nur	Nt	ICF
Reproductive	651	27	0.96
Respiratory	143	7	0.957
Gastrointestinal	454	24	0.949
Antipyretic	148	9	0.946
Parasitic	385	22	0.945
General weakness	43	4	0.928
Eye	98	9	0.917
Dermatological	329	29	0.914
Osteological	113	13	0.892
Urinary problem	86	13	0.858

Table 7. Top 20 medicinal plant species with highest citation frequency

Botanical name	Local name	n	Cf%
Trachyspermum ammi (L.) Sprague	Juwano	247	10.08
Myristica fragrans Houtt.	Jaifal	111	4.53
Sesamum indicum L.	Kalo til	109	4.44
Saccharum officinarum L.	Ukhu	107	4.36
Zea mays L.	Makai	97	3.95
Artemisia indica L.	Titepati	95	3.87
Cannabis sativa L.	Ganja	89	3.63
Azadirachta indica A.Juss.	Neem	81	3.30
Solanum melongena L.	Bhenta	80	3.26
Artocarpus heterophyllus Lam.	Rukh katar	77	3.14
Marsdenia tenacissima (Roxb.) Moon	Bilajor	68	2.77
Carica papaya L.	Mewa	64	2.61
Melia azedarach L.	Bakaino	62	2.53
Aloe vera (L.) Burm.f.	Ghyukumari	60	2.44
Curcuma longa L.	Besar	55	2.24
Piper nigrum L.	Marich	47	1.91
Musa x paradisiaca L.	Malboe kera	41	1.67
Brassica nigra L.	Tori	41	1.67
Trigonella foenum-graecum L.	Methi	39	1.59
Pisum sativum L.	Simi	36	1.46

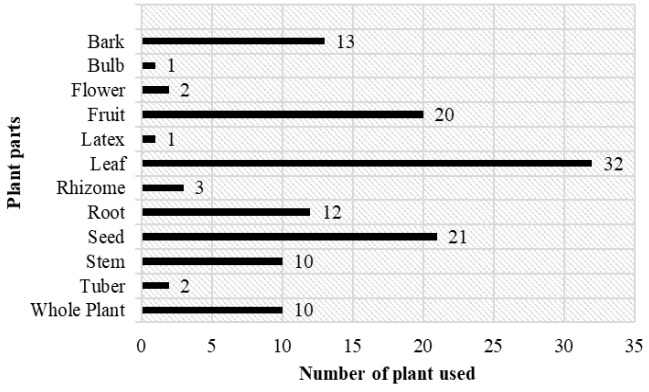


Figure 3. Different plant parts used

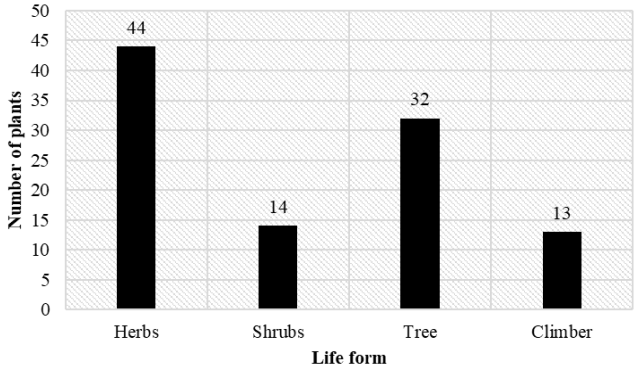


Figure 4. Life form of medicinal plants

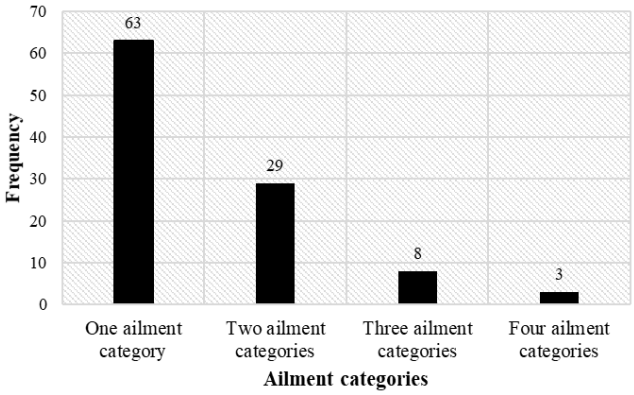


Figure 5. Number of plant species used for number of ailment categories

In conclusion, traditional medicine is widely practiced by the people of Chhatradev Rural Municipality, Arghakhanchi district as it has become a part of social life and culture, and modern medicine implies a higher cost. People are dependable on the locally available indigenous plant species for the treatment of animals. As the people of younger generations are not acquainted with the knowledge on the use of medicinal plants, the actions should be taken for the documentation of uses of plants, to save the knowledge of ethnoveterinary medicinal plants from generation to generation, and increase the interest of young generation for the study of ethnoveterinary medicinal plants. It is of utmost necessity to involve the community in preserving and rational use of medicinal plants at the local level. Conservation programs should be done from the government level, and study on the use of medicinal plants as well as animals for treating animal diseases is recommended to a bigger extent.

ACKNOWLEDGEMENTS

We would like to thank Dr. Subodh Khanal for his guidance, and help during the preparation of the manuscript. We are indebted to all the respondents for sharing their indigenous knowledge and cooperation during the fieldwork.

REFERENCES

Acharya KP, Acharya M. 2010. Traditional knowledge on medicinal plants used for the treatment of livestock diseases in Sardikhola VDC, Kaski, Nepal. J Med Plants Res 4 (2): 235-239. DOI: 10.17660/ActaHortic.2014.1023.4.

Acharya R, Marasini D, Acharya DP. 2015. Traditional knowledge on medicinal plants used for the treatment of domestic cattle in Dhikura village of Arghakhanchi district, Nepal. Adv J Med Plant Res 2 (1): 6-16.

Adeniran LA, Okpi S, Anjorin TS, Ajagbonna OP. 2020. Medicinal plants used in ethnoveterinary practices in the Federal Capital Territory, North-Central Nigeria. J Med Plant Res 14 (8): 377-388. DOI: 10.5897/JMPR2020.6975.

Aryal K, Dhimal M, Pandey A, Pandey A, Dhungana R, Khaniya N, Mehta R, Karki K. 2016. Knowledge Diversity and Healing Practices of Traditional Medicine in Nepal, Nepal Health Research Council, Government of Nepal. http://nhrc.gov.np/publication/knowledge-diversity-and-healing-practices-of-traditional-medicine-in-nepal/

Aziz MA, Khan AH, Adnan M, Ullah H. 2018. Traditional uses of medicinal plants used by Indigenous communities for veterinary practices at Bajaur Agency, Pakistan. J Ethnobiol Ethnomed 14 (11): 1-18. DOI: 10.1186/s13002-018-0212-0.

Bartha, SG, Quave CL, Balogh L, Papp N. 2015. Ethnoveterinary practices of Covasna County, Transylvania, Romania. J Ethnobiol Ethnomed 11 (1): 1-22. DOI: 10.1186/s13002-015-0020-8.

Baskota N, Doj Raj K. 2013. Study on Ethnoveterinary Practices in Jumla. In: 9th National Proceedings of Livestock and Fisheries Research, Nepal 277-282.

CBS. 2012. National Population and Housing Census 2011. In Central Bureau of Statistics, Government of Nepal, Kathmandu.

DPR. 2015. Plant resources. In: Lamichchane D, Basukala S, Tandukar U, Lakhey PB (eds.). Bulletin of Department of Plant Resources 37 (1). Department of Plant Resources (DPR), Kathmandu, Nepal.

Hassan N, Nisar M, Kakar SUR, Ul Hassan F, Zhiwei Zhong S, Nong L, Ijaz Khan M, Shuaib M, Wang D, Deli Wang C, Zhong Z. 2017. Determination of informant consensus factor of medicinal plants used as therapy in district Dir Lower Pakistan. J Med Plants Stud 5 (4): 183-188.

Heinrich M, Ankli A, Frei B, Weimann C, Sticher O. 1998. Medicinal plants in Mexico: Healers' consensus and cultural importance. Soc Sci Med 47 (11): 1859-1871. DOI: 10.1016/s0277-9536 (98)00181-6

ISE. 2006. International Society of Ethnobiology Code of Ethics (with 2008 additions). http: //ethnobiology.net/code-of-ethics/

IUCN Nepal. 2000. National Register of Medicinal Plants. IUCN Nepal. https: //portals.iucn.org/library/sites/library/files/documents/2000-058.pdf

Katerere DR, Luseba D. 2010. Ethnoveterinary Botanical Medicine: Herbal Medicines for Animal Health. CRC Press.

Khan MA, Islam MK, Siraj MA, Saha S, Barman AK, Awang K, Rahman MM, Shilpi JA, Jahan R, Islam E, Rahmatullah M. 2015. Ethnomedicinal survey of various communities residing in Garo Hills of Durgapur, Bangladesh. J Ethnobiol Ethnomed 11 (1): DOI: 10.1186/s13002-015-0033-3

Malla B, Chhetri R. 2012. Ethnoveterinary practices of some plant species by ethnic people of Parbat District, Nepal. KU J Sci Eng Technol 8 (1): 44-50. DOI: 10.3126/kuset.v8i1.6042.

MOAD. 2017. Statistical Information on Nepalese Agriculture 2073/74 (2016/17). Ministry of Agriculture and Livestock Development, Nepal, Kathmandu.

NAVS. 2015. Ethno-veterinary Medicine: A Concept for Sustainable Livestock Production. National Academy of Veterinary Science, New Delhi, India.

Pan S, Litscher G, Gao S, Zhou S, Yu Z, Chen H, Zhang S, Tang M, Sun J, Ko K. 2014. Historical Perspective of Traditional Indigenous Medical Practices : The Current Renaissance and Conservation of Herbal Resources. Evid Based Compl Altern Med 2014: 1-20. DOI: 10.1155/2014/525340

Petrovska BB. 2012. Historical review of medicinal plants' usage. Pharmacogn Rev 6 (11): 1-5. DOI: 10.4103/0973-7847.95849

Phondani PC, Maikhuri RK, Kala CP. 2010. Ethnoveterinary uses of medicinal plants among traditional herbal healers in Alaknanda catchment of Uttarakhand, India. Afr J Tradit Compl Altern Med 7 (3): 195-206. DOI: 10.4314/ajtcam.v7i3.54775

Poffenberger M, McGean B, Khare A, Campbell J. 2010. Community Forest Economy and Use Patterns : Participatory Rural Appraisal (PRA) Methods in South Gujarat, India. In Field Method Manual Volume 2. Society for Promotion of Wastelands Development, New Delhi.

POWO. 2017. Plants of the World. Royal Botanic Gardens, Kew. http: //www.plantsoftheworldonline.org/

Raut B, Shrestha AP. 2012. Ethnoveterinary Practices in Western Morang, Nepal. Intl J Pharm Sci 3 (1): 182-188.

Ryan A. 2014. Documenting Traditional Medical Knowledge. World Intellectual Property Organization, Geneva.

Subedi R. 2017. Ethnobotanical Study of Panchase Protected Forest, Kaski District, Central Nepal. [Dissertation]. Tribhuvan University, Kirtipur. [Nepal]

Tariq A, Mussarat S, Adnan M, Abdelsalam NM, Ullah R, Khan AL. 2014. Ethnoveterinary study of medicinal plants in a tribal society of Sulaiman Range. Sci World J 2014: 1-10. DOI: 10.1155/2014/127526

Weckmüller H, Barriocanal C, Maneja R, Boada M. 2019. Factors affecting traditional medicinal plant knowledge of the Waorani, Ecuador. Susted 11: 1-12. DOI: 10.3390/su11164460.

NUSANTARA BIOSCIENCE
Vol. 13, No. 1, pp. 41-46
May 2021

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n130106

Impact of cognitive-behavioral therapy on daily living skills of high functioning autistic children with anxiety disorders

ATALLAH KHALAF ALENEZI[1,♥], KHADIGA ABD-ELGIED GOMEA HASSAN[1,2], TAHANY EL-SAYED EL-SAYED AMR[1,3], ABDULELLAH ALSOLAIS[4]

[1]College of Applied Medical Sciences at Shaqra, Shaqra University. Shaqra, Saudi Arabia. Tel./fax. +966-55-5182868, ♥email: atta@su.edu.sa
[2]Department of Pediatric Nursing, Faculty of Nursing, Port Said University, Port Said, Egypt
[3]Department of Maternal and Newborn Health Nursing, Faculty of Nursing, Menoufia University, Egypt
[4]College of Applied Medical Sciences at Dawadmi, Shaqra University. Shaqra, Saudi Arabia

Manuscript received: 5 August 2020. Revision accepted: 17 January 2021.

Abstract. *Alenezi AK, Hassan KAG, Amr TEE, Alsolais A. 2021. Impact of cognitive-behavioral therapy on daily living skills of high functioning autistic children with anxiety disorders. Nusantara Bioscience 13: 41-46.* Cognitively Behavioral Therapy (CBT) is a psychotherapeutic intervention that is used to improve mental health. It is known to have positive effect on the daily living skills of autistic children with anxiety disorders. The aim of the current study was to evaluate the effect of cognitive-behavioral therapy on daily living skills of high functioning autistic children with anxiety disorders. A Quasi-experimental research design was adopted in this research. A purposive sample of 100 autistic children (50 in intervention group and 50 in control group) aged between 7-8 years were included in this study. Three main tools were used: Vineland Adaptive Behavior Scale used to measure the everyday living skills of children, Parent-Child Interaction Questionnaire (PACHIQ), and Diagnostic and Statistical Manual of Mental Disorders (DSM-V) for assessing anxiety disorder in children and adolescents 6-18 years. The results showed an improvement in children's daily life skills and slight reductions in caregiver's participation in the daily life skills of children. There was a significant difference between intervention group and control groups ($p < 0.001$). The current results exhibit that CBT may help in increasing autistic children's independence towards daily living skills.

Keywords: Anxiety disorders, autistic disorders, cognitive behavioral therapy, daily living skills

INTRODUCTION

Autism Disorders (AD) is a persistent deficit social conversation, and social interaction as manifested by lack of mutual conversation and emotional reciprocity, lack of non verbal communication, and lack of developing, sustaining, and emphatic relationship (DSM-5 2013). The cause of autism is unknown. However, a lot of predisposing agents, ecological and biological factors and physical defects, have been associated with autism.

About eighty percent of children with autism have anxiety disorders (Duvekot et al. 2017). Some researchers reported that anxiety disorders interfere with daily living practice; so autistic children are more liable to have poorly developed on daily self-care which makes them more needed for suitable intervention (Bishop et al. 2013). A recent study suggested that children who are fully dependent on their parents in basic daily self-care are at high risk to remain anxious during their childhood life (Huffman et al. 2011). Many strategies have been used to improve clinical features of autism such as promoting socialization and communication with others. Till now, there is no effective intervention for improving basic daily life activity for a child with high function autism, who has seventy or more degrees of an intelligence quotient (Gillis et al. 2016). Basic daily life activities are essential tasks for human survival, which cover daily care, composed of individual skills, all activities in house or school and community (Huffman et al. 2011). In usual autistic children do not carry out daily self-activity even if they are able to do it.

Most mothers mention several difficulties in training daily life activities for their children suffering from autism (Gillis et al. 2016; Lord et al. 2005). Family-based cognitive-behavioral therapy is an effective intervention for managing anxiety disturbances in well-child and child with autism (Scherer and Dawson 2011). CBT is an intervention model in which new skills are established by enhancing the child and his family through logic and convincing manner and Socratic questioning (Stone et al. 2010). Hence, the current study examines the impact of the primary parent-based CBT intervention on parental perceptions of daily life skills of the child with autism and synchronous anxiety disturbances.

MATERIALS AND METHODS

Study design

A non-randomized Quasi-experimental pretest-posttest design was used in this study. The impact of primary parent-based CBT on parental perceptions of daily life skills of the child with autism and synchronous anxiety disturbances was determined.

Setting and sample

This study was carried out in Al Amal Complex for Mental Health in Dammam City, Saudi Arabia. The intervention took place between 20 October 2018 to 16 April 2019. A sample of 100 children, aged between 7 to 13 years were included in this study.

Ethical consideration

A written informed consent was obtained from manager of the facility and parents of children (mostly their mothers), who was primarily accountable for supervision of the children's everyday skills and were willing to participate in this study. Before conducting the study, confidentiality and anonymity of the children were assured during collection of the data. Parents of children were assured that the data will not be reused in another research without their consent. The aim of this study was explained to members of the facility, who were directly associated with children.

Subjects and selection method

The sample used in this study were children suffering from autism, who were visiting for follow-up at Al Amal Complex for Mental Health in Dammam city, Saudi Arabia.

Inclusion criteria

Autistic children, Asperger disorders or Pervasive Developmental Disorder - Not Otherwise Specified. Autistic children suffering from separation anxiety or social phobia, or obsessive-compulsive disorder.

Exclusion criteria

Autistic children their verbal Intelligence Quotient less than 70. Autistic children who participated in other behavioral therapy. Autistic children who received psychiatric drugs or changed dosage during the study. Autistic children or parents disable to share in the current study

Research hypothesis

Children with autism acquire an improvement in basic daily life skills and reductions of parent's participation in daily life skills after CBT intervention.

Method

Ethical approval was obtained from College of Applied Medical Science at Shaqra and official permission for conducting the study was secured at Al Amal Complex for Mental Health in Dammam city, Saudi Arabia. Upon approval, the daily living skills of children were measured using the Vineland Adaptive Behavior Scale, and the relationship of the child and parent was evaluated with the use of the Parent-Child Interaction Questionnaire (PACHIQ). The researchers developed a suitable intervention program from literature for children with autism and their parents to improved their basic daily skills. This basic daily life skills program was developed to promote child's skills independently (cognition reorganization) that included a) Focusing on child's establishing trust and self-fulfillment (b) Focusing on social customs that enable the child to act alone. c) Giving rewards with each attempt even if the child do simple steps. A hierarchy plan for developing child's daily life skills (gradation of contact) was used. Families were instructed to assist and encourage any attempts from their children towards independence and give their feedback about this new strategy. A pilot study was carried out on 5 children with autism to examine validity of the questionnaires and to test research feasibility, clarity, and objectivity of the tools. The sample included in the pilot study was excluded from the study sample, and necessary modifications were done accordingly.

The sample of 100 autistic children was divided equally into two main groups; intervention and control of 50 children each. The autistic children in the intervention group were divided into five subgroups, with 10 children for each practitioner. There were no dropouts in the study. These practitioners were in the clinically psychiatric training area at College of Applied Medical Science at Shaqra University and they had experience of dealing with autistic children. Practitioners attended 10 hours of primary activity of the intervention, studied management guide, watched videos of CBT, performed management with pilot study children, and attended 10 workshops with clinical researchers who established the program. Practitioners implemented 8 sessions in 8 weeks, session per week for each subgroup in the study group. Each session continued 70 minutes (about 25 minutes with the children and 45 minutes with the mothers /parents) and the treatment fidelity was assessed by researchers.

Instruments

The study instruments had three tools. The first tool was a structured questionnaire sheet. It included items related to socio-demographic characteristics of children and their families, such as age, child order, occupation, and level of education of their parents. The second tool is The Vineland Adaptive Behaviour Scales, (Vineland-2) (Community-University Partnership for the Study of Children, Youth, and Families 2012) that measures the personality and social skills of individuals from birth through adulthood. Because adaptive behavior refers to an individual's typical performance of daily tasks essential for personality and social adequacy, this scale assesses what the individuals indeed do, not what they are capable to do. The Vineland-2 assesses adaptive behavior in 4 areas: Communication, Daily life Skills, Socialite, and Motor Skills. Additionally, it provides an accumulation mark that epitomizes the person's achievement over 4 areas of investigation (Community-University Partnership for the Study of Children, Youth, and Families 2012).

The third tool is the Parent-Child Interaction Questionnaire (PACHIQ). It assesses how fathers and mothers see the relation with their children (PACHIQ-Parents form), and how the child assesses his/her relation with his/her mother/father (PACHIQ-Children form). It contains 21 elements in the parents form and 25 elements in the children form, about communications strategies and emotional effects (Lange et al., 2002). The researcher

evaluated children with autism for anxiety disturbance and daily life activities before and immediately after the intervention and at the end of three months from intervention. Diagnostic and Statistical Manual mental disorders (DSM-V) was used to assess anxiety disorder in children and adolescents 6-18 years. This scale composed of 38 sections contains panic somatic, separation anxiety, general anxiety, social phobia, and school phobia (Community-University Partnership for the Study of Children, Youth, and Families 2012). The data was analyzed using Chi^2 test using GraphPad Instat software.

RESULT AND DISCUSSION

Table 1 shows that most (95%) of the autistic children were between 7 and 13 years of age. The mean age was 7.1 ± 1.316 days. As regards to educational level, most of them (59%) were at the primary phase. All the autistic children (100%) had siblings and 3 % of them had affected siblings with autism. Table 2 shows that majority (97%) of fathers of autistic children had secondary education and majority of them were employed. In addition, most (90%) of mothers of autistic children had a high level of education and 7% of them were house-makers. About 86% of families were in the middle level of income.

Table 2 shows that majority of families of autistic children faced behavioral, emotional, and physical problems. Also, most (93%) of them had financial problems. The behavioral and emotional problems were expected. However, such high percentage of physical problems (98%) seemed to be high. Saudi Arabia is one the richest countries in the world due to natural resources and many of the Saudis live a comfortable life. Surprisingly, about 93% of the respondents told they have financial issues.

Table 3 shows that there was a statistically significant difference between intervention and control group at pre-intervention, post-intervention, and at the end of three months from intervention. The intervention produced a significant improvement in daily life activities and this was increased further in the 3-months follow-up ($P<0.001$).

Table 4 shows that there was a statistically significant difference between intervention and control group immediately and at the end of three months from intervention ($P<0.001$).

Table 1 showed that most (95%) of the autistic children were between 7 and 13 years of age. The mean age was 7.1 ± 1.316 days. As regards to educational level, most of them (59%) were at primary phase. All autistic children (100%) had siblings and 3 % of them had affected siblings with autism. Majority (97%) of fathers of autistic children had secondary education and the majority of them worked. In addition, most (90%) of mothers of autistic children had high level of education and 7% of them were housewives. Also, the comments (86%) of families were in the middle level of income. There was no significant difference in the socio-demographic characters between intervention and control group.

Table 1. Socio-demographic characteristics of autistic children and parents

Items		Intervention group (n=50)		Control group (n=50)		Total (n=100)	
		No	%	No	%	No	%
Age	7- less than 13 years	2	4	3	6	5	5
	13-18 years						
Mean +/- SD = 7.1 ± 1.316							
Family members	3-5 members	47	94	49	98	96	96
	More than 5	3	6	1	2	4	4
Mean +/- SD = 3.94 +/- 0.848							
Sex	Boy	34	68	35	70	69	69
	Girl	16	32	15	30	13	13
Educational level	Primary	31	62	28	56	59	59
	Preparatory	19	38	22	44	41	41
Having sibling	Yes	50	100	50	100	50	100
	No	0	0	0	0	0	0
Child order	1^{st} - 3^{rd}	25	50	43	86	68	68
	4^{th} -9^{th}	25	50	7	14	32	32
Affected sibling	Yes	1	2	2	4	3	3
	No	49	98	48	96	97	97
Father education	Secondary	49	98	48	96	97	97
	High	1	2	2	4	3	3
Father occupation	Do not work	2	10	0	100	98	98
	Work	48	90	50	2	1	1
Mother education	Illiterate	0	0	1	2	1	1
	Primary	3	15	0	0	3	3
	Secondary	5	25	1	2	6	6
	High	42	60	48	96	90	90
Mother occupation	Housewife	3	15	4	20	7	7
	Work	47	85	46	80	93	93
Family income	Low	4	20	3	15	7	7
	Middle	42	60	44	70	86	86
	High	4	20	3	15	7	7

Table 2. Shows that the majority of families of autistic children faced behavioral, emotional and physical problems. Also, most (93%) of them had financial problems

Items		Intervention group (n=50)		Control group (n=50)		Total (n=100)	
		No	%	No	%	No	%
Behavioral problem	Yes	49	98	48	96	47	97
	No	1	2	2	4	3	3
Emotional problem	Yes	49	98	49	96	48	98
	No	1	2	1	4	2	2
Physical problems	Yes	49	49	49	98	48	98
	No	1	1	1	2	2	2
Financial Problems	Yes	46	46	47	85	93	93
	No	4	4	3	15	7	7

Discussion

This study found that CBT produced improvements in daily living skills of autistic children skills that received cognitive therapy and completed three months program. The CBT performances provide adaptation skills to autistic study group children and reassure the primary parent for helping their children to be more independent. However, it should be noted that factors such as incorrect reports from primary parents may influence the authenticity of the results.

Table 3. Impact of CBT on basic daily life activities of autistic children (Intervention and control groups)

Basic activities of daily living	PI[a] (n-100)		II[b] (n-100)		3months follow up (n=99)	
	IG N=50(%)	CG[d] N=50(%)	IG N=50(%)	CG N=50(%)	IG N=50(%)	CG N=49(%)
Bathing and showering:						
Need no support	0	0	0	0	0	0
Slight support	8(16)	5(10)	42(84)	5(10)	38(76)	4(8.2)
Complete support	42(84)	45(90)	8(16)	45(90)	12(24)	45(91.8)
	(0.467) P>0.001		(6.568)* P<0.001		(7.451)* P<0.001	
Dressing:						
Need no support	0	0	0	0	0	0
Slight support	38(76)	35(70)	45(90)	35(70)	40(80)	33(67.3)
Complete support	12(24)	15(30)	5(10)	15(30)	10(20)	16 (32.7)
	(0.346) P>0.001		(3.851)* P<0.001		(7.695)* P<0.001	
Self- feeding:						
Need no support	0	0	0	0	0	0
Slight support	15(30)	10(20)	42(84)	10(20)	30(60)	10(20.4)
Complete support	35(70)	40(80)	8(16)	40(80)	20(40)	39(79.6)
	(0.717) P>0.001		(5.724)* P<0.001		(6.603)* P<0.001	
Toilet hygiene:						
Need no support	0	0	0	0	0	0
Slight support	40(80)	35 (70)	43(86)	35(70)	40(80)	35(71.4)
Complete support	10(20)	15(30)	7(14)	15(30)	10(20)	14(28.6)
	(0.717) P>0.001		(5.480)* P<0.001		(6.474)* P<0.001	
Oral care:						
Need no support	0	0	0	0	0	0
Slight support	38(76)	37(74)	40(80)	37(74)	30(60)	36(73.5)
Complete support	12(24)	13(26)	10(20)	13(26)	20(40)	13(26.5)
	(1.050) P>0.001		(4.873)* P<0.001		(5.173)* P<0.001	
Total daily living skills						
Need no support	0	0	0	0	0	0
Slight support	35 (70)	37(74)	45(90)	37(74)	40(80)	37(75.5)
Complete support	15(30)	13(26)	5(10)	13(26)	10(20)	12(24.5)
	(0.406) P>0.001		(5.853)* P<0.001		(9.846)* P<0.001	

Note: [a]PI: Before Intervention, [b]II: Post-intervention. [c]IG: Intervention Group, [d]CG: Control Group, *Statistically Significant Difference

Table 4. Relation between daily living skills and anxiety disorders among autistic children. (Intervention and control groups)

	BI[a] (n-100)		II[b] (n-100)		At the end of 3months (n=99)	
	CG[c] n=50(%)	IG[d] n=50(%)	CG n=50(%)	IG n=50(%)	CG n=49(%)	IG n=50(%)
Total daily living skills	(0.41) P>0.001		(5.86)* P<0.001		(9.85)* P<0.001	
Parent–Child Interaction	(5.601)* P<0.001		(3.449)* P<0.001		(0.406) P>0.001	
Anxiety Disorders	(5.457)* P<0.001		(8.177)* P<0.001		(0.677) P>0.001	

Note: [a]PI: Before Intervention, [b]II: Post-intervention. [c]SG: Intervention Group, [d]CG: Control Group, *Statistically Significant Difference

The primary parents of children in the study group recognized an increase in basic daily living skills that are applied on a regular basis by children, such as oral care, toilet hygiene, dressing, self-feeding, and easy health care, compared with autistic children in control group. Earlier reports suggest that without general external support and guidance, autistic children and their primary parents may find obstacles in developing daily living skills that match children's chronological age (Drahota et al. 2010; Duvekot et al. 2017; Estes et al. 2015).

Additionally, primary parent encouragements, beliefs about intervention and expectations, and intervention obedience can play an important role in the modification of basic daily living skills marks at post-test. CBT needs primary parents to perform a dynamic role in implementing the program. Primary parents should perform correct, regular intervention in self-sufficiently, manner. Reportss of parental belief suggests that managing reliability is significantly accompanied with parent motivation to practice intervention procedures, and parent expectancy of intervention results anticipate parental intervention adherences (Estes et al. 2015; Oswald et al. 2016; National Autistic Society, 2016).

As a final point, primary parents of autistic children in the study group stated that their participation in their child's basic daily self-care skills significantly reduced in comparison with primary parents of autistic children in the control group. A moderate influence was established in pre-test and post-test scores related to the participation of primary parents of autistic children in the study group, whereas an insignificant influence was found for primary parents of children in the control group. These are significant outcomes to the CBT Program because primary parent conducts, such as participation in their children's basic daily living skills, have been concerned as an element in the preservation of anxiety and lessened daily living skills (Lord et al. 2005; Drahota et al. 2010). Relating to Wood's model of parental invasiveness, if care-providers take over tasks that children could be accomplished independently, they limit children's mastery and aid dependence and sustained anxiety. Otherwise, children with daily living skill insufficiencies may grow anxious due to their extreme dependence on care-provider. Consequently, reductions in parent invasiveness in their children's basic living skills develop independence and may help in skill achievement and mastery, as well as possibly lowering anxiety level (Duvekot et al. 2017; Estes et al. 2015). In the circumstance, there is a relationship between modifications in anxiety level and daily living changes score, representing various relationships between these concepts. So, elevating daily living skills may be associated with falling in anxiety levels. Fascinatingly, when testing the power of the correlation, correlation is not strong in the study group as opposed to the control group, which would be predictable in a right intervention association. It is evident that primary parental perception of study outcomes of CBT program was seen throughout the study period.

Finally, the study has slight limitations; parent's observations were used to judge the activities of their children as well as their personal participation in the children's self-care skills. As the parents are responsible for the intervention program of CBT, parents' reports might have been subjective by their wishes to achieve the aims of this study. Future studies such as a randomized controlled trial are recommended to support our results.

Parents' CBT program provides statistical and clinically significant in basic daily life activities between autistic children. Parents observed that their autistic children who included in the program were capable to carry out basic daily life activities alone when the program finishes. Parents who included in the program stated that they decreased their participation in their children's personal care, which may provide them with extra time for doing other tasks.

ACKNOWLEDGEMENTS

The authors are thankful to the staff of Al Amal Complex (Dammam) and staff of College of Applied Medical Sciences for their help.

REFERENCES

Begeer S, Mandell D, Wijnker-Holmes B, Venderbosch S, Rem D, Stekelenburg F, Koot HM. 2013. Sex differences in the timing of identification among children and adults with autism spectrum disorders. J Autism Dev Disord 43 (5): 1151-1156. DOI: 10.1007/s10803-012-1656-z.

Bishop SL, Hus V, Duncan A, Huerta M, Gotham K, Pickles A, Kreiger A, Buja A, Lund S, Lord C. 2013. Subcategories of restricted and repetitive behaviors in children with autism spectrum disorders. J Autism Dev Disord 43 (6): 1287-1297. DOI: 10.1007/s10803-012-1671-0.

Community-University Partnership for the Study of Children, Youth, and Families. 2012 Review of the Vineland Adaptive Behavior Scales-Second Edition (Vineland-II). Edmonton, Alberta, Canada.

Diagnostic and statistical manual of mental disorders: DSM-5. 2013. American Psychiatric Publishing, Washington (D.C.).

Drahota A, Wood JJ, Hwang WC, Langley A, Piacentini JC. 2010. Adaptive functioning in school-aged children: Linkages
with trait anxiety and anxiety disorders. J Autism Dev Discord 41 (3): 257-265.

Duvekot J, Hoopen LW, Slappendel G, van der Ende J, Verhulst FC, van der Sijde A, Greaves-Lord K. 2017. Design and cohort characteristics of the social spectrum study: A multicenter study of the autism spectrum among clinically referred children. J Autism Dev Disord 47 (1): 33-48. DOI: 10.1007/s10803-016-2919-x

Estes A, Zwaigenbaum L, Gu H, St John T, Paterson S, Elison JT, Hazlett H, Botteron K, Dager SR, Schultz RT, Kostopoulos P, Evans A, Dawson G, Eliason J, Alvarez S, Piven J, IBIS network. 2015. Behavioral, cognitive, and adaptive development in infants with autism spectrum disorder in the first 2 years of life. J Neurodev Disord 7 (1): 24. DOI: 10.1186/s11689-015-9117-6.

Gillis JM, Roth ME, Sevlever M. 2016. Personal Hygiene. In J. K. Luiselli (Ed.), Behavioral Health Promotion and Intervention for People with Intellectual and Developmental Disabilities. Springer, Switzerland.

Huffman LC, Sutcliffe TL, Tanner IS, Feldman HM. 2011. Management of symptoms in children with autism spectrum disorders: A comprehensive review of pharmacologic and complementary-alternative medicine treatments. J Dev Behav Pediatr 32 (1): 56-68. DOI: 10.1097/DBP.0b013e3182040acf.

Lange A, Evers A, Jansen H, Dolan C. 2002. PACHIQ-R: The parent-child interaction questionnaire--revised. fam process. 41 (4): 709-722. DOI: 10.1111/j.1545-5300.2002.00709.x.

Lord C, Wagner A, Rogers S, Szatmari P, Aman M, Charman T, Dawson G, Durand VM, Grossman L, Guthrie D, Harris S, Kasari C, Marcus L, Murphy S, Odom S, Pickles A, Scahill L, Shaw E, Siegel B, Sigman M, Stone W, Smith T, Yoder P. 2005. Challenges in evaluating psychosocial interventions for autistic spectrum disorders. J Autism Dev Disord 35 (6): 695-711. DOI: 10.1007/s10803-005-0017-6

Maughan AL, Weiss JA. 2017. Parental outcomes following participation in cognitive behavior therapy for children with autism spectrum disorder. J Autism Dev Disord 47 (10): 3166-3179. DOI: 10.1007/s10803-017-3224-z

National Autistic Society. 2016. https://www.autism.org.uk/about/what-is/asd.aspx. 2016

Oswald TM, Winter-Messiers MA, Gibson B, Schmidt AM, Herr CM, Solomon M. Sex Differences in internalizing problems during adolescence in autism spectrum disorder. J Autism Dev Disord 46 (2): 624-636. DOI: 10.1007/s10803-015-2608-1

Scherer SW, Dawson G. 2011. Risk factors for autism: translating genomic discoveries into diagnostics. Hum Genet 130 (1): 123-148. DOI: 10.1007/s00439-011-1037-2

Stone W, Ruble L, Coonrod E, Hepburn S, Pennington M, Burnette C, Bainbridge NB. 2010. Treatment and Research Institute for Autism Spectrum Disorders 2nd ed. Vanderbilt Kennedy Center, Nashville, TS.

NUSANTARA BIOSCIENCE
Vol. 13, No. 1, pp. 47-51
May 2021

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n130107

Short Communication: *Serratia rubidaea* as contaminant in laboratory environment

DEWI PETI VIRGIANTI
Program of Medical Laboratory Technique, Sekolah Tinggi Ilmu Kesehatan Bakti Tunas Husada. Jl. Cilolohan 36 Tasikmalaya 46115, West Java, Indonesia. Tel.: +62-81312928444, email: dewivirgianti@gmail.com

Manuscript received: 11 September 2020. Revision accepted: 19 January 2021.

Abstract. *Virgianti DP. 2021. Short Communication:* Serratia rubidaea *as contaminant in laboratory environment. Nusantara Bioscience 13: 47-51.* There have been many cases of bacterial contamination in the laboratory. The bacterial genera identified as contaminants are *Bacillus, Staphylococcus, Micrococcus, Pseudomonas, Shigella* and *Serratia*. These bacteria are classified as non-pathogenic and pathogenic bacteria that can interfere with the test and potentially develop false-positive results. The present research has shown that red-colored contaminant bacteria develop in unused sterile media in our laboratory. Based on related information, *Serratia marcescens* is a red bacterial species that have been reported as a contaminant in the laboratory. The purpose of this study was to identifiy contaminant bacteria at the molecular level. Based on the phylogenetic characterization using the 16S rDNA gene region, this red contaminant bacterium was identified as *Serratia rubidaea*.

Keywords: Contamination, environment, gene barcoding, red bacteria, *Serratia*

INTRODUCTION

Contamination is an important concern in biological laboratories. They can be categorized into three major groups physical, chemical, and biological. Bacteria, mold, yeast, viruses, and mycoplasma are the most common biological contaminants (Abatenh et al. 2018). Bacterial contamination can occur in numerous laboratories, including animal and plant laboratories (Li et al. 2018), health faculty microbiology laboratories (Lutpiatina 2015), university microbiology laboratories (Ghayoor et al. 2015), as well as in hospital microbiology laboratories (Ng et al. 2011; Konar and Das 2013). Sources of contamination can come from a variety of sources, including air and surfaces (Konar and Das 2013). Sources of contaminated items include tables, floors, clothing, laboratory surfaces such as incubators, microscopes, computers, phones, and water taps (Ng et al. 2011; Ghayoor et al. 2015). In addition, contamination can derive from the body parts of laboratory staff, such as the hands (Konar and Das 2013; Ng et al 2011).

According to Li et al. (2018), the number of microbial contaminants in animal laboratories and bacterial laboratories is found to be higher than in plant laboratories. These contaminant bacteria are pathogenic and non-pathogenic. Common bacterial genera as contaminants in the laboratory include *Bacillus, Staphylococcus,* and *Micrococcus* (Ghayoor et al. 2015; Konar and Das 2013). Pathogenic bacteria such as Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa, Salmonella spp.* and *Enterobacteriaceae* have also been identified as contaminants, especially in hospital microbiology laboratories, potentially giving false-positive test results (Ng et al. 2011). Environmental and human influences have a major impact on the diversity and dynamics of microbial contaminants (Li et al. 2018), as well as the usage of gloves and handwashing, which are very successful in minimizing pathogenic bacterial contamination in the hospital microbiology laboratory (Ng et al. 2011). In addition, proper disinfection and sterilization are needed to eliminate contaminant microorganisms and personal hygiene of laboratory staff is also required (Konar and Das 2013).

In a health education laboratory where microbiology is practiced, contaminant bacteria can interfere with the learning process. The genus *Bacillus* is the main contaminant of the laboratory (Lutpiatina 2015). In the present research, red bacterial isolates were collected from contaminated media that were kept in the washing area of the glassware in a medical school laboratory. In current reports, red bacteria were found as contaminated bacteria in clinical microbiology laboratory of hospital and identified as *Serratia marcescens*. The contamination caused a pseudo-outbreak as a result of diagnosis. These contaminant bacteria are found in saline solutions, soaps and disinfectants used in laboratories. This pseudo-outbreak emphasizes the importance of laboratory worker ability to perform specimen processing procedures (Dundar et al. 2009). *Serratia* typically causes neonatal nosocomial outbreaks. This outbreak is associated with personal hygiene, such as hand hygiene (Zingg et al. 2017) and environmental hygiene, such as the value of clean air conditioning, which can become a nosocomial reservoir (Uduman et al. 2002). The genus *Serratia* is a Enterobacteriaceae member that produces prodigiosin, a non-diffusible red pigment. The habitat of *Serratia* includes air, water, and soil. It can also be associated with plants, insects, and other animals (Grimont and Grimont 1978).

Information about the diversity of the contaminant bacteria in the laboratory and the origin of their spread is very essential. Therefore, this study emphasized the molecular characterization of contaminant red bacteria isolated from the culture media in the laboratory.

MATERIALS AND METHODS

Procedure

Isolation of red bacteria

Red bacteria were isolated from the contaminated culture medium of Sabouraud Dextrose Agar (SDA) to the Nutrient Agar (NA) medium. The red bacterium was called Bakteri Merah as the code.

Identification of red bacteria

Identification of red bacteria was based on morphological and molecular characterization. Morphological characterization was performed through observation of colony and Gram staining. Molecular characterization was performed by barcoding the 16S rRNA gene using 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') as universal primer for bacteria. DNA genome extraction was carried out using the Fungal/Bacterial Miniprep Kit (Zymo Research, D6005). PCR amplification was conducted with MyTaq HS Red Mix (Bioline) with 1 x 25µL master mix PCR composition consisting of 9.5 µL ddH2O, 12.5 µL My Taq Red Mix, 2x, 1 µL 10 µmol/µL 27F primers, 1 µL 10 µmol/µL 1492R primer and 1µL DNA template. PCR was performed with the Agilent SureCycler 8800 Thermocycler. PCR was performed at an initial denaturation step of 95 °C for 1 min, followed by 35 cycles at 95 °C for 15 sec, 52 °C for 15 sec, and a final extension step at 72 °C for 72 sec. PCR products have been purified with the Zymoclean Gel DNA Recovery Kit (Zymo Research). Sequencing was carried out in two ways, carried out commercially by Genetika Science Indonesia. Sequential data assembly was done with BLAST through http:/www.ncbi.nlm.nih.gov/BLAST/.

Evolution analysis was conducted with MEGA7 (Kumar et al. 2016) and using the Neighbor-Joining method to obtain a percentage of the tree repetition in which the taxa cluster was associated with the bootstrap test (1000 repetitions). The evolutionary distance was calculated using the Maximum Composite Likelihood method.

RESULTS AND DISCUSSION

Purification of red bacteria

Contaminated SDA medium was sterilized unused medium, but was previously cut into laminar air flow with aseptic tools for other purposes. Contamination occurs when the medium was placed in washing room of glasswares located inside the laboratory. The pure culture of red bacterial colonies showed characteristics of round, medium-sized colonies of 2-3 mm, flat edges, convex elevations, and pink to red color (Figure. 1). Gram staining results exhibited that the bacterium was cocobacili Gram-negative.

Phylogenetic analysis

Molecular identification was performed to get a confirmed identification of the bacteria. The 16S rRNA sequence was successfully amplified. The PCR product was shown by DNA fragment at 1500 bp (Figure 2). Evolutionary analysis conducted by MEGA7 showed the red bacteria were clustered into *S. rubidaea* **(**Figure 3). The clade of *S. rubidaea* was distinctly distinguished from the other species of *Serratia*. According to the phylogenetic tree as described in figure 3, the most closely related strains were *S. rubidaea* NR_024644 strain JCM 1240 and *S. rubidaea* NR_114232 strain NBRC 103169. A similar result was obtained from BLASTN result that the similarity of the strain reached 99.65% identity with *S. rubidaea* NR_024644 strain JCM 1240 and 99.58% with *S. rubidaea* NR_114232 strain NBRC 103169. Meanwhile, other clades include other *Serratia* species namely *S. ficara* , *S. odorifera, S. marcescens, S. ureilytica* and the other genus of *Cedecea*. Based on NCBI information, *S. rubidaea* NR_024644 strain JCM 1240 is Japan's bacterial strain used as comparative reference in the journal of investigation of the origin of intracellular aerobic gut bacteria symbiont of the *Buchnera* aphid (Harada et al. 1995).

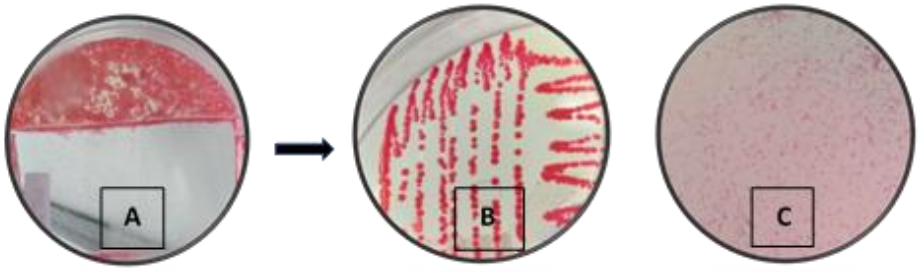


Figure 1. Contaminants of red bacteria grown in unused culture medium (A), pure colonies of red bacteria (B) Cocobacilli Gram-negative bacteria (C)

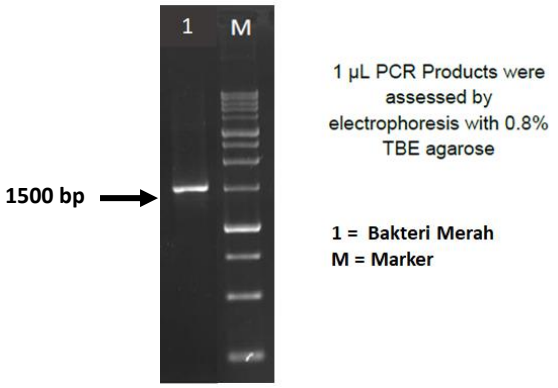


Figure 2. L PCR products were assessed by electrophoresis with 1% TBE agarose. Red bacteria (Bakteri Merah) (1) and marker (M).

The genus *Serratia* belongs to the Enterobacteriaceae family and the Gammaproteobacteria class. The genus consists of 15 species, namely *S. entomophila, S. ficaria, S. fonticola, S. glossinae, S. grimesii, S. liquefaciens, S. marcescens, S. nematodiphila, S. odorifera, S. plymuthica, S. proteamaculans, S. quinivorans, S. rubidaea, S. symbiotica,* and *S. ureilytica* (http://www.catalogueoflife.org) . *S. rubidaea* was first described in 1940 as *Bacterium rubidaea* and also as *Serratia marinarubra*, but was reclassified as *S. rubidaea* (Ewing et al. 1973). There are three subspecies in it: *S. rubidaea subsp. burdigalensis, S. rubidaea subsp. rubidaea,* and *S. rubidaea subsp. colindalensis* (Grimont and Grimont 2006).

The habitat of *S. rubidaea* is not known for certain, but it has reported to have been isolated from foods such as coconuts (Siva et al. 2012), tomato salad (Abd-Alla et al. 2011), green chillies, and milk (Al-Mijalli 2014; Immanuel et al. 2008). It has also been identified as phytopathogen in tulips (Stoyanova and Bogatzevska 2011), epiphytic of *Chaetomorpha media* seaweed (Pawar et al. 2015) and isolated from soil (Nalini and Parthasarathi 2013).

The presence of *S. rubidaea* in clinical samples is uncommon, but may cause opportunistic infection in several patients who have invasive procedures of surgery or receiving broad-spectrum antimicrobials (Ursua et al. 1996; Gentille et al. 2014; Litterio et al 2002; Yao et al 2016). *S. rubidaea* has been reported in several research journals, including as a causes of nosocomial urinary tract infections (Menezes et al. 2004), and causes reddish urine color (Kumar et al. 2013). The red pigment of *S. rubidaea* known as prodigiosin might be associated with urinary tract infection (UTI) and leading to reddish discoloration of urine. This case has been documented in diabetic patients who presented with burning micturition and reddish discoloration (Kumar et al. 2013). As nosocomial urinary tract infection agent, the frequency of *S. rubidaea* appearance in urine samples is the lowest compared to *S. liquifaciens* and *S. odorifera* (Menezes et al. 2004). The damaged physical structure of hospital facilities, including surgery and obstetric birthing center, leads increasing predisposition of bacterimia in neonatal caused by *S. rubidaea* (Karkey et al. 2018). In addition, it has been reported to cause sepsis, although the case was rare (Okada et al. 2002). Six species of *Serratia*, namely *Serratia ficaria, Serratia fonticola, Serratia odorifera, Serratia plymuthica, Serratia rubidaea* and *Serratia entomophila* are uncommon in clinical studies, but almost all *Serratia* have been isolated from human clinical specimens and virulence-associated properties have also been reported (Stock et al. 2003).

S. rubidea capable of producing extracellular enzymes that play an important role in its opportunistic pathogenicity. Protease, lipase, alkaline phosphatase and polygalacturonase enzymes were detected in *S. rubidaea* which produce at several optimum temperatures (Al-Mijalli 2014). Besides the enzymes, *S. rubidaea* is a source of red pigment prodigiosin, which is very important in biotechnology and pharmacology. Siva et al. (2012) documented the production of pigment prodigiosin in *S. rubidaea* was higher than *S. marcescens*. Prodigiosin has potential clinical significance as it is stated to have antibacterial (Ibrahim et al. 2014), antifungal (Shaikh 2016), antiprotozoal/antimalaria (Papireddy et al. 2011) and promising anticancer activity (Lin et al. 2019; Li et al. 2018).

The cases of *S. rubidaea* contamination have never been documented in laboratories, but this finding proves that *S. rubidaea* can contaminate the stored media of microbial culture in the laboratory environment. Contaminated culture media was sterile media that was no longer used and placed in the laboratory without wrapping in a glassware washing area. It is suspected that the source of the contaminant comes from vector insects such as *Drosophila melanogaster*, aphid, mites or ants. The vector can enter the unwrapped Petri dishes through the gaps (Pease 1937).

Some *Serratia* species have been associated with insects. *S. marcescens*, has been associated with a number of insects such as *Nilaparvata lugens* Ståll (Priyatno et al. 2011), *Plutella xylostella* (Indiragandhi et al. 2011), *Myrmica* ants, and *Maculinea* (Salvo et al. 2019). *S. entomophila* also plays a role in the chemical cross-talk in larval stage between of *Maculinea* and also in the *Myrmica ants* (Salvo et al. 2019).

In conclusion, *S. rubidaea* has been identified as contaminant bacteria in laboratories. This result provided further knowledge of the variety of contaminant bacteria obtained from the laboratory environment, such as insects as vectors.

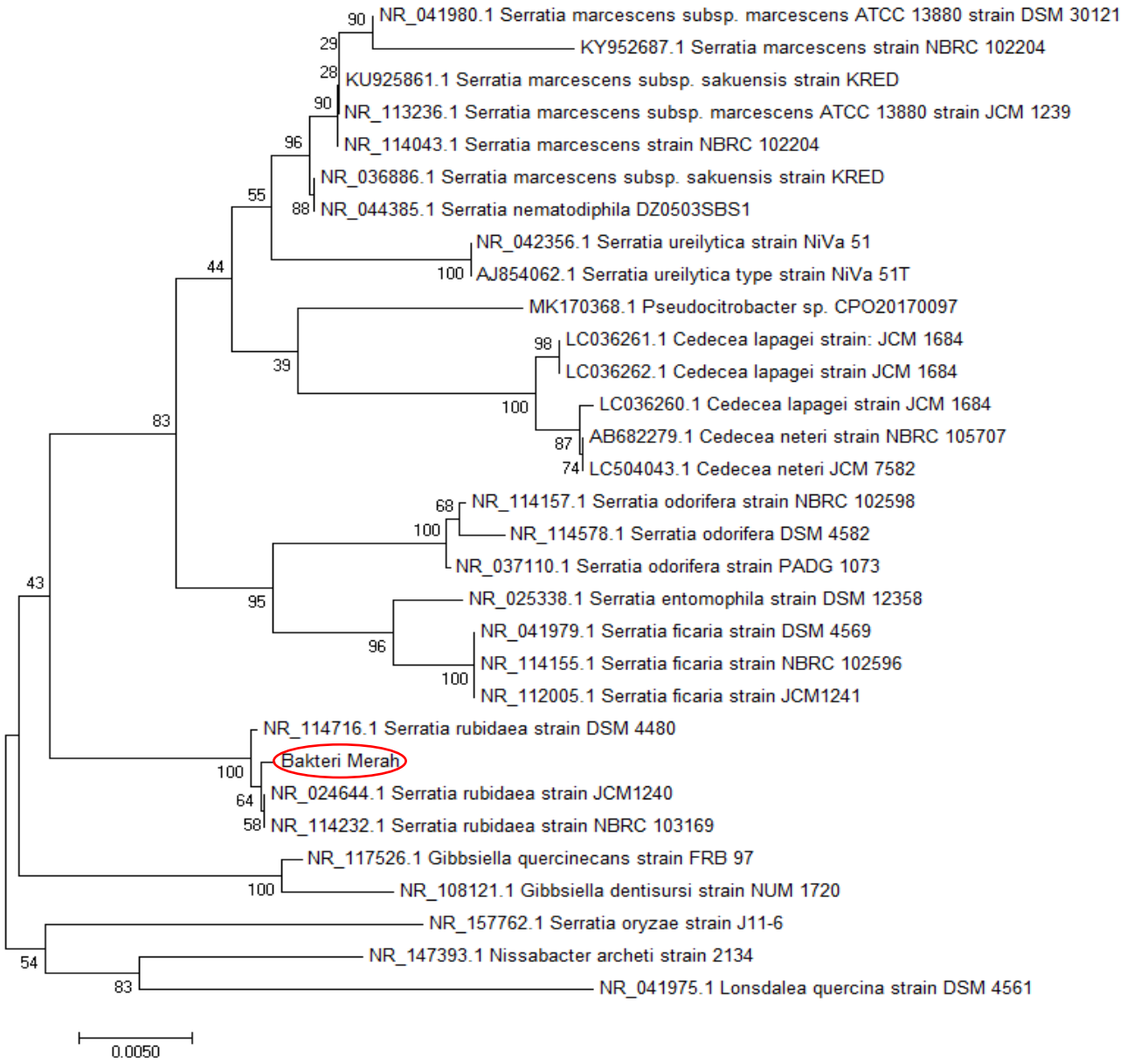


Figure 3. Neighbor-joining dendrograms based on 16S rRNA sequences show a phylogenetic relationship between red bacteria (Bakteri Merah) and a sequence of bacteria obtained from BLAST results. Bootstrap values based on 1000 repetitions are displayed on branch nodes. Phylogenetic trees recognize red bacterial isolates in one clade as *Serratia rubidaea*

ACKNOWLEDGEMENTS

This research was funded by P3M STIKes BTH Tasikmalaya, Indonesia.

REFERENCE

Abatenh E, Gizaw B, Tsegaye Z. 2018. Contamination in a microbiological laboratory. Int J Res Stud Biosci 6 (4): 7-13. DOI: 10.20431/2349-0365.0604002.

Abd-Alla MH, Bashandy SR, Schnell S, Ratering S. 2011. Isolation and characterization of *Serratia rubidaea* from dark brown spots of tomato fruits. Phytoparasitica 39 (2): 175-183. DOI: 10.1007/S12600-011-0148-6.

Al-Mijalli SHS. 2014. Isolation and characterization of plant and human pathogenic bacteria from green pepper (*Capsicum annum* L.) in Riyadh, Saudi Arabia. Biotech 4: 337-344. DOI 10.1007/s13205-013-0136-2.

Dundar D, Meric M, Vahaboglu H, Willke A. 2009. Pseudo-outbreak of *Serratia marcescens* in a tertiary care hospital. New Microbiol 32: 273-276.

Ewing WH, Davis BR, Fife MA, Lessel EF. 1973. Biochemical characterization of *Serratia liquefaciens* (Grimes and Hennerty) Bascomb et al. (formerly *Enterobacter liquefaciens*) and *Serratia rubidaea* comb. nov. and designation of type and neotype strains. Int J Syst Bacteriol 23: 217-225.

Gentille D, Pérez M, Centelles MJ. 2014. Bacteremia by a *Serratia rubidaea* with an atypical quinolones resistance phenotype. Rev Chilena Infectol 31 (3): 351-352.

Ghayoor M, Qadoos A, Bahadar S, Hayat A, Daud M, Hassan A, Ali F, Zeb A, Ur Rahman K, Wahab A, Khattak ZF, Khattak B. 2015.

Isolation and identification of common contaminants bacteria from working area in microbiology laboratory. J Bio-Mol Sci 3 (2): 74-78.

Grimont F, Grimont PAD. 2006. The genus *Serratia*. Prokaryotes 6: 219-244. DOI: 10.1007/0-387-30746-x_11.

Grimont PAD, Grimont F. 1978. The genus *Serratia*. Ann Rev Microbiol 32: 221-248.

Harada H, Oyaizu H, Ishikawa H. 1996. A consideration about the origin of aphid Intracellular symbiont in connection with gut bacterial flora. J Gen Appl Microbiol 42: 17-26.

Ibrahim D, Nazari TF, Kassim J, Lim SH. 2014. Prodigiosin-an antibacterial red pigment produced by *Serratia marcescens* IBRL USM 84 associated with a marine sponge *Xestospongia testudinaria*. J Appl Pharm Sci 4: 1-6. DOI: 10.7324/JAPS.2014.40101.

Immanuel G, Esakkiraj P, Jebadhas A, Lyapparaj P, Palevesam A. 2008. Investigation of lipase production by milk isolate *Serratia rubidaea*. Food Technol Biotechnol 46 (1): 60-65.

Indiragandhi P, Anandham R, Sa TM. 2011. Functional significance of insect gut bacteria and their role in host insect processes, development, and crop production. In: Maheshwari DK (ed.) Bacteria in Agrobiology: Plant Growth Responses. DOI: 10.1007/978-3-642-20332-9_14. Springer-Verlag Berlin Heidelberg.

Karkey A, Joshi N, Chalised S, Joshi S, Shrestha S, Nguyen TNT, Dongol S, Basnyat B, Baker S, Boinett CJ. 2018. Outbreaks of *Serratia marcescens* and *Serratia rubidaea* bacteremia in a central Kathmandu hospital following the 2015 earthquakes. Trans R Soc Trop Med Hyg 112: 467-472. DOI: 10.1093/trstmh/try077.

Konar J, Das S. 2013. Common contaminants of bacteriology laboratory: microbiological paramores**.** Int J Pharm Sci Invention 2 (11): 2319-6718.

Kumar S, Bandyopadhyay M, Chatterjee M, Mukhopadhyay P, Pal S, Poddar S, Banerjee P. 2013. Red discoloration of urine caused by *Serratia rubidae*: A rare case. Avicenna J Med 3 (1): 20-22. DOI: 10.4103/2231-0770.112790.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33: 1870-1874.

Litterio ML, Arazi S, Hernandez C. 2012. Isolation of Serratia rubidaea from a mixed infection after a horse bite. Rev Argent Microbiol 44: 272-274.

Li D, Liu J, Wang X, Kong D, Dua W, Li H, Hse CY, Shupe T, Zhou D, Zhao K. 2018. Biological potential and mechanism of prodigiosin from *Serratia marcescens* Subsp. lawsoniana in human choriocarcinoma and prostate cancer cell lines. Int J Mol Sci 19 (11): 3465. DOI: 10.3390/ijms19113465.

Li X, Zhu X, Wang W, Ning K. 2018. Microbial contamination screening and interpretation for biological laboratory environments. bioRxiv 439570. DOI: 10.1101/439570.

Lin PB, Shen J, Ou PY, Liu LY, Chen ZY, Chu FJ, Wang J, Jin XB. 2019. Prodigiosin isolated from *Serratia marcescens* in the *Periplaneta americana* gut and its apoptosis-inducing activity in HeLa cells. Oncol Rep 41 (6): 3377-3385. DOI: 10.3892/or.2019.7089.

Lutpiatina L. 2015. Produk urikase dari *Bacillus sp*. kontaminan laboratorium. Med Lab Technol J 1 (2): 96-101. [Indonesian]

Menezes EA, Cezafar FC, de Sena Andrade MS, de Paula Rocha MVA, Cunha FA. 2004. Frequency of *Serratia* sp in urine infections of intern patients in the Santa Casa de Misericórdia in Fortaleza. Revista da Sociadade Brasileira de Medicina Tropical 37: 70-71.

Nalini S, Parthasarathi R. 2013. Biosurfactant production by *Serratia rubidaea* SNAU02 isolated from hydrocarbon-contaminated soil and its physicochemical characterization. Bioresour Technol 147: 619-622. DOI: 10.1016/j.biortech.2013.08.041.

Ng LSY, Teh WT, Ng SK, Eng LC, Tan TY. 2011. Bacterial contamination of hands and the environment in a microbiology laboratory. J Hosp Infect 78: 231-233. DOI: 10.1016/j.jhin.2011.01.025.

Okada T, Yokota E, Matsumoto I. 2002. Community-acquired sepsis by *Serratia rubidaea*. Kansenshogaku Zasshi. J Jpn Assoc Infect Dis 76 (2): 109-112.

Papireddy K, Smilkstein M, Kelly JX, Salem SM, Alhamadsheh M, Haynes SW, Challis GL, Reynolds KA. 2011. Antimalarial activity of natural and synthetic prodiginines. J Med Chem 54: 5296-5306. DOI: 10.1021/jm200543y.

Pawar R, Mohandass C, Sivaperumal E, Sabu E, Rajasabapathy R, Jagtap T. 2015. Epiphytic marine pigmented bacteria: A prospective source of natural antioxidants. Braz J Microbiol 46 (1): 29-39. DOI: 10.1590/S1517-838246120130353.

Pease D. 1937. The insect menace in the bacteriology laboratory. J Bacteriol 33 (6): 619-624.

Priyatno TP , Dahliani YA, Suryadi Y, Samudra IM, Susilowati DN, Rusmana I, Wibowo BS, Irwan C. 2011. Identifikasi entomopatogen bakteri merah pada wereng batang coklat (*Nilaparvata lugens* Stål.). Jurnal Agro*Biogen* 7 (2): 85-95. [Indonesian]

Salvo MD, Calcagnile M, Talà A, Tredici SM, Maffei ME, Schönrogge K, Barbero F, Alifano P. 2019. The microbiome of the Maculinea-Myrmica host-parasite interaction. Sci Rep 9: 8048. DOI: 10.1038/s41598-019-44514-7.

Siva R, Subha K, Bhakta D, Ghosh AR, Babu S. 2012. Characterization and enhanced production of prodigiosin from the spoiled coconut. Appl Biochem Biotechnol 166: 187-196. DOI: 10.1007/s12010-011-9415-8.

Shaikh Z. 2016. Biosynthesis of prodigiosin and its applications. IOSR J Pharm Biol Sci 11: 1-28. DOI: https://doi.org/10.9790/3008-1106050128.

Stock I, Burak S, Sherwood KJ, Gruger T, Wiedemann B. 2003. Natural antimicrobial susceptibilities of strains of 'unusual' *Serratia* species: *S. ficaria, S. fonticola, S. odorifera, S. plymuthica* and *S. rubidaea*. J Antimicrob Chemother 51 (4):865-85.

Stoyanova M, Bogatzevska N. 2011. Phytopathogenic *Serratia rubidaea* Isolated from Tulips. Sci Technol 1 (6): 20-24.

Uduman SA, Farrukh AS, Nath KNR, Zuhair YH, Ifrah A, Khawla AD, Sunita P. 2002. An outbreak of *Serratia marcescens* infection in a special-care baby unit of a community hospital in United Arab Emirates: the importance of the air conditioner duct as a nosocomial reservoir. J Hosp Infect 52 (3): 175-180. DOI: 10.1053/jhin.2002.1308.

Ursua PR, Unzaga MJ, Melero P, Iturburu I, Ezpeleta C, Cisterna R. 1996. *Serratia rubidaea* as an invasive pathogen. J Clin Microbiol 34 (1): 216-217. DOI: 10.1128/JCM.34.1.216-217.1996.

Yao X, Sun Q, Liu W, Yin X, Pei G, Wang Y, An X, Mi Z, Luo Y, Tong Y, Chen S. 2016. Complete genome sequence of *Serratia rubidaea* isolated in China. Genome Announc 4 (2): e00283. DOI: 10.1128/genomeA.00283-16.

Zingg W, Soulake I, Baud D, Huttner B, Pfister R, Renzi G, Pittet D, Schrenzel J, Francois P. 2017. Management and investigation of a *Serratia marcescens* outbreak in a neonatal unit in Switzerland – the role of hand hygiene and whole-genome sequencing. Antimicrob Resist Infect Control 6 (125): 1-6. DOI: 10.1186/s13756-017-0285-x.

NUSANTARA BIOSCIENCE
Vol. 13, No. 1, pp. 53-61
May 2021

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n130108

Preliminary QTL detection for *Corynespora* Leaf Fall disease resistance in rubber plant

FETRINA OKTAVIA[1,♥], SUDARSONO[2], DINY DINARTI[2]

[1]Indonesian Rubber Research Institute. Jl. Raya Palembang-Pangkalan Balai Km. 29, Sembawa, Banyuasin 309553, South Sumatra, Indonesia. Tel.: +62-711-7439493, Fax.: +62-711-7439282, ♥email: fetrina_oktavia@yahoo.com

[2]Department of Agriculture, Institut Pertanian Bogor. Jl. Meranti, IPB University Campus Dramaga, Bogor 16680, West Java, Indonesia

Manuscript received: 20 November 2020. Revision accepted: 21 January 2021.

Abstract. *Oktavia F, Sudarsono, Dinarti D. 2021. Preliminary QTL detection for Corynespora Leaf Fall disease resistance in rubber plant. Nusantara Bioscience 13: 53-61. Corynespora* Leaf Fall (CLF) disease caused by *Corynespora cassiicola* fungus is one of the most important diseases of rubber trees. Conventional breeding to obtain the resistant rubber clones was constrained by the long time required for selection processes. This study was aimed to identify resistant loci linked with CLF disease on the rubber tree. Analysis was conducted on 104 F1 of BPM 1 x RRIM 600. The resistance evaluation of the population had been done against two *C. cassiicola* isolates and the molecular analysis was generated by using 28 selected SSR markers. There was a phenotypic variation in F1 resistance to both isolates with leaf wilting intensities ranged from 5.2% to 33.4% on CC-06 isolate, and from 3.5% to 36.4% on CC-22 isolate. There was no QTL identified on the genetic linkage map, but a single marker analysis showed that some loci associated with CLF disease. The associated loci can be used as a preliminary information to develop molecular markers linked with resistance to CLF disease to assist the rubber breeding programs.

Keywords: CLF disease, *Hevea brasiliensis*, MAS, progeny, rubber breeding

Abbreviations: CLF: *Corynespora* Leaf Fall; QTL: Quantitative Trait Loci; MAS: Marker Assisted Selection

INTRODUCTION

Rubber tree (*Hevea brasiliensis*) is one of the ten members of the *Hevea* genus which became the main cultivated species in the natural rubber producers. The plants coming from South America are growing well in Southeast Asia and it contributes more than 90% of the natural rubber production in the world. However, this potential was restricted by *Corynespora* leaf fall (CLF) disease caused by the *Corynespora cassiicola* fungus which causes a decrease in latex production, and in severe conditions, the disease can lead to plant death. CLF disease has caused serious damage in almost all of the centers of rubber plantations in Southeast Asia and Africa.

Some efforts have been done to control the CLF disease and chemical control is commonly used to control the disease. Although the chemical method becomes the first choice in the field, it is not the best solution. Chemical control is not only expensive but its application also causes a negative impact on the environment. Continuous use of pesticides can cause the pathogen to develop resistance against the pesticide, thus the pesticide is no longer effective against the pathogen.

Use of high and stable resistant clones is one of the most effective and economical ways to control the CLF disease. Breeding activities to produce new resistant clones have been done by breeders and there are some problems mainly related to the limited area and the lengthy time of selection. One alternative to overcome these problems is the use of *Marker Assisted Selection* (MAS).

MAS can help accelerate the breeding programs mainly on the perennial crops which have a long life cycle. To obtain an accurate MAS, information on and the availability of genetic linkage maps are required. The development of genetic mapping studies on the perennial crops which are cross-pollinated and heterozygous are not as advanced as that in horticultural crops. This is because perennial plants such as Hevea have a long life cycle and low ability to produce seeds due to inbreeding depression, so the preparation of progeny for genetic studies is very difficult (Lespinasse et al. 2000). These limitations also caused only F1 progenies obtained from crossing of the heterozygous parents are possible to be prepared as a mapping population of which these conditions may cause segregation of alleles in one locus. The generated data can be analyzed as a double pseudotestcross and preparation of the genetic linkage map for each parent has to be carried out separately (Grattapaglia and Sederoff 1994). The use of F1 population that was obtained from a cross had been widely used as a mapping population to construct a linkage map as was reported in *Eucalyptus* (Grattapaglia and Sederoff 1994), tea plant (Hackett et al. 2000; Ma et al. 2014), and rubber tree (Le Guen et al. 2003; Le-Guen et al. 2007; Le Guen et al. 2011; Le Guen et al. 2013; Rattanawong et al. 2009; Novalina 2013; Souza et al. 2011; Souza et al. 2013; Conson et al. 2018; Rosa et al. 2018; An et al. 2019).

In this study, we reported the construction of a genetic linkage map by using F1 population that was obtained from crossing of BPM 1 and RRIM 600 clones. The availability of genetic linkage maps and information about the molecular markers related to the loci that control resistance to CLF disease can be used as a marker for early detection of resistant accessions so that it will accelerate the selection process in breeding programs to obtain new superior clones in the future.

MATERIALS AND METHODS

Evaluation of CLF disease resistance

104 F1 progenies which consist of 30 plants and 74 embryos were obtained from crossings the CLF disease-resistant rubber clone (BPM 1) and the susceptible clone (RRIM 600) (Oktavia et al. 2016). The CLF disease resistance level of 30 F1 plants was tested by their resistance to the toxin filtrate produced by the two isolates of *C. Cassiicola*, namely CC-06 and CC-22 collected from GT 1 and RRIM 600 rubber clones in the Sembawa Research Centre, Palembang. The isolates were classified as highly virulent isolates based on previous study (Oktavia et al. 2017). Procedures of toxin production and resistance evaluation were based on Breton et al. (2000) with some modifications. Resistance evaluation was conducted in the laboratory-based on the activity of the toxin filtrate of isolate using immature leaves (B2C growth stage). The leaves were taken from plants grown in the greenhouse and then soaked in water for one night (16 hours). Subsequently, 125 ml toxin filtrate (concentration of 5 mg L^{-1}) was inserted in the tray covered with perforated styrofoam (3 cm diameter) and the petiole was inserted into the hole until submerged in toxin filtrate. As a control, sterile water was used instead of toxin filtrate, and all treatments were incubated at room temperature for 48 hours. Each treatment was repeated three times.

Assessment of plant resistance level was based on the water loss estimation due to toxin activity that was observed 48 hours after soaking the leaves in the toxin filtrate. Water loss estimation was calculated as the leaf wilting intensity (LWI) which showed the percentage of difference in weight of the leaves before and after the treatment with the toxin minus the control.

Linkage map analysis

Total DNA was extracted from 104 rubber leaf samples according to the procedure described by Orozco-Castillo et al. (1994). The DNA stocks were either dissolved in TE for storage at -20 ^{o}C in the freezer or diluted in ddH_2O as the working solution.

PCR amplification was carried out in a total volume of 12.5 µl by using the selected SSR primers. The PCR reaction mixture consisted of 2 µl of 25 ng μl^{-1} DNA template, 0.75 µl (10 nm) each of the forward and reverse primer, 2.75 µl ddH_2O and 6.25 µl of PCR mix (Kapa Biosystem Inc. USA). The Amplifications were performed in a DNA thermal cycler (Model T-100 Thermal Cycler, Bio-Rad, USA). The amplification program was as follow: one cycle of pre-denaturation at 95 ^{o}C for 3 minutes, followed by 35 cycles of amplification consist of denaturation at 95 ^{o}C for 15 seconds, primer annealing at 53-56 ^{o}C for 15 seconds, and primer extension at 72^{o}C for 30 seconds, and terminated by one cycle of final primer extension at 72 ^{o}C for 3 min. The amplified PCR products were evaluated in 1% agarose in TBE buffer horizontal gel electrophoresis. All of the samples that positively produced PCR product were subsequently separated in a vertical denaturing SDS polyacrylamide gel electrophoresis (SDS PAGE) containing 7 M urea, by using a single gel dedicated manual sequencer (Cole-Parmer®). The observed allelic patterns of the individual accession were visualized by staining the gel in a silver nitrate according to procedure developed by Creste et al. (2001).

The SSR primers used to construct genetic linkage map were obtained from two steps of selection. The first selection was polymorphism of 135 SSR primers (An et al. 2013; Cubry et al. 2014; Le Guen et al. 2011; Li et al. 2012; Mantello et al. 2014; Silva et al. 2014; Triwitayakorn et al. 2011) on both parental clones. Subsequently, the selected primers were used to amplify all individuals in the mapping population. The progenies that carried a specific allele from the resistant parent were indicated as a heterozygous genotype (H) and the ones that did not carry the allele were characterized as a homozygous genotype (A). Furthermore, the loci in the population having segregation of H:A that fitted the ratio of 1:1 based on chi-square test were used to construct the linkage map by converting the frequency of recombination into map distance (centiMorgan = cM). Construction of the linkage map was done using the Kosambi mapping function employing the MapMaker / EXP 3.0 program of the model backcross on the value of LOD minimum 3.0 and 2.0 with fractions of recombinant of 0.5 (Lincoln and Landers 1993). Identification of QTLs linked to CLF disease was done based on the observed resistance level and molecular data by using QGene 4.0 software (Joehanes and Nelson 2008).

Identification of SSRs markers associated with CLF disease resistance

The association between SSR markers with CLF disease resistance was determined by using a single marker analysis (Champoux et al. 1995; Collard et al. 2005). The analysis was performed by combining the disease resistance data and SSR markers data employing SPSS 20 statistical package. This method was done by using analysis of variance, where the plant accessions scoring result was the independent variable (X) and the level of resistance as the dependent variable (Y). Analysis of variance was conducted on each of the SSR markers. Linear model of each variance was $yij = \mu + Xi + \varepsilon ij$ where: yij: resistance variable on the accession of the i-th and j-th repetition, µ: the average general, Xi: the effect of accession to-i, and εij: environmental effect.

RESULTS AND DISCUSSION

Resistance evaluation of mapping population to CLF disease

One of the problems to prepare a mapping population on the rubber plant is a low success rate of hand pollination to produce F1 progenies. In this study, 6031 crosses were made between BPM 1 and RRIM 600 clones, and resulted in only 30 F1 plants and 74 F1 embryos which were collected from seeds with a crossing success rate of 0.005%. Thus, only 30 F1 plants were evaluated.

Cassiicolin toxin is the main effector in the pathogenicity of *C. cassiicola* isolate, so that it can be used as an evaluation method for resistance to CLF disease. Evaluation of parental clones against two *C. cassiicola* isolates showed that the resistant parent clone BPM 1 decreased in water content of 6.9% in CC-06 and 7.8% in CC-22, respectively, while the susceptible parent RRIM 600 had a water content reduction of 33.6% in CC-06 and 41.3% in CC-22 due to the toxin activity, respectively. This indicated that the parental clones used to obtain the mapping population had a contrasting resistance level to CLF disease (Figure 1). These results could support the mapping of quantitative character loci related to resistance of the rubber plant to CLF disease. McCouch and Tanksley (1991) stated that the selection of the parents having a resistant and susceptible extreme response was one of the important factors determining the success of genetic mapping to be able to obtain a segregating progeny in those two parental crossings.

Figure 1 also shows the resistance level of F1 plant population to the two *C. cassiicola* isolates ranged from 5.2% - 33.4% to CC-06 and 3.5% - 36.4% to CC-22 isolates, respectively. The differences in the resistance level to both isolates could be caused by the differences in virulence level of the isolates caused by specific interactions between the plants and the isolates. Lieberei (2007) stated that the disease occurrence can be influenced by many factors such as the level of genetic variability of pathogens and plants, environment and interaction between them. These factors can cause a plant genotype to become resistant to one isolate but susceptible to the other. Three progenies had a better resistance level to both isolates as compared to the resistant parental clone of BPM 1, i.e., F1.1, F1.5, and F1.10. While the F1.27 progeny had a better resistance just to the CC-06 isolate, and F1.11, F1.12, F1.14, F1.23, F1.28, and F1.29 progenies had a better resistance level to CC-22 isolate.

The information about genetic studies of rubber plant resistance to CLF disease was still limitedly reported. Tan and Tan (1996) reported that resistance to CLF disease was complex that is regulated by polygenic, and on the other hand, Hadi and Hartana (2004) reported that the resistance was oligogenic with an epistatic effect. The characters that were regulated by polygenic (quantitative) had a normal distribution, on the contrary, the characters that were regulated by monogenic or oligogenic (qualitative) have a skewed distribution. Figure 2 shows the distribution of leaf wilting intensity of F1 progeny to two *C. cassiicola* isolates. The shape of distribution does not look like a normally distributed trait, and it showed that resistance to CLF disease was regulated by a few major and minor genes (oligogenic).

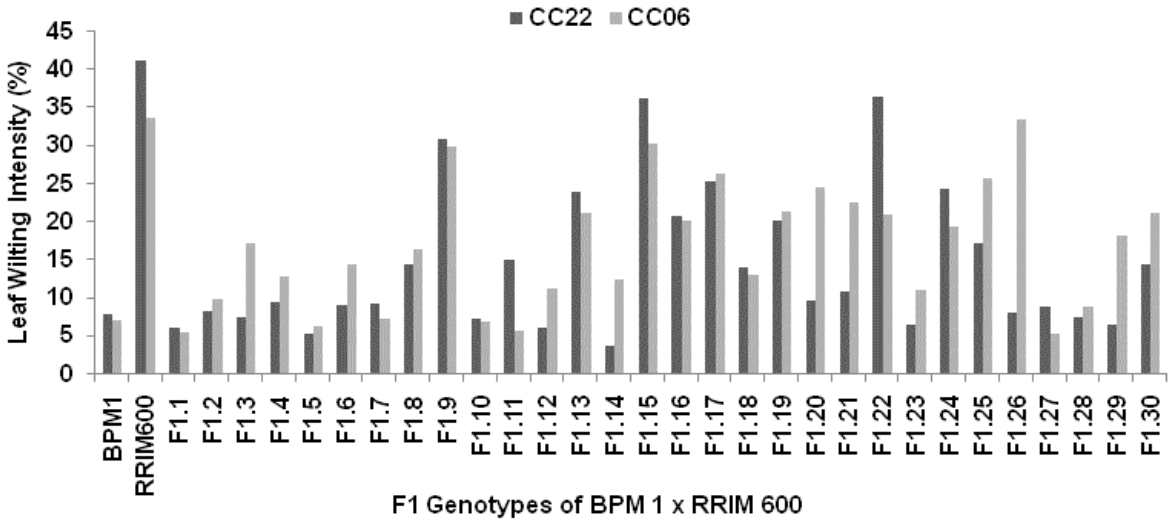


Figure 1. Leaf wilting intensity of 30 F1 progenies of BPM 1 x RRIM 600 to toxin filtrate of *C. cassiicola* isolates

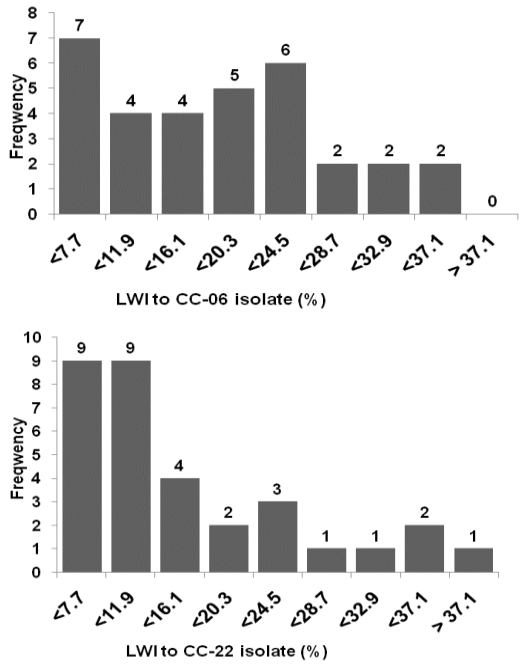


Figure 2. The distribution of leaf wilting intensity (LWI) of mapping population to two of *C. cassiicola* isolates

Selection of SSR markers for construction of genetic linkage map

The first step selection of 135 SSR loci on both parental clones revealed 31 SSR markers that produced polymorphic loci (22.9%). These loci were heterozygous in the resistant parent clone (BPM 1) and homozygous in the susceptible parent clone (RRIM 600), or heterozygous on both clones with different positions of at least one allele. The percentage of polymorphic loci in this study was higher than that of previous studies on the RRIM 600 and RRII 105 clones (18.27%) (Triwitayakorn et al. 2011) but was lower than that produced from primer selection conducted on PB 260 and RO 38 clones (46.15%) (Lespinase et al. 2000) as well as on PB 217 with PR 255 clones (51%) (Souza et al. 2013). These differences are caused by the different types of clones used in the selection, where polymorphic loci in one pair of clones could become monomorphic in other clones due to the differences in the clone's genetic background. In addition, the different type and number of SSR markers selected also caused difference in the percentage of polymorphic loci among studies.

The first step selected markers were used to amplify all population members consisting of 104 F_1 (30 plants and 74 embryos) and their parents. Allele obtained only in the resistant parent clone (BPM 1) was considered as a specific allele that is assumed to be associated with CLF disease resistance in the rubber plant. Individuals carried a specific allele were grouped as individuals with H genotype and those without the specific allele were grouped as individuals with A genotype.

The second step of primer selection was based on the results of PCR amplification of the whole population that was analyzed by using chi-square test. The results showed that 28 of SSR loci had H and A genotypes with segregation ratio of 1:1, while the others did not fit the 1:1 ratio (Table 1). The loci with segregation ratio of 1:1 were used to generate the genetic linkage maps.

Figure 3 shows the results of linkage analysis of 28 SSR loci segregation ratio of 1:1 in the population using the MapMaker / EXP software. In the LOD 3 FR 0.5, 4 linkage groups were obtained, consisted of eight linkage loci, while the remaining 20 loci segregated independently. Two loci distributed on each linkage group with group size range of 30-47.8 cM (Figure 3.A). If the LOD was lowered to 2 with FR 0.5, there was an increasing number of linkage loci to be 11 loci while the remaining 17 loci segregated independently. There was also a decreasing number of linkage groups into two groups, six loci distributed in group 1 and five loci in group 2 with a distance range of linkage groups of 203-228.5 cM (Figure 3.B). This showed that the linkage groups 3 and 4 on LOD 3 were merged into linkage group 1 in LOD 2 with the addition of two bridge loci between EHBla-2 and mHbCIRA-2715. Two loci that were previously independently segregated and then changed to linked were HB-52 and EHB-122. While the linkage groups 1 and 2 on LOD 3 merged to linkage group 2 on LOD 2 with the addition of one linked locus of gSSR-165. These showed that the decrease of LOD limit can increase the number of linked loci, but the chances of the possibility of recombination also increased.

The number of linkage groups obtained in this study was lower compared to the number of rubber plant chromosomes. Similarly, the locus density on each linkage group, which in the closest distance between locus was about 30 cM. It was due to low number of individuals in population and loci used. The same results were also reported in other studies that obtained 2 linkage groups in the construction of genetic linkage map on 22 F1 of PB 260 x PN 711 by using 94 RAPD markers (Novalina and Sagala 2013). Compared to previous studies, the genetic map generated in this study was able to detect a small part of the rubber plant genome. Lespinasse et al. (2000) reported 18 linkage groups with a markers density every 3 cM on the analysis of 106 F1 progenies of PB 260 x RO38 using 717 loci consisting of 301 RFLP, 388 AFLP, 18 SSR, and 10 isozymes. Le-Guen et al. (2007) used 234 SSR markers on the same population and obtained markers density every 10-15 cM. Likewise, the analysis of 81 F1 plants of RRIM 600 x RRII 105 using 97 SSR loci obtained 23 linkage groups with interval between loci 11.9 cM (Triwitayakorn et al. 2011). While Le Guen et al. (2011) on 351 F1 progeny of PB 260 x MDF 180 using 203 SSR, 96 AFLP, and 1 STS markers produced 18 of linkage groups. Using of 1,079 markers on 146 F1 of GT 1 x RRIM 701, Conson et al. (2018) identified 38 QTLs on 18 linkage groups with an average marker density of 3.5 cM. One of the SSR loci in one of the linkage groups was also found in this study. The locus was called mHbCIRA 2715 currently on the linkage group 3 with a distance of 0 cM, and this locus was in linkage group 12 with a distance of 70.8 cM.

Table 1. Chi-square analysis of 30 SSR loci segregated into 1:1 ratio on the mapping population

Locus	Accession no.		Segregation	X^2	P
	H	A			
EHB-069	49	55	1:1	0.35	Ns
EHB-070	43	55	1:1	1.47	Ns
EHB-079	58	46	1:1	1.38	Ns
EHB-081	67	32	1:1	12.37	S
EHB-087	62	42	1:1	3.85	Ns
EHB-088	45	57	1:1	1.41	Ns
EHB-113	53	51	1:1	0.04	Ns
EHB-122	58	46	1:1	1.38	Ns
EHB-133	53	50	1:1	0.09	Ns
EHB-151	62	42	1:1	3.85	Ns
HB-52	53	47	1:1	0.36	Ns
HB-68	37	58	1:1	4.64	S
HB-78	46	57	1:1	1.17	Ns
HESR-032	44	57	1:1	1.67	Ns
EHBp-15	44	56	1:1	1.44	Ns
EHBp-18	54	49	1:1	0.24	Ns
EHBla-2	60	46	1:1	2.96	Ns
EHBc-34	55	48	1:1	0.48	Ns
SSRH-103	52	37	1:1	2.53	Ns
SSRH-358	58	43	1:1	2.23	Ns
SSRH-548	56	43	1:1	1.71	Ns
gSSR-165	60	43	1:1	2.81	Ns
gSSR-268	44	60	1:1	2.46	Ns
HBE-329	49	50	1:1	0.01	Ns
P070	55	46	1:1	0.80	Ns
mHbCIRA-2715	58	46	1:1	1.38	Ns
mHbCIRA-2425	52	52	1:1	0.00	Ns
gSSR-194	54	50	1:1	0.15	Ns
gSSR-212	52	45	1:1	0.51	Ns
HESR-029	51	50	1:1	0.01	Ns

Note: P (5%) on the df 1: 3.84. Ns: nonsignificant, number of the H and A genotypes are not significantly different (segregation of alleles 1:1). S: significant, number of the H genotype is significantly different from A genotype (segregation of alleles deviated from 1:1 ratio)

Based on the information of genetic linkage map generated, the identification of QTLs position on the linkage map can be conducted by using information of the resistance phenotype and SSR data. Analysis using QGene program showed that with a total distance of 387.4 cM and markers density of 35.3 cM, this analysis could not identify the presence of QTLs linked with CLF disease resistance on the linkage map. A few of the linkage group numbers and a low locus density were major limited factors on the QTLs identification process. Other studies reported that the presence of QTLs associated with SALB (South American Leaf Blight) disease was identified on 18 linkage groups with a markers density of 10-15 cM (Le Guen et al. 2007) and 8-10.6 cM (Le Guen et al. 2011). Likewise, the QTLs associated with the growth of rubber plants were identified on 23 linkage groups with a markers density of 10 cM (Souza et al. 2013), 21 linkage groups with 5 cM (Rosa et al. 2018), 18 linkage groups with 3.5 cM (Conson et al. 2018) and 15 linkage groups with 3.0 cM (An et al. 2019).

Association of molecular markers with phenotypic characters could be identified by using a single marker analysis without the information of genetic linkage map (Champoux et al. 1995; Collard et al. 2005). However, the information obtained was limited to the association between the markers with the phenotypic character, while their position and distance of the QTLs from the marker were unknown. This might be because markers and the resistance character were not in the same chromosome, or they may be in the same chromosome but they had a long-distance so that it would not be segregated together (Collard et al. 2005).

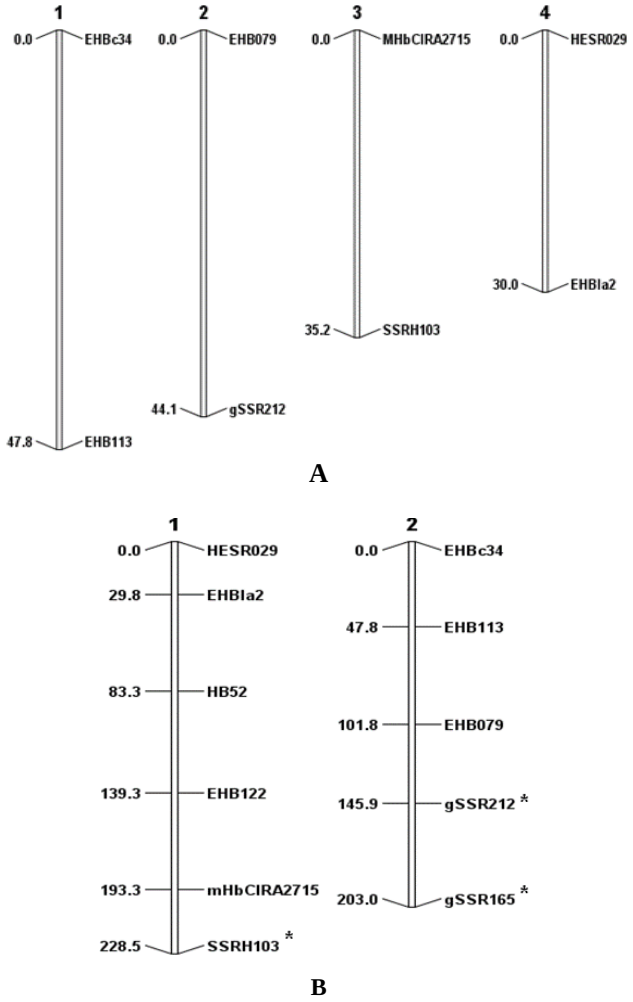


Figure 3. Genetic linkage map of F1 population from BPM 1 x RRIM 600 clones resulted from Qgene analysis. A. Analysis on the LOD (Log of Odd) 3 with RF (Recombinant Fraction) 0.5 and B. Analysis on the LOD 2 RF 0.5. Distance between locus in centi Morgan (cM). * is associated locus with CLF disease based on single-marker analysis.

Identification of SSR marker associated with CLF disease resistance

The single-marker analysis by using SPSS program successfully identified the presence of SSR loci associated with the resistance of the rubber plant to *C. cassiicola* isolates (Table 2). The result showed that there were four loci associated with resistance to both isolates, one locus only associated with CC-06 isolate and five loci associated only with CC-22 isolate ($P < 0.05$). Loci associated with resistance to the CC-06 isolate were EHB-70, EHB-081, EHBp 18 SSRH-548, and HB-68, while loci associated with CC-22 isolate were gSSR-165, HBE-329, EHB-070, SSRH-103, HB-78, EHB-081, EHBp-18, SSRH-548, and gSSR-212. Among these, EHB-70, EHB-081, EHBp-18, and SSRH-548 loci are associated with resistance to the two isolates used.

The number loci associated with resistance to CC-22 isolate were higher than that of CC-06 isolate. This may be correlated with the host origin of the isolates; the CC-22 isolate was obtained from RRIM 600 clone. This clone was the susceptible parent clone, so it was expected that there was a specific relationship between the population resistance with isolate tested. The locus association with resistance to CC-22 isolate was also highly significant ($P < 0.01$).

Furthermore, four of the ten loci associated with resistance to CLF disease were an EST-SSR (Expressed Sequence Taq-Simple Sequence Repeat) which was designed based on the cDNA. These loci were HBE-329 from latex cDNA (Feng et al. 2009), EHB-070 and EHB-081 from apical meristem cDNA (Triwitayakorn et al. 2011), and EHBp-18 from bark cDNA (Silva et al. 2014). The HBE-329, EHB-070, and EHB-081 were designed by using cDNA of RRIM 600 clone, while EHBp-18 was from cDNA of PB 217 clone.

Figure 4 shows the combinations of allele from five loci associated with CC-06 isolate. Each locus has a variation of allele combinations with a different average leaf wilting percentage, where the lower the percentage would cause the higher the plant resistance level. Based on the average resistance value of each allele combination, the selected associated allele with the resistance to CLF disease was estimated. Estimated loci carried the selected allele were red-circled. Resistance to CLF disease at the EHB-070 and HB-68 was estimated to be controlled by the A1 allele, whereas in the EHB-081, EHBp-18 and SSRH-548 were controlled by the A2 allele.

Likewise, ten of loci associated with the CC-22 isolate have allele variations with a different average leaf wilting percentage. A1 allele at the SSRH 548, SSRH 103, HB 78, and gSSR 212 was predicted to be associated with the resistance to CC-22 isolate, while EHB 70, EHB 081, EHBp 18, and HBE 329 were estimated to be associated with the A2 allele and A3 allele at the gSSR 165 locus (Figure 5).

There were differences of allele associated with resistance to CC-06 and CC-22 isolates on the EHB 070 and SSRH 548 loci. However, based on the average value of resistance to both isolates, it was expected that A1 and A2 were the associate alleles on the EHB 070 and SSRH 548 loci, respectively (Figure 6). Although they can not be used as direct markers for resistance selection tools, the presence of these alleles could be used as early information to develop the molecular markers linkage with CLF disease resistance. To improve the accuracy of obtained data to allow the loci could be used in marker-assisted selection, it is necessary to make further analysis by increasing the number of mapping population and loci analyzed.

Table 2. Results of single marker analysis of SSR locus with resistance of mapping population to two of *C. cassiicola* isolates

Locus	Response to isolate		
	F R-CC-06	F R-CC-22	F R-Average
gSSR-268	1.381 ns	0.563 ns	0.976 ns
gSSR-194	1.974 ns	2.360 ns	2.536 ns
EHB-87	0.229 ns	1.505 ns	0.898 ns
EHB-113	0.717 ns	0.038 ns	0.308 ns
SSRH-358	0.254 ns	0.064 ns	0.109 ns
HB-52	0.156 ns	0.906 ns	0.311 ns
HESR-032	0.790 ns	0.519 ns	0.657 ns
EHBc-34	1.048 ns	2.069 ns	1.814 ns
EHB-069	0.267 ns	1.426 ns	0.716 ns
EHB-122	0.166 ns	0.441 ns	0.175 ns
EHB-079	1.028 ns	0.279 ns	0.64 ns
EHB-088	0.774 ns	1.011 ns	0.996 ns
P-070	1.704 ns	1.054 ns	1.216 ns
gSSR-165	1.992 ns	7.677 **	5.461 *
EHB-151	0.542 ns	0.563 ns	0.618 ns
mHbCIRA-2425	0.613 ns	1.283 ns	1.144 ns
HESR-029	0.662 ns	0.318 ns	0.563 ns
EHBla-2	0.119 ns	0.301 ns	0.190 ns
HBE-329	1.890 ns	4.878 **	3.908 *
EHB-133	0.949 ns	2.349 ns	1.585 ns
EHB-070	3.407 *	7.847 **	6.701 **
mHbCIRA-2715	0.664 ns	0.634 ns	0.652 ns
SSRH-103	2.442 ns	8.044 **	6.026 **
HB-78	2.864 ns	8.962 **	6.529 **
EHB-081	3.866 **	8.092 **	8.494 **
EHBp-15	2.079 ns	1.348 ns	1.536 ns
EHBp-18	3.469 *	7.755 **	6.73 **
gSSR-212	1.892 ns	5.044 **	3.723 *
SSRH-548	2.812 *	13.31 **	7.886 **
HB-68	5.516 **	2.250 ns	3.596 *

Note: F R-CC-06 and F R-CC-22: F value of resistance to *C. casiicola* isolate of CC-06 and CC-22. ns: non significant, **: significant in α 0.01, *: significant at α 0.05

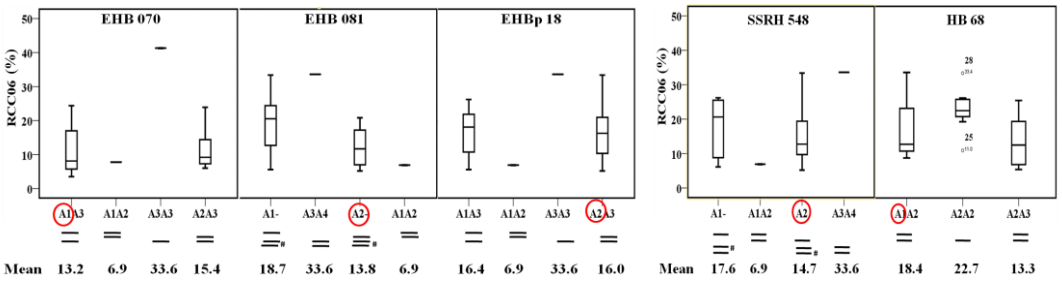


Figure 4. Allele variations and mean resistance level of mapping population to *C. cassiicola* isolate CC-06 on the 5 SSR locus associated with CLF disease. The lower proportion of allele variation is the parents alleles. The circled alleles are the alleles predicted to be associated with CLF disease. # shows the position of one of the allele pairs

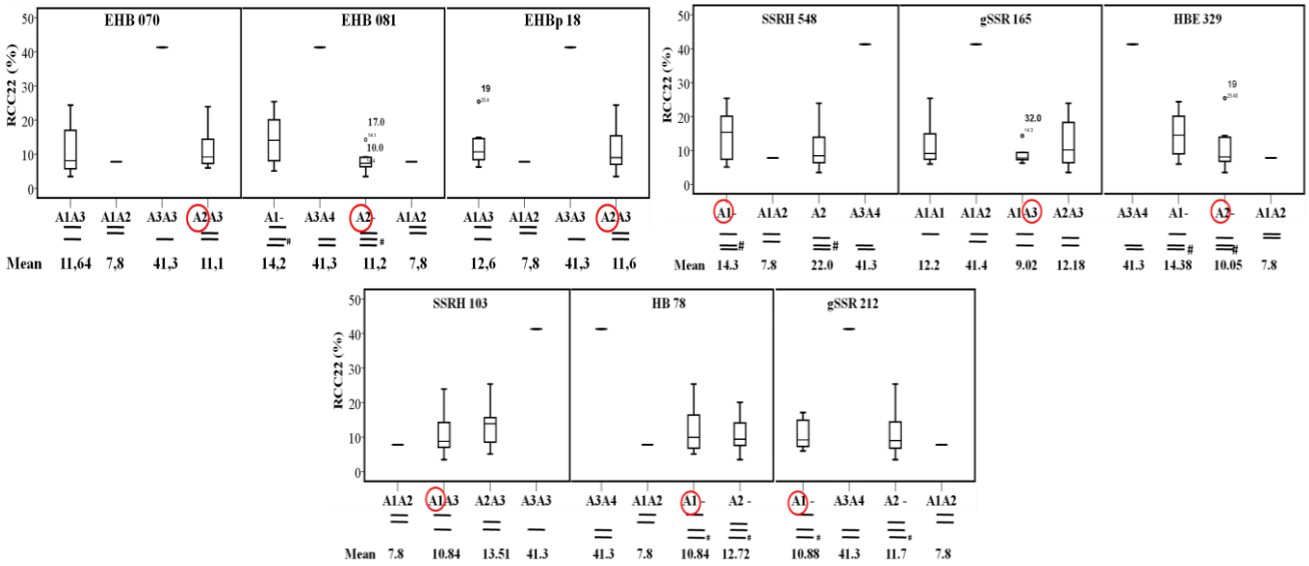


Figure 5. Allele variations and mean resistance level of mapping population to CC-22 isolate on 9 of SSR locus associated with CLF disease. The lower proportion of allele variation is the parents alleles. The red circled alleles are the alleles predicted to be associated with CLF disease. # shows the position of one of the allele pair

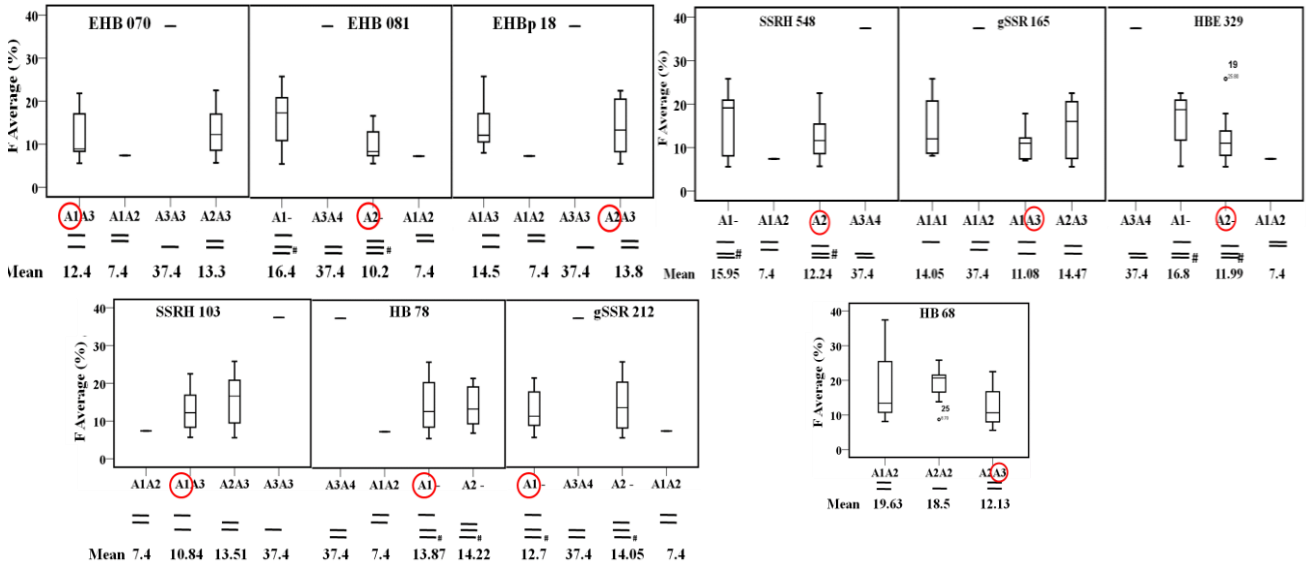


Figure 6. Allele variations and mean resistance level of mapping population of rubber plant to *C. cassiicola* isolates CC-06 and CC-2 on the 10 of SSR locus associated with CLF disease. The lower proportion of allele variation is the parents alleles. The circled alleles are the alleles predicted to be associated with CLF disease. # shows the position of one of the allele pairs.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Dr. Kuswanhadi and Dr. Thomas Wijaya for the meaningful language correction of the manuscript and our thankful for the financial support from the Indonesian Rubber Research Institute.

REFERENCES

An ZW, Li YC, Zhai QL, Xie LL, Zhao YH, Huang HS. 2013. Development and characterization of novel expressed sequence tag-derived simple sequence repeat markers in *Hevea brasiliensis* (rubber tree). Genet Mol Res 12 (4): 5905 5910. DOI: 10.4238/2013.November.22.18

An Z, Zhao Y, Zhang X, Huang X, Hua Y, Cheng H, Li X, Huang H. 2019. A high-density genetic map and QTL mapping on growth and latex yield-related traits in *Hevea brasiliensis* Müll. Arg. Industrial Crops Prod 32: 440-448. DOI: 10.1016/j.indcrop.2019.03.002

Breton F, Sanier C, d'Auzac A. 2000. Role of cassiicolin a host-selective toxin in pathogenicity of *Corynespora cassiicola*, causal agent of a leaf fall disease of *Hevea.* J Rubb Res 3 (2): 115-128.

Champoux MC, Wang G, Sarkarung S, Mackill DJ, O'Toole JC, Huang N, McCouch SR. 1995. Locating genes associated with root morphology and drought avoidance in rice via linkage to molecular markers. Theor Appl Genet 90 (7-8): 969-981.

Collard BCY, Jahufer MZZ, Brouwer JB, Pang ECK. 2005. An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. Euphytica 142*:* 169-196. DOI: 10.1007/s10681-005-1681-5

Conson ARO, Taniguti CH, Amadeu RR, Andreotti IAA, de Souza LM, dos Santos LHB, Rosa JRBF, Mantello CC, da Silva CC, José Scaloppi Junior E, Ribeiro RV, Le Guen V, Garcia AAF, de Souza Gonçalves P, de Souza AP. 2018. High-resolution genetic map and QTL analysis of growth-related traits of *Hevea brasiliensis* cultivated under suboptimal temperature and humidity conditions. Front Plant Sci 9: 1255. DOI: 10.3389/fpls.2018.01255

Creste S, Neto AT, Figueira A. 2001. Detection of single sequence repeats polymorphism in denaturing polyacrylamide sequencing gels by silver staining. Plant Mol Bio*l* Rep 19: 299-306. DOI: 10.1007/BF02772828

Cubry P, Pujade-Renaud V, Garcia D, Espeout S, Le Guen V, Granet F, Seguin M. 2014. Development and characterization of a new set of 164 polymorphic EST-SSR markers for diversity and breeding studies in rubber tree (*Hevea brasiliensis* Müll. Arg.). Plant Breed 133 (3): 419-426. DOI: 10.1111/pbr.12158

Feng SP, Li WG, Huang HS, Wang JY, Wu YT. 2009. Development, characterization and cross-species/genera transferability of EST-SSR markers for rubber tree (*Hevea brasiliensis*). Mol Breed 23: 85-97. DOI: 10.1007/s11032-008-9216-0

Grattapaglia D, Sederoff R. 1994. Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using pseudo-test-cross-mapping strategy and RAPD markers. Genetics 137: 1121-1137.

Hackett CA, Wachiraà FN, Paul S, Powell W, Waugh R. 2000. Construction of a genetic linkage map for *Camellia sinensis* (tea). Heredity 85: 346-355. DOI: 10.1046/j.1365-2540.2000.00769.x

Hadi H, Hartana A. 2004. Analysis of molecular marker for *Hevea* rubber resistance to *Corynespora* leaf fall disease. Indon Nat Rub Res 22 (2): 47-56.

Joehanes R, Nelson JC. 2008. Genetics and population analysis: QGene 4.0, an extensible Java QTL-analysis platform. Bio Appl Note 24 (23): 2788-2789. DOI: 10.1093/bioinformatics/btn523

Le-Guen V, Garcia D, Mattos C, Doare F, Lespinasse D, Seguin M. 2007. Bypassing of a phylogenic *Microcyclus ulei* resistance in rubber tree analyzed by QTL detection. New Phytologist 173: 335-345.

Le Guen V, Garcia D, Doaré F, Mattos C, Condina V, Couturier C, Seguin M. 2011. A rubber tree's durable resistance to *Microcyclus ulei* is conferred by a qualitative gene and a major quantitative resistance factor. Tree Genet Genome 7: 877-889. DOI: 10.1007/s11295-011-0381-7

Le Guen V, Garcia D, Mattos C, Fouet O, Doaré F, Condina V, Seguin M 2013. A newly identified locus controls complete resistance to *Microcyclus ulei* in the Fx2784 rubber clone. Tree Genet Genome 9 (3): 805-812. DOI: 10.1007/s11295-013-0599-7

Le Guen V, Gay C, Xiong TC, Souza LM, Rodier-Goud M, Seguin M. 2011. Development and characterization of 296 new polymorphic microsatellite markers for rubber tree (*Hevea brasiliensis*). Plant Breed 130 (2): 294-296. DOI: 10.1111/j.1439-0523.2010.01774.x

Le Guen V, Lespinasse D, Oliver G, Rodier-Goud M, Pinard F, Seguin M. 2003. Molecular mapping of genes conferring field resistance to South American Leaf Blight (*Microcyclus ulei*) in rubber tree. Theor Appl Genet 108 (1): 160-167. DOI: 10.1007/s00122-003-1407-9

Lespinasse D, Rodier-Goud M, Grivet L, Leconte A, Legnate H, Seguin M. 2000. A saturated genetic linkage map of rubber tree (*Hevea* spp.) based on RFLP, AFLP, microsatellite and isozyme markers. Theor Appl Genet 100: 127-138. DOI: 10.1007/s001220050018

Li D, Deng Z, Qin B, Liu X, Men Z. 2012. De novo assembly and characterization of bark transcriptome using Illumina sequencing and development of EST-SSR markers in rubber tree (*Hevea brasiliensis* Muell. Arg.). BMC Genomics 13 (1): 192-205. DOI: 10.1186/1471-2164-13-192

Lieberei R. 2007. South American Leaf Blight of the Rubber Tree (*Hevea* spp.): New steps in plant domestication using physiological features and molecular markers. Annals Bot 100 (6): 1125-1142. DOI: 10.1093/aob/mcm133

Lincoln SE, Landers SL. 1993. Constructing Genetic Linkage Maps with MAPMAKER/EXP Version 3.0: A Tutorial and Reference Manual: A Whitehead Institute for Biomedical Research. Technical Report, Cambridge.

Ma JQ, Yao MZ, Wang XC, Jin JQ, Wang XM, Chen L. 2014. Construction of SSR-based genetic map and identification of QTLs for catechins content in tea plant (*Camellia sinensis*). PLoS One 9 (3): e93131. DOI: 10.1371/journal.pone.0093131

Mantello CC, Cardoso-Silva CB, da-Silva CC, de-Souza LM, Scaloppi-Junior EJ, de Souza Gonçalves P, de Souza AP. 2014. De novo assembly and transcriptome analysis of the rubber tree (*Hevea brasiliensis*) and SNP markers development for rubber biosynthesis pathways. PLoS One 9 (7): e102665. DOI: 10.1371/journal.pone.0102665

McCouch SR, Tanksley SD. 1991. Development and use of restriction fragment length polymorphism in rice breeding and genetics. In GS. Khus & G. H. Toenissen (Eds.), Rice Biotechnology*:* Biotech Agr 8: 109-133). Phillipina: IRRI.

Novalina, Sagala AD. 2013. Construction of *Hevea brasiliensis* genetic linkage map and identification of quantitative trait loci using RAPD markers. Inter J Adv Sci Engineer Inform Tech 3 (1): 71-75.

Orozco-Castillo, Chalmers C, Waugh R, Powell W. 1994. Detection of genetic diversity and selective gene introgression in coffee using RAPD markers. Theor Appl Genet 87: 934-940. DOI: 10.1007/BF00225787

Rattanawong R, Prapan K, Lekawipat N, Seguin M, Clement-Demange A. 2009. Detection of QTLs association with growth, latex production and rubber quality for the development of Marker Assisted Selection (*Hevea brasiliensis*). IRRDB Workshop on Genome and Transcriptome.

Rosa JRBF, Mantello CC, Garcia D. 2018. QTL detection for growth and latex production in a full-sib rubber tree population cultivated under suboptimal climate conditions. BMC Plant Biol 18 (223): 1-16. DOI: 10.1186/s12870-018-1450-y

Silva CC, Mantello CC, Campos T, Souza L. M, Gonçalves PS, Souza AP. 2014. Leaf-, panel and latex-expressed sequenced tags from the rubber tree (*Hevea brasiliensis*) under cold-stressed and suboptimal growing conditions: The development of gene-targeted functional markers for stress response. Mol Breed 34 (3): 1035-1053. DOI: 10.1007/s11032-014-0095-2

Souza L, Gazaffi R, Mantello C, Silva C, Garcia D, Le Guen, V. 2013. QTL mapping of growth-related traits in a full-sib family of rubber tree (*Hevea brasiliensis*) evaluated in a sub-tropical climate. PLoS One 8 (4): e61238. DOI: 10.1371/journal.pone.0061238

Souza L, Mantello C, Suzuki F, Rodrigo G, Le-Guen V. 2011. Development of a genetic linkage map of rubber (*Hevea brasiliensis*) based on microsatellite markers. Paper presented at the IUFRO tree biotechnology conference. 26 Jun-2 Jul 2011, Arraial D'Ajuda, Bahia, Brazil.

Tan A, Tan AM. 1996. Genetic studies of leaf diseases resistance in Hevea. J Nat Rubber Res 11 (2): 108-114.

Triwitayakorn K, Chatkulkawin P, Kanjanawattanawong S, Sraphet S, Yoocha T, Sangsrakru D, Tangphatsornruang S. 2011. Transcriptome sequencing of *Hevea brasiliensis* for development of microsatellite markers and construction of a genetic linkage map. DNA Res 18: 471-482. DOI: 10.1093/dnares/dsr034

NUSANTARA BIOSCIENCE
Vol. 13, No. 1, pp. 61-67
May 2021

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n130109

Development of a core collection from Sri Lankan traditional rice (*Oryza sativa*) varieties for phenotypic and genetic diversity

SHYAMA R. WEERAKOON♥, S. SOMARATNE
Department of Botany, The Open University of Sri Lanka, Nawala, Nugegoda 10250, Sri Lanka. ♥email: shyamaweerakoon@gmail.com

Manuscript received: 22 November 2020. Revision accepted: 26 January 2021.

Abstract. *Weerakoon SR, Somaratne S. 2021. Development of a core collection from Sri Lankan traditional rice (Oryza sativa) varieties for phenotypic and genetic diversity. Nusantara Bioscience 13: 61-67.* A collection of over 2000 traditional rice varieties are conserved at Gene Bank, Plant Genetics Resource Center, Sri Lanka. *Oryza sativa* varieties grown in Sri Lanka from ancient times to the middle of the last century are known as traditional rice. These varieties show adaptability to biotic and abiotic stresses and, an important component of biodiversity of Sri Lanka. A detailed understanding of the diversity of traditional rice varieties is essential for effective utilization of rice genetic resources and identification of potential parents possessing valuable genetic traits for future crop improvement. Study objectives were phenotypic and molecular characterization of one-hundred traditional rice varieties and to identify a core collection for phenotypic and genetic diversity. Rice varieties were grown in a plant house following RCBD with 4 replicates and 5 plants per replicate. Thirty-two agro-morphological characters were observed/collected. Genomic DNA was extracted from 20-days-old seedlings. Thirty-three microsatellite (Simple Sequence Repeat-SSR) primer pairs were used to assay genetic variation and PCR products were subjected to fragment analysis by capillary electrophoresis. Descriptive statistics and basic inferential statistical analyses were performed to access variation of agro-morphological characters among rice varieties. Cluster analysis and Multidimensional scaling produced 07 groups which were further analyzed using Classification and Regression Analysis to extract the diagnostic agro-morphological features. Groups of rice varieties were characterized by lemma palea color, awn color at maturity, seedling height, and flag-leaf angle. Traditional varieties represent distant clusters on agro-morphological features. Molecular analyses revealed all 33 loci displayed polymorphism (66.7-96.9%) among 100 traditional rice varieties with a total of 387 alleles identified with an average of 11.72 alleles per variety. All varieties were genetically structured into fifteen well-separated groups. UPGMA analysis based on Jaccard's similarity separated varieties into 05 major clusters. Genetic diversity information is useful in the efficient use of Sri Lankan rice germplasm and managing *in situ* and *ex situ* germplasm collections in conserving traditional rice varieties.

Keywords: Agro-morphological characterization, core collection, molecular characterization, *Oryza sativa*, traditional rice varieties

INTRODUCTION

Comprehensive knowledge of genetic diversity and population structure of germplasm collections is important for crop improvement. Rice (*Oryza sativa* L.) is the staple food in Sri Lanka, cultivated as a wetland crop in all the districts of the island. According to Department of Agriculture of Sri Lanka reports, rice is the single most important crop occupying 34% percent (0.77 million ha) of the total cultivated area on the island. Rice provides 45% total calories and 40% total protein requirement of an average Sri Lankan (Crop recommendations 2014). Approximately 75% of the rice lands in Sri Lanka are located within the inland valley systems with varying form and size, and the balance of 25% is in coastal plains and associated flood plains (Panabokke 1996). Out of total cultivated amount, around 99% of the area is cultivated with new improved rice varieties. The remaining area is cultivated with traditional rice varieties with low yield. Although new improved varieties produce comparatively higher yields, local and export market demand for traditional rice varieties is higher for their grain qualities, such as high fiber content, despite the lower production (Wickramasinghe and Noda 2008). Moreover, farmer's perceptions (Efisue et al. 2008), improvement of system sustainability (Abeyratne 1956), and the higher adaptability to problem soils (Mandal et al. 1999) further increased interest in traditional rice varieties.

The overall population structure of global rice germplasm has been well characterized. However, detailed analyses on country-specific basis have only been recently begun (Thomson et al. 2007). The extent of genetic variability that exists in a gene pool is an important factor for genetic improvement in rice. Sri Lanka's rice gene pool consists of many abiotic and biotic stress tolerant traits with diverse agronomical characters (Ranawake and Amarasinghe 2014).

Oryza sativa varieties which have been grown in Sri Lanka from ancient times to the middle of the last century are known as traditional rice varieties. A landrace is a dynamic population of a cultivated plant that has historical origin, distinct identity and lacks formal crop improvement, as well as often being genetically diverse, locally adapted, and associated with traditional farming systems (Camacho Villa et al. 2005; Choudhury et al. 2013). Sri Lanka is considered as one of the secondary diversity centers for rice genetic resources (Kobayashi et al. 1991). In ancient times, farmers cultivated traditional rice varieties, due to their adaptability to Sri Lankan soil types, climate, geography, and harsh environmental conditions such as

flood, drought, soil salinity, iron toxicity, pests, and diseases. The traditional rice varieties have a historical origin, distinct identity, and lack formal crop improvement, as well as often being genetically diverse, locally adapted and associated with traditional farming systems (Camacho Villa et al. 2005).

Rice varietal improvement by incorporating germplasm from traditional rice may lead to important advances as these varieties seem to harbor significantly higher genetic and phenotypic diversity than the cultivated rice (Bentota and Weerasinghe 2005; Atwell et al. 2014). Moreover, traditional rice varieties are one important component of the biodiversity of Sri Lanka.

Genetic fingerprinting of these varieties is essential to distinguish them and to characterize accessions. Molecular markers such as simple sequence repeats (SSR) and microsatellites are useful for assessing genetic variations within conserved gene pool in rice. To date, more than two thousand SSR markers of cultivated rice are available and these provide a powerful tool for studying close relatives. SSRs are simple, tandemly repeated, nucleotide sequence motifs flanked by unique sequences (Roa et al. 2000) and have become useful markers for genetic diversity analysis because they detect high levels of allelic diversity, occur frequently throughout plant genomes, and are easily assayed by PCR. SSR markers have been extensively used to identify genetic variation among rice species to analyze genetic structure within cultivated rice (Ren et al. 2005), and to evaluate genetic diversity among strains of wild rice (Shishido et al. 2006) and among cultivars of cultivated rice (Yu et al. 2003). Further, microsatellites have been used for studies of parentage (Roa et al. 2000), genetic mapping and breeding, gene flow, genetic diversity and population differentiation (Cho et al. 2000). Genetic differentiation among selected Sri Lankan traditional rice (*Oryza sativa*) varieties and wild rice species were conducted using AFLP markers by Rajkumar et al. (2011).

Despite the limited studies conducted, there is still a strong need for more detailed characterization of the responses and acclimatization mechanisms of rice under stresses that are occurring in farmers' fields. Thus, integration of crop agro-morphology and molecular approaches to dissect complex environment tolerance traits is essential. The objectives of the present study are; phenotypic characterization and Molecular analysis of traditional rice varieties collected from PGRC, Sri Lanka, and identification of a core collection of traditional rice varieties.

MATERIALS AND METHODS

Collection of seed material

One hundred (100) Sri Lankan traditional rice varieties collected from PGRC, Sri Lanka were used in the study.

Germination of seeds

Seeds were kept at 50 °C for 5 days to break the dormancy. Then seeds were kept in 70% alcohol for 2 minutes and washed properly with distilled water and dipped in 2% Clorox for about 30 minutes and again washed properly with distilled water. Then seeds were kept in an incubator at 35 °C for 7 days under dark conditions. The experiment was conducted at seedling stage. Germinated seeds were planted in plastic trays filled with soil collected from paddy fields.

Agro-morphological characterization

The study was carried out in a plant house at The Open University of Sri Lanka, Nawala in the Low country Wet Zone, Western Province of Sri Lanka. The average ambient temperature was 28-32 °C and average relative humidity was 80-85% in the experiment site during the experiment period. The soil was collected from paddy field and filled into pots (5 kg pot^{-1}).

The germinated seeds were allowed for 2 weeks for reaching the seedling stage and were planted in pots according to Randomized Complete Block design with 4 replicates and 5 plants per replicate. Fertilizer management and the other crop management practices were followed according to the recommendations of Department of Agriculture. Thirty-two (32) agro-morphological characters were observed/collected as explained in PGRC Characterization Catalogue on Rice Germplasm (1999).

Statistical analyses

Data collected on agro-morphological characters of traditional rice varieties were analyzed using different statistical procedures. Prior to the analysis, data were converted to unique type, *i.e.* Nominal data. Descriptive statistics included frequency analysis, and cross-tabulation. Inferential statistical analyses were carried out using χ^2 test and multivariate analytical techniques such as cluster analysis (CA) and Multidimensional scaling (MDS) to explore the statistical procedural variations in the outcomes. These procedures were used to examine the patterns of grouping of traditional rice varieties according to their agro-morphological features. Based on the grouping patterns reflected in the analyses, rice varieties were grouped into seven (07) categories such as A, B, C, D, E, F, and G (Table 1). In addition, classification and regression analysis was performed to extract the diagnostic agro-morphological features of the groups of traditional rice varieties include in the study.

Molecular characterization

A total of 100 traditional rice varieties collected from PGRC, Sri Lanka were used in the study. The green leaves were individually collected from 20-day-old seedlings for DNA extraction. The gDNA was extracted using the Plant genomic DNA kit (Biomed DL114-01) following by the CTAB protocol. Thirty-three microsatellite (Simple Sequence Repeat-SSR) primer pairs were used to assay genetic variation. The DNA amplification was carried out using a 2,720-thermal cycler (Applied Biosystems). PCR products were subjected to fragment analysis by capillary electrophoresis using an Applied Biosystems 3130×l DNA analyzer (Applied Biosystems).

RESULTS AND DISCUSSION

A long history of traditional rice production across diverse environments in Sri Lanka has led to a diverse array of traditional rice varieties. However, the relative importance and influence of yield-related traits on grain yield have changed over time due to rice improvement. The yield potential of a rice variety is a theoretical concept determined by a complex series of interactions with the components of the environment it is exposed to. Even though recommendations of crop varieties are done based on grain yield, there are other important traits related to grain quality and/or agronomy which are not related to grain yield (Samita et al. 2005). Under such circumstances, selection based on yield can lead to the loss of these important characters. Therefore, classification using multiple morphological characteristics is important to identify adaptation of a variety and to improve the evaluation of varieties for potential adaptation (Lin and Binns 1985; Lin et al. 1986). At present, there is still a lack of information on morphological diversity, how the diversity has changed with rice improvement, and its impact on grain yield of traditional and improved rice varieties in Sri Lanka.

Traditional rice varieties represent important genetic reservoirs with valuable traits and there is an urgent need to provide proper incentives and encourage the farmers to cultivate these to help in the *in situ* conservation of this important gene pool. The selected rice cultivars with abiotic stress tolerance have the potential for direct introduction as cultivars or utilization in the breeding programs. Sri Lankan traditional rice varieties were found to be biotic and abiotic stress tolerant showing exceptional levels of tolerance which play an important role in rice breeding programs (Madurangi et al. 2012; Munasinghe et al. 2017).

However, these varieties are rapidly being lost due to favor of agronomically improved rice varieties. Gene Bank of Plant Genetics Resource Center (PGRC) conserves a collection of over 2000 traditional rice accessions. Only a few studies have so far been conducted on genetic diversity among Sri Lankan traditional rice (Rajkumar et al. 2011; Ranawake and Amarasinghe 2014; Wijayawardhana et al. 2015; Bandara et al. 2017) and there is a need to conduct a comprehensive study.

Studies conducted by Wijayaeardhana et al. (2015) and Bandara et al. (2017) using Sri Lankan Traditional rice varieties revealed an effective use of agro-morphological characters based on Characterization Catalogue on Rice Germplasm descriptors to characterize Sri Lankan traditional rice varieties. A similar method was adopted in the present study using 32 Rice Germplasm descriptors explained in PGRC Characterization Catalogue on Rice Germplasm (1999) for agro-morphological characterization of 100 traditional rice varieties.

Microsatellites have been used for studies of genetic diversity and population differentiation in rice germplasm. Genetic differentiation among selected Sri Lankan traditional rice (*Oryza sativa*) varieties and wild rice species were conducted using AFLP markers by Rajkumar et al. (2011) and Molecular Characterization of Accessions from a Traditional Rice Cultivar, *Suwandel* using SSR markers by Gunasena et al. (2015). In the present study, 33 SSR markers were used to assay the genetic variation among 100 traditional rice varieties.

The present study revealed that traditional rice varieties represent distant clusters based on agro-morphological features, particularly on lemma-palea color, awn color at maturity, seedling height, and flag-leaf-angle. These findings were further confirmed by molecular studies indicating a comparatively high level of genetic differentiation among individuals of selected traditional rice varieties.

Agro-morphological characterization

Descriptive statistics indicated that variation of agro-morphological characters across rice varieties are negligible and certain characters such as presence/absence of awn, characteristics of awn are restricted to certain rice varieties.

However, result of the Kruskal-Wallis test showed that most of the agro-morphological characters significantly vary across rice varieties ($p < 0.05$). However, the variation of stem color among the rice varieties was not statistically significant ($p > 0.05$).

The result of CA and MDS indicated that majority of rice varieties are clustered into seven (7) groups in the dendrogram (Figure 1) and biplot of MDS dimensions and certain rice varieties indicated a grouping tendency. It is clear from the MDS biplot that the verities included in the study grouped into seven categories. The characters lemma-palea color, awn color at maturity, seedling height, and flag-leaf-angle are most likely determine the grouping in the dendrogram.

The characterization of these seven categories was confirmed by the results of CART (Classification and Regression Tree Analysis) analysis (Figure 2). Similar to clustering pattern of the dendrogram (Figure 1), according to the results of CART analysis, traditional rice varieties which consist of seven groups can be categorized by the characters; lemma-palea color, awn color at maturity, seedling height, and flag-leaf angle (Table 2).

Molecular characterization

According to the present study, all 33 loci displayed polymorphism (66.7-96.9 %) among 100 traditional rice varieties with a total of 387 alleles identified with an average of 11.72 alleles per variety. The AMOVA results showed that 34% of the variation distributed among accessions, 59% among individuals, and 7% within individuals indicating a comparatively high level of genetic differentiation among individuals of selected rice varieties. Structure analysis results illustrated that all 100 varieties were genetically structured into five (05) well-separated groups, high ΔK peak was recorded at K=15, K= 5, K= 19 and K= 2 respectively (Figure 3).

Table 1. Groups of traditional rice varieties resulted from the CA, PCA and MDS on agro-morphological data

Group and number of rice varieties	Rice variety (accession no.)
A (33)	*Murungabala wee* (3246), *Kanni murunga* (3260), *Periavellai* (3279), *Suduru samba* (3333), *Vanam* (3488), *Pokuru wee* (3499), *Maha ma wee* (3551), *Polon wee* (3553), *Muthu samba* (3564), *Pokkali* (3573), *El wee* (3578), *Seedevi* (3605), *Lumbini* (3613), *Maha wee* (3618), *Gal pa wee* (3341), *Kiri naran* (3350), *Kalundai* (3381), *Eth samba* (3383), *Moddai karuppan* (3388) *Wanni dahanala* (2053), *Hattapas dawas wee* (2051), *Dahanala* (2049), *Herath* (2048), *Bala samba* (2047), *Herath banda* (2063), *Handiran* (2057), *Gona baru* (2056), *Dingiri menika* (2055), *Demas* (2054), *Weda heenati* (2340), *Hal sudu wee* (2110), *Dik wee* (2109), *Heen dik wee* (3191)
B (03)	*Kalu heenati* (3471), *Pihatu wee* (3403), *Kurulu wee* (4903)
C (02)	*Kattagarang* (3176*), Rathu heenati* (3390)
D (02)	*Galu Sulai* (4616), *Thirissa* (3186)
E (02)	*Kalu hondarawalu* (4622*)* and *Yalalu* (4606)
F (27)	*Moothuki El* (3180), *Kuru hondarawalu* (3184), *Gangala* (3185), *Khombila* (3188), *Wanduru wee* (2046), *Balamurunga kayan (*2045), *Batapola wee* (2036), *Madayal (*3475), *Rankiri* (3476), *Sudugoda wee* (3477), *Japan sulai (*3393), *Devereddiri (*3398), *Atta wee* (02035), *Wanni heenati* (3401), *Batapola El* (2038), *Magoda El (*4905), *Nara wee* (4908), *Niyan wee* (4909), *Pathmawee* (4912), *Sudugalkada* (3983), *Welihandiran (*3916), *Mahamenik* (3923), *Manikkam* (3901), *Rath El* (3705), *Gambada Samba* (3714), *Goda heenati (*3724), *Kuruluthuda* (4553)
G (31)	*Soothuru wee* (3179), *HathiEl* (3183), *Molligoda* (4770), *kurulu wee* (4541), *MadaEl* (3177), *Yalihanthiran* (3187), *Pushmaraga* (3979), *Andikulan* (3189), *Mahakuru wee* (3190), *Puwakmalata samba* (3486), *Gires* (3193), *Katharamana* (3194), *Bala Murunga* (2108), *Hetada* (2069), *Heen murunga* (2979), *Mas samba* (2349), *Duru wee* (2990), *Polayal* (3071), *Thanthiri Balan* (3072), *Ratnawalu* (4916), *Weda heenati* (4917), *Pachchaperumal* (5383), *Black gora* (5387), *Malawariya* (5527), *Gallkatta* (3195), *Nandu heenati* (3197), *Arnolis wee* (3198), *Rathkuda* (3231), *Ralukuda* (3232), *Rathawalu* (3233), *Liyanweli* (4904)

Table 2. Characterization of groups of traditional rice varieties

Rice group	Flag-leaf-angle	Awn color at maturity	Lemma palea color	Seedling height (height class)
A	2	4	3	2
B	1	Absent	9	2
C	2	4	3	3
D	4	4	7	3
E	1	6	3	2
F	2	6	2	3
G	4	4	2	3

Note: Flag-leaf-angle 1. Erect, 2. Intermediate, 3. Horizontal, 4. Descending; Awn color at maturity 1. Straw, 2. Gold, 3. Brown (tawny), 4. Red, 5. Purple, 6. Black; Lemma Palea color 0. Straw, 1. Gold, 2. Brown spots on straw, 3. Brown furrows on straw, 4. Brown, 5. Reddish to light purple, 6. Purple spots on straw, 7. Purple furrows on straw, 8. Purple, 9. Black, 10. White; Seedling height (Height class) 1. < 20 cm, 2. >=20, 3. < 25 cm and 4. >=25 cm.

UPGMA analysis based on Jaccard's similarity separated the varieties into five (5) major clusters (Figure 4). A cophenetic correlation with r=0.786 strongly supported the clustering pattern of UPGMA dendrogram. A principal coordinate analysis (PCoA) also confirmed the UPGMA clusters. Varieties referred to the same cluster showed similar morphological characteristics (e.g. flag-leaf-angle, lema palea color, etc.) while varieties that are identified as morphologically distinct appeared genetically separated.

Breeding a new variety based on phenotypic characters may take more than a decade and, even then the release of an improved variety cannot be guaranteed. Molecular markers make this procedure more efficient and expedite the selection process in rice breeding. Hence, molecular markers are widely used as an efficient tool to characterize genetic variability. A similar study was conducted by Rajkumar et al. (2011) on the genetic diversity of 46 traditional rice (*Oryza sativa*) varieties in Sri Lanka using Amplified Fragment Length Polymorphism (AFLP) markers with ten primer combinations. A UPGMA analysis based on Jaccard's similarity separated the accessions into four major clusters. Traditional rice varieties referred to the same cluster showed similar morphological characteristics (e.g. height, grain color, etc.) while varieties that are known to be morphologically distinct appeared genetically separated.

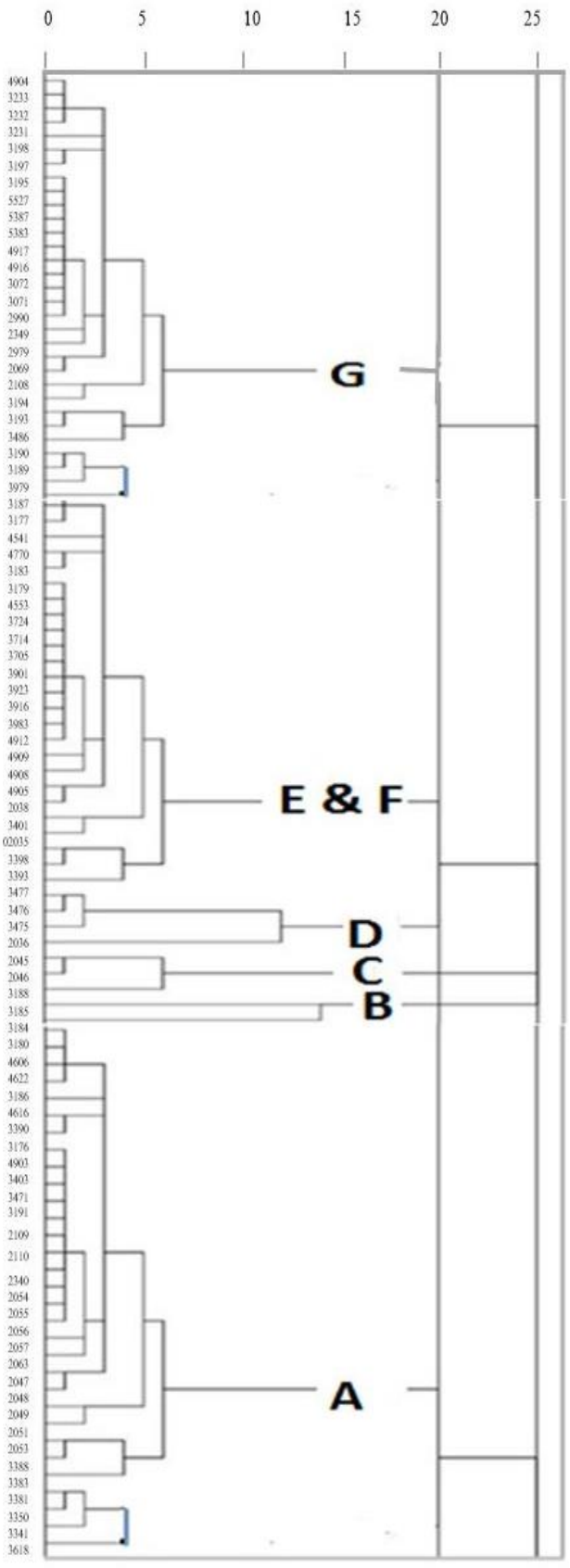


Figure 1. Dendrogram resulted from the agro-morphological characters of 100 traditional rice varieties

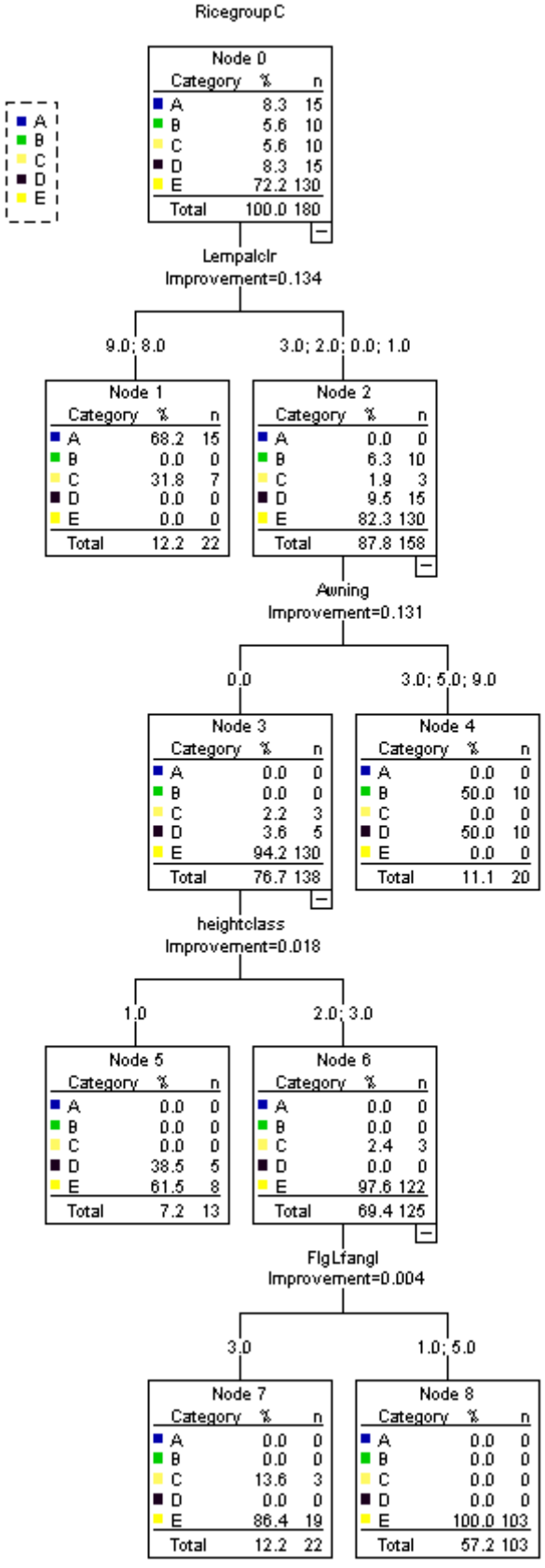


Figure 2. Summary of the characterization of the groups of rice varieties resulted from the CA and MDS Analysis using CART analysis

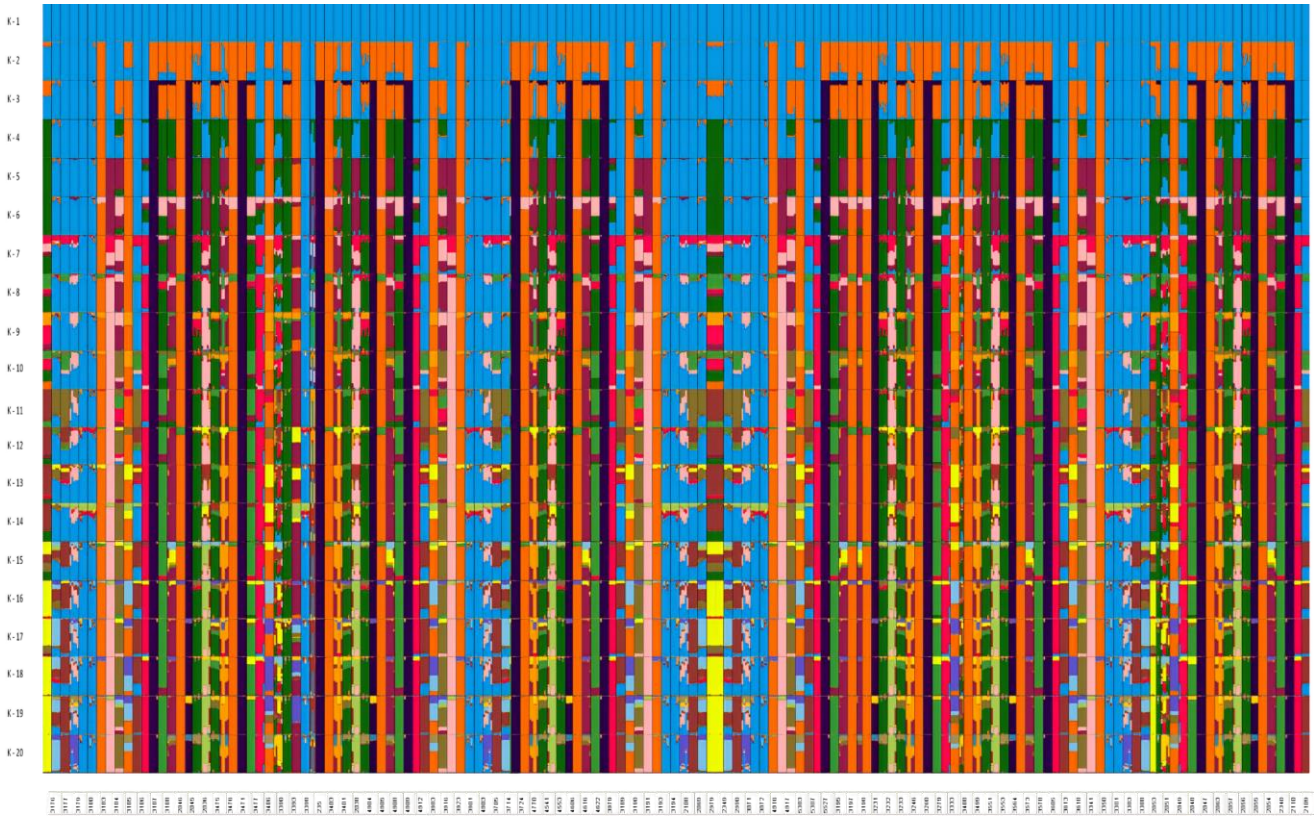


Figure 3. Structure results bimodal-based population assignment at *K* from 1 to 20. Each vertical bar represents an individual (100 varieties), with its assignment probability to genetic clusters represented by different colors

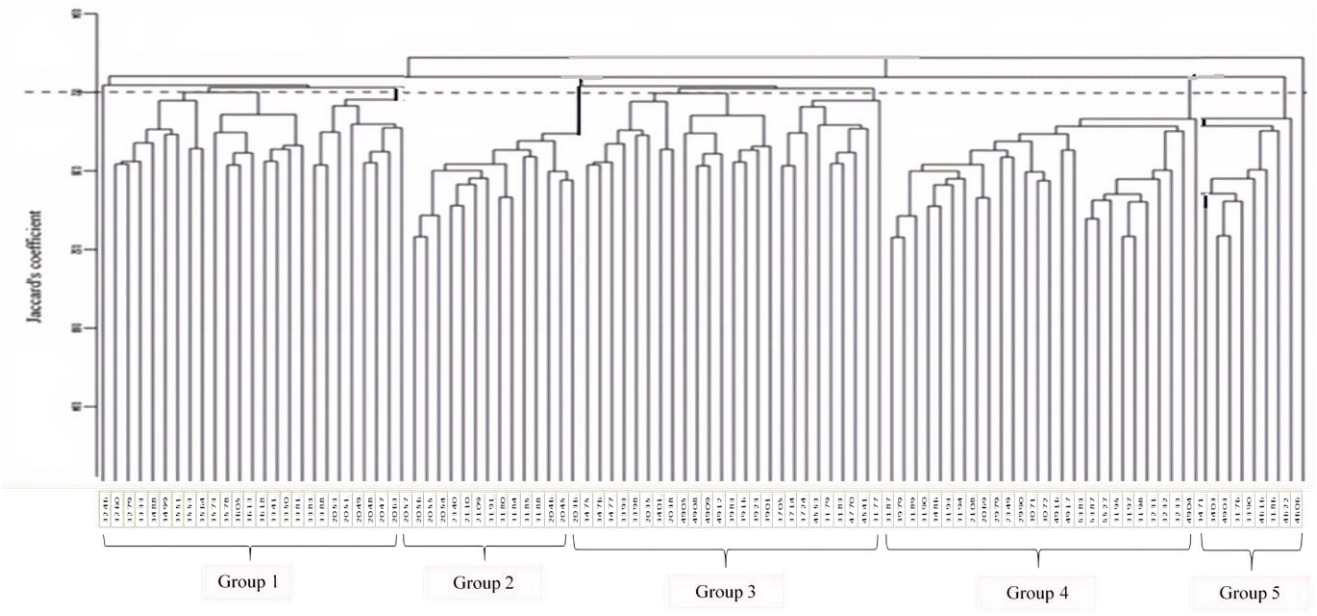


Figure 4. The UPGMA dendrogram showing genetic diversity among 100 Sri Lankan traditional rice varieties based on Jaccard's similarity coefficient. (Group 1-25 varieties, Group 2-14 varieties, Group 3-26 varieties, Group 4-26 varieties, Group 5-9 varieties)

In conclusion, the genetic diversity observed among the traditional rice varieties is significantly high. Genetic diversity assessment at agro-morphological level provides reliable information for the selection of germplasm to develop new rice varieties and in the conservation of traditional rice genetic resources for future breeding programs. The studied 100 traditional rice varieties consist of seven (07) groups which can be characterized by the

characters, lemma palea color, awn color at maturity, seedling height, and flag-leaf-angle. However, genetic diversity assessment at the molecular level provides reliable information for selection of germplasm in the development of new rice varieties and in conservation of traditional rice genetic resources. Structure analysis and UPGMA analysis based on Jaccard's similarity separated the tested 100 rice varieties into five (5) major clusters. All methods of analysis produced similar results confirming the reliability of data used in this study. Therefore, the genetic diversity information obtained will be useful in the efficient use of Sri Lankan rice germplasm collection in breeding programs. In addition, this information will be useful in management of *in situ* and *ex situ* germplasm collections in conservation programs for traditional rice varieties. Studies on genetic differentiation are also important to avoid duplication of traditional rice varieties in the Gene Bank at PGRC.

ACKNOWLEDGEMENTS

The authors wish to thank the Faculty of Natural Sciences Research Grant, The Open University of Sri Lanka for financial support. Technical assistance by Ms. Kalpni Widanapathirana and Ms. Chamodi Sandanayake is gratefully acknowledged.

REFERENCES

Abeyratne EF. 1956. Dryland farming in Ceylon. Trop Agric 112 (3): 191-229.

Atwell BJ, Wang H, Scafaro AP. 2014. Could abiotic stress tolerance in wild relatives of rice be used to improve *Oryza sativa*?. Plant Sci 215-216: 48-58. DOI: 10.1016/j.plantsci.2013.10.007

Bandara MMK, Herath HMVG, Wickramasinghe HAM, Bamunuarachchige TC. 2017. Assessment of morphological characters of selected traditional Sri Lankan rice varieties (*Oryza sativa* L.). J Dry Zone Agric 2017: 21-28.

Bentota AP, Weerasinghe BGDS. 2005. Iron toxicity tolerance in some traditional rice varieties of Sri Lanka. Annals of the Sri Lanka Department of Agriculture, Department of Agriculture, Peradeniya, Sri Lanka 7: 337-340.

Camacho Villa TC, Maxted N, Scholten M, Ford-Lloyd B. 2005. Defining and identifying crop landraces. Plant Genetic Resources: Characterisation and Evaluation 3 (3): 373-384.

Cho YG, Ishii T, Temnykh S, Chen X, Lipovich L, McCouch SR, Park WD, Ayres N, Cartinhour S. 2000. Diversity of microsatellites derived from genomic libraries and GeneBank sequences in rice (*Oryza sativa* L.). Theor Appl Genet 100: 713-722. DOI: 10.1007/s001220051343

Crop Recommendations. 2014. Department of Agriculture, Sri Lanka.

Choudhury B, Khan ML, Dayanandan S. 2013. Genetic structure and diversity of indigenous rice (*Oryza sativa*) varieties in the Eastern Himalayan region of Northeast India. SpringerPlus 2: 228. DOI: 10.1186/2193-1801-2-228

Efisue A, Tongoona P, Derera J, Langyintuo A, Laing M, Ubi B. 2008. Farmers' perceptions on rice varieties in Sikasso region of Mali and their implications for rice breeding. J Agronom Crop Sci 194 (5): 393-400. DOI: 10.1111/j.1439-037X.2008.00324.x

Gunasena PGSD, Wasala SK, Sumanasingha VA. 2015. Molecular characterization of accessions from a traditional rice cultivar, *Suwandel* conserved at plant genetic resources centre, Sri Lanka. Trop Agric Res 27 (1): 103-109.

Kobayashi N, Ikeda R, Vaughan DA. Shigenaga S. 1991. Resistance to tungro in some wild relatives of rice. Int Rice Res Newslett 16 (4): 13.

Lin CS, Binns MR. 1985. Procedural approach 12 for assessing cultivar-location data: Pair-wise genotype-environment interactions of test cultivars with checks. Canadian J Plant Sci 65 (4): 1065-1071.

Lin CS, Binns MR, Lefkovitch LP. 1986. Stability analysis: Where do we stand?. Crop Sci 26 (5): 894-900. DOI: 10.2135/cropsci1986.0011183X002600050012x

Mandal AB, Pramanik SC, Chowdhury B, Bandyopadhyay AK. 1999. Salt-tolerant Pokkali somaclones: Performance under normal and saline soils in Bay Islands. Field Crops Res 61 (1): 13-21. DOI: 10.1016/S0378-4290(98)00145-2

Madurangi SAP, Ranawake AL, Dahanayake N. 2012. Evaluation of level of drought tolerance in traditional rice cultivars in Sri Lanka at the seedling stage. Proceedings of the International Symposium on Agriculture and Environment 2012, Ruhuna, Sri Lanka, 29 November 2012.

Munasinghe SP, Somaratne S, Weerakoon SR. 2017. Screening of Sri Lankan rice (*Oryza sativa* L.) landraces for drought tolerance. Trop Agric Res 28 (2): 183-191.

Panabokke CR. 1996. Soils and agro-ecological environments of Sri Lanka. Natural Resources Series-Natural Resources and Energy Authority. Maitland Place, Colombo.

PGRC Characterization Catalogue on Rice Germplasm. 1999. Plant Genetics Resource Center (PGRC), Gannoruwa, Sri Lanka.

Ranawake AL, Amarasinghe UGS. 2014. Relationship of yield and yield-related traits of some traditional rice cultivars in Sri Lanka as described by correlation analysis. J Sci Res Rep 3 (18): 2395-2403. DOI: 10.9734/JSRR/2014/12050

Rajkumar G, Weerasena J, Fernando K, Liyanage A, Silva R. 2011. Genetic differentiation among Sri Lankan traditional rice (*Oryza sativa*) varieties and wild rice species by AFLP markers. NORDIC J Bot. DOI: 10.1111/j.1756-1051.2011.00998.x.

Ren F, Lu BR, Li S, Huang J, Zhu Y. 2005. A comparative study of genetic relationships among the AA-genome *Oryza* species using RAPD and SSR markers. Theoretical Appl Genet 108 (1): 113-120. DOI: 10.1007/s00122-003-1414-x

Roa AC, Chavarriaga-Aguirre P, Duque MC, Maya MM, Bonierbale MW, Iglesias C, Tohme J. 2000. Cross-species amplification of Cassava (*Manihot esculenta*) (Euphorbiaceae) microsatellites: Allelic poplymorphism and degree of relationship. Am J Bot 87:1647-1655.

Samita S, Anputhas M, Abeysiriwardena DS de Z. 2005. Accounting for multi traits in recommending rice varieties for diverse environments. Exp Agric 41 (2): 213-225.

Shishido R, Kikuchi M, Nomura K, Ikehash H. 2006. Evaluation of genetic diversity of wild rice (*Oryza rufipogon* Griff.) in Myanmar using simple sequence repeats (SSRs). Genet Resour Crop Evol 53 (1): 179-186. DOI: 10.1007/s10722-004-3226-5

Thomson M, Septiningsih E, Suwardjo F, Santoso T, Silitonga T, McCouch S. 2007. Genetic diversity analysis of traditional and improved Indonesian rice (*Oryza sativa* L.) germplasm using microsatellite markers. Theor Appl Genet 114 (3): 559-568. DOI: 10.1007/s00122-006-0457-1

Yu SB, Xu WJ, Vijayakumar CHM, Ali J, Fu BY, Xu JL, Jiang YZ, Marghirang R, Domingo J, Aqino C, Virmani SS, Li ZK. 2003. Molecular diversity and multilocus organization of the parental lines used in the International Rice Molecular Breeding Program. Theor Appl Genet 108 (1): 131-140. DOI: 10.1007/s00122-003-1400-3

Wickramasinghe HAM, Noda T. 2008. Physicochemical properties of starches from Sri Lankan rice varieties. Food Sci Technol Res 14 (1): 49-54.

Wijayawardhana HCD, Herath HMVG, Weerasinghe PA, Herath HMDAK. 2015. Morphological variation in selected Sri Lankan rice (*Oryza sativa* L.) accessions in relation to the vegetative parameters. Trop Agric Res 26 (2): 380-389.

NUSANTARA BIOSCIENCE
Vol. 13, No. 1, pp. 68-72
May 2021

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n130110

Short Communication: Insect detection using a machine learning model

SOMJIT HOMCHAN, YASH MUNNALAL GUPTA♥

Department of Biology, Faculty of Science, Naresuan University. 99 Moo 9 Phitsanulok-Nakhonsawan Road, Phitsanulok 65000, Thailand.
Tel.: +66-55-963112, Fax.: +66-55-963113, ♥email: yashmunnalalg@nu.ac.th, yash.610@live.com

Manuscript received: 14 January 2021. Revision accepted: 3 February 2021.

Abstract. *Homchan S, Gupta YM. 2021. Short Communication: Insect detection using a machine learning model. Nusantara Bioscience 13: 68-72.* The key step in characterizing any organisms and their gender highly relies on correct identification of specimens. Here we aim to classify insect and their sex by supervised machine learning (ML) model. In the present preliminary study, we used a newly developed graphical user interface (GUI) based platform to create a machine learning model for classifying two economically important cricket species. This study aims to develop ML model for *Acheta domesticus* and *Gryllus bimaculatus* species classification and sexing. An experimental investigation was conducted to use Google teachable machine GTM for preliminary cricket species detection and sexing using pre-processed 2646 still images. An alternative method for image processing is used to extract still images from high-resolution video for optimum accuracy. Out of the 2646 images, 2247 were used for training ML model and 399 were used for testing the trained model. The prediction accuracy of trained model had 100 % accuracy to identify both species and their sex. The developed trained model can be integrated into the mobile application for cricket species classification and sexing. The present study may guide professionals in the field of life science to develop ML models based on image classification, and serve as an example for researchers and taxonomists to employ machine learning for species classification and sexing in the preliminary analysis. Apart from our main goals, the paper also intends to provide the possibility of ML models in biological studies and to conduct the preliminary assessment of biodiversity.

Keywords: Biodiversity, crickets, image recognition, insect, machine learning

INTRODUCTION

Classifying specimens/organisms is an initial and essential step for any biological studies. The scientific classification of a species demands prior knowledge for identification. However, automatic identification using machine learning model can be used to reduce time, cost, and labor. ML-based classifier is a rewarding tool identify organism and have been employed for insect identification (Yang et al. 2015). Various algorithms have been developed for insect classification (Ding and Taylor 2016; Kang et al. 2014), but all previously proposed methods are extremely dependent on algorithm development for optimal artificial neuron model for insect detection (Kaya and Kayci 2014). Nevertheless, these methods are also manual and time-consuming to develop insect detection algorithm (Larios et al. 2011; Kaya and Kayci 2014).

Artificial intelligence (AI) is revolutionizing human lifestyle and it has been integrated into many aspects (Deshpande and Kumar 2018). Machine learning (ML) is rapidly evolving and emerging in daily life. The bridge between users and developers is also expanding. It is essential to enlarge the involvement of professionals from different fields to apply their skills and develop a custom ML model without the need for coding/scripting skills. ML tools typically require technical skills that often include writing scripts to train classification models based on the input dataset. Particularly, professionals from life sciences are usually unfamiliar with certain skills that require and align with machine learning tools, which typically access with scripting computer language. Although perverse studies have successfully demonstrated a specific insect classification system, it has certain limitations in terms of complex algorithm development process and usability in the real world. To date, various algorithms have been used and introduced for insect detection, but require scripting and algorithm testing to perform machine learning tasks (Kasinathan et al. 2020). Therefore, the present research concentrates on the investigation and usability of the graphical user interface systems to produce a machine learning model for biological images.

Google teachable machine (GTM) is a fairly new graphical user interface (GUI) based tool introduced in 2019 and it does not require coding or scripting skills to build ML models (Carney et al. 2020). GTM is based on TensorFlow, which is Javascript library of ML algorithms. Using the standalone TensorFlow library requires scripting skills in computer language like Python or JavaScript, which is eliminated in GTM and easy to access with GUI. Recently, GTM is being used for teaching purposes (Toivonen et al. 2020) and even has been used to design an educational program (Yu et al. 2019). ML algorisms have been applied for insect and crop pest classification (Tuda and Luna-Maldonado 2020; Ayan et al. 2020), but GTM has never been evaluated to develop ML model for insect classification purposes.

Insects are being consumed in several Asian countries including Thailand. These edible insects, especially cricket species are very popular in Thailand for a few decades (Hanboonsong et al. 2013). Edible insects are very popular among locals and easily recognizable due to their morphological features. However, it is difficult to distinguish closely related species. Previous studies of pest detection research had to deal with the image processing system. However, the classification model needed several technical steps (Miranda et al. 2014). The image processing for machine learning has evolved and can be done without scripting skills. Researchers have utilized GTM for classifying images (Ji and Jun 2020). Herein, we intended to employ GTM for preliminary cricket detection. Nevertheless, the same strategy can be adapted for pest detection and also can be integrated as a standalone application.

Machine learning has been adapted for numerous studies in life science. ML has been applied for insect detection using audio analysis (Silva et al. 2013), and also for the assessment of crop damage caused by insects using ML-driven drones (Puig et al. 2015). Even in November 2020, authors attempted ML approach to determine three insect species and their sex using still images (Tuda and Luna-Maldonado 2020). However, researchers also stated that an automated and online system yet to be developed. Moreover, determining and training model for ML proposes could be difficult, and integrating the ML-driven model requires skills that researchers from different filed often do not possess. Herein, we aim to use GTM for insect species and gender determination that does not require specific scripting or arithmetical skills. The GTM is a web-based machine learning system and also provides a script that can be integrated into mobile or web-based applications.

Crickets are economically very important species and widely consumed by local people in Thailand

(Gupta et al. 2020). Therefore, in the present preliminary study, we have used two edible cricket species to train machine learning model using GTM. Moreover, the sex-ratio is important in cricket breeding facilities due to the polyandrous nature of female crickets, the present ML model might serve as a preliminary tool to maintain sex-ratio in the captive population. The trained algorithm from the present study can be utilized for application development or can be accessed using web-link. To our knowledge, this is the very first attempt to use GTM for insect species detection and sex, which will also encourage researchers to use machine learning platforms like GTM.

MATERIALS AND METHODS

Data collection and pre-processing

Two cricket species, *Acheta domesticus* and *Gryllus bimaculatus* were collected from cricket breeding facilities. Initially, these crickets were scientifically identified and used by our research team for population genetic studies (Gupta et al. 2020). In the current application, females and males from both cricket species were used to capture images from all directions. For the Image pre-processing, specimens were placed on white paper and 4K video at 30 frames per second (fps) was taken from all directions. Recorded 4K videos were used to extract frames for machine learning using Google Teachable Machine (GTM). GTM uses TensorFlow.js (Javascript library for machine learning). Extracted JPEG images were rectangular with the resolution of 500 dots per inch (DPI) having the dimension of 1280 X 720, JPEG images were separated into four different folders before uploading them into four classes on GTM server. Total dataset of 2,646 JPEG image files was created before using them for machine learning.

Training machine learning model on GTM

Pre-processed images were used and uploaded to GTM server. The training was repeated 100 times (epoch value). For deep learning with high accuracy, the batch size was set to 16 to divide data into smaller batches during training for each repetition. 85 % of the dataset was used to train the model and the other 15% of dataset was never been used for training but used to check the performance and accuracy of the trained model. Previously, the randomized splitting method was also used to divide the pest image dataset prior to machine learning (Ayan et al. 2020).

To evaluate the model, the advanced parameters were used at GTM platform to compute accuracy per cricket species class, accuracy per repetition, loss per repetition, and to generate confusion matrix.

Sample data and JavaScript availability:

Https://github.com/yashmgupta/Insect-species-detection-

RESULTS AND DISCUSSION

Firstly, to increase the accuracy and capture the image from all directions, 4K (around 4000 pixels in the horizontal direction) video was taken and the process to obtain JPEG files of still images having a resolution of 500 DPI with 24-bit depth. Recently, researchers used a scanning method to obtain 600 JPEG files of insects (Tuda and Luna-Maldonado 2020). In the present study, we used an alternative and less time-consuming method to generate pre-processed image files for machine learning purposes. Moreover, scanning insect specimens cannot capture the visual of a specimen from all directions that might affect the accuracy of the trained model for species or sex determination using a machine learning model. Usually, the train model accuracy increases by increasing the numbers and the quality of images (Ji and Jun 2020). To reduce time consumption for data pre-processing and increase the probability of training machine learning model with the highest accuracy, we developed an alternative method, which resulted in 2646 pre-pressed image files for deep machine learning. Hereto, 2247 images were used for training the model with GTM and 399 images were used as test samples to check the performance of the trained model.

The results show that the trained model has a 100% probability for categorizing cricket images to correct species and sex. The 399 cricket images were employed to check accuracy. There was no instance where a single cricket image was classified for more than one insect species or sex. In the present supervised machine learning test, for all four classes, the accuracy was one (1.00) as shown in Table 1. This means all samples used to test the trained model were successfully classified according to their features (species and sex) Figure 1.

Default options of GTM for epochs are fifty, but in present supervised machine learning model, the whole dataset went through the training a hundred times for better prediction and accuracy (as mentioned in method section). The training and testing accuracy per epoch is shown in Figure 2 (A). Both accuracies reached their threshold after only seven epochs of training with the whole dataset. In the present machine learning model, the prediction for correct cricket species and sex becomes practically perfect after the 7^{th} epoch, as loss per each became zero at the 20th epoch and remained stagnant till the last epoch, Figure 2 (B). When the prediction is perfect, the accuracy becomes one, and after that prediction, if the confidence is hundred percent, the loss becomes zero (Carney et al. 2020). The validation result matches and confirms that our ML model learned appropriately without underfit or overfit.

As described in the method section, we extracted still images from high-resolution video to be converted into still images for training ML model to capture morphological keys of cricket species. Recently, researchers have used scanners for image processing (Tuda and Luna-Maldonado 2020), but we have altered the conventional method of pre-processing still images for classification. The ML model based on image classifications has been applied to map tree species (Wang et al. 2018; Lim et al. 2019), and using objected-based analysis to classify deciduous tree species (Franklin and Ahmed 2018).

Insects, including crickets, are relatively small, which often makes them harder to identify or determine their sex without experience or expertise. However, the most common visible feature in sexing for cricket is the presence of an ovipositor in female crickets. Our application of GTM for primary identification based on image classification confirms that the machine learning model trained for cricket species identification and sexing. Previously, researchers suggested applying molecular biology techniques for sustainable insect breeding for food and feed (Gupta and Homchan 2019). Herein, we are suggesting that the developed ML model can also be applied to maintain sex-ratio in the breeding facility.

Besides, this study is the first step towards educating researchers to use machine learning from GTM platform in biological sciences. Result of the trained model for cricket species classification using GTM is encouraging. The developed algorithm can be excess via an online platform, or can be used with a mobile application (developed to run ML model in the real environment). This research was concerned with the usability of GTM for research purposes; however, the findings suggest that this approach can be useful for developing algorism using an online platform for insect detection with direct practical relevance. Moreover, TensorFlow is huge library of machine learning algorithms extensively used for research purposing, including drug discovery and image classification (Abadi et al. 2016). GTM uses pretrained neural network of mobilenet models from TensorFlow library, which has separate 28 layers for the image classification with high accuracy (Howard et al. 2017). The finding also has important implications for assisting active sample collectors without prior knowledge about cricket or any other insect species for assessment of biodiversity or population analysis.

The proposed method can be adapted for several biological science applications and can be readily used in practice for preliminary work. In the present study, particular attention is paid to develop an adaptable approach for ML practice in biological science where observation-based assessment is needed (e.g. on-site cricket sample collection). On the basis of the promising results of two cricket species classification and sexing, the ML model with other cricket species will be developed and investigated in future research.

Table 1. Accuracy per class of cricket species and their gender.

Classes	Accuracy	Test samples
ADF	1.00	118
ADM	1.00	118
GBF	1.00	78
GBM	1.00	85

Note: ADF: *Acheta domesticus* female, ADM: *Acheta domesticus* male, GBF: *Gryllus bimaculatus* female, GBM: *Gryllus bimaculatus* male

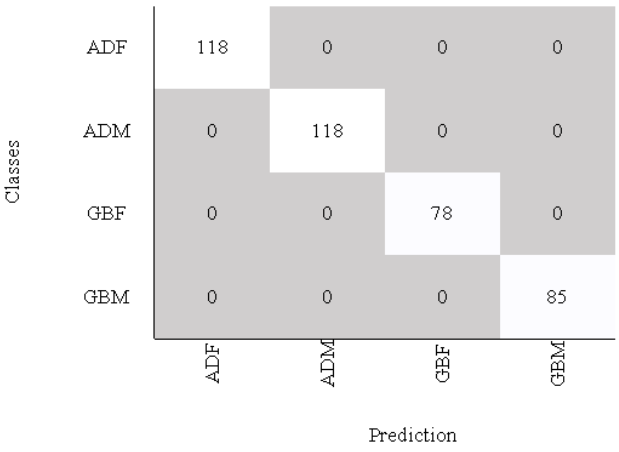


Figure 1. Confusion matrix of classes according to the provided samples (Y-axis) and predicted classes based on the trained model (X-axis). The trained model showed no misclassification for 399 samples that new-before-seen by ML model.

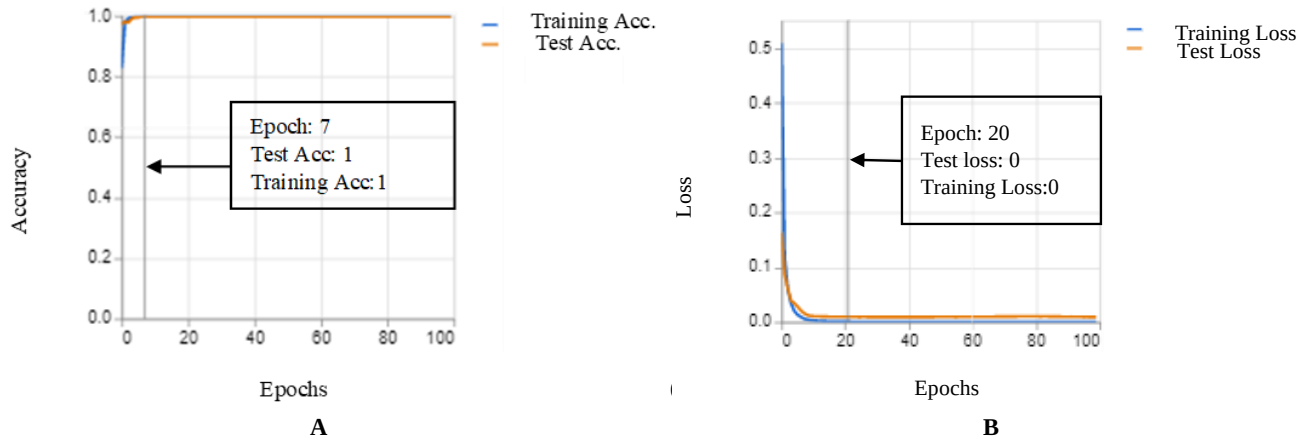


Figure 2. Plots for training and testing dataset result validation: A. Plot of training and test accuracy against epochs. B. The plot of training loss and test loss against epochs

The present study was designed to determine the usability of machine learning platform like GTM for preliminary species detection and sexing. The result of the investigation shows that the ML model can be developed with ease using GTM and incorporated in mobile and website or can be directly excess with the shareable link. The key strengths of this study are its user-friendly online ML platform and accessibility after training the cricket classification model. Although the current ML model is based on two cricket species, the findings suggest that ML model can be developed for classification of several cricket species with different morphological features. A reasonable approach to capture details is extracting still images from the video; therefore, we suggest a similar to this one should carry out before building the ML model. Taken together, our results suggest that ML model can be trained on the GTM platform for image-based classification in biological sciences, including insect classification.

ACKNOWLEDGEMENTS

We would like to thank Ms. Satwika Nindya Kirana for polishing English and giving valuable comments. This research has been supported by Department of Biology, Faculty of Science, Naresuan University, Thailand.

REFERENCES

Abadi M, Agarwal A, Barham P, Brevdo E, Chen Z, Citro C, Corrado GS, Davis A, Dean J, Devin M, Ghemawat S, Goodfellow I, Harp A, Irving G, Isard M, Jia Y, Jozefowicz R, Kaiser L, Kudlur M, Levenberg J, Mane D, Monga R, Moore S, Murray D, Olah C, Schuster M, Shlens J, Steiner B, Sutskever I, Talwar K, Tucker P, Vanhoucke V, Vasudevan V, Viegas F, Vinyals O, Warden P, Wattenberg M, Wicke M, Yu Y, Zheng Y. 2016. Tensorflow: Large-scale machine learning on heterogeneous distributed systems. Arxiv:1603.04467.

Ayan E, Erbay H, Varçın F. 2020. Crop pest classification with a genetic algorithm-based weighted ensemble of deep convolutional neural networks. Comput Electron Agric 179: 105809. DOI: 10.1016/j.compag.2020.105809.

Carney M, Webster B, Alvarado I, Phillips K, Howell N, Griffith J, Jongejan J, Pitaru A, Chen A. 2020. Teachable Machine: Approachable web-based tool for exploring machine learning classification. In Proceedings of the 2020 CHI Conference on Human Factors in Computing Systems. Honolulu, 25-30 April 2020. [USA] DOI: 10.1145/3334480.3382839.

Ding W, Taylor G. 2016. Automatic moth detection from trap images for pest management. Comput Electron Agric 123: 17-28. DOI: 10.1016/j.compag.2016.02.003.

Deshpande A, Kumar M 2018. Artificial Intelligence for Big Data: Complete Guide to Automating Big Data Solutions Using Artificial Intelligence Techniques. Packt Publishing Ltd., Birmingham, UK.

Franklin SE, Ahmed OS. 2018. Deciduous tree species classification using object-based analysis and machine learning with unmanned aerial vehicle multispectral data. Int J Remote Sens 39(15-16): 5236-5245. DOI: 10.1080/01431161.2017.1363442.

Gupta YM, Homchan S. 2019. Preventive measures and sustainable practices for insect breeding. J Biol Sci 5 (4): 1-4.

Gupta YM, Tanasarnpaiboon S, Buddhachat K, Peyachoknagul S, Inthim P, Homchan S. 2020. Development of microsatellite markers for the house cricket, *Acheta domesticus* (Orthoptera: Gryllidae). Biodiversitas 21(9): 4094-4099. DOI: 10.13057/biodiv/d210921.

Hanboonsong Y, Jamjanya T, Durst PB. 2013. Six-Legged Livestock: Edible Insect Farming, Collection and Marketing In Thailand. FAO Regional Office for Asia and the Pacific, Bangkok.

Howard AG, Zhu M, Chen B, Kalenichenko D, Wang W, Weyand T, Andreetto M, Adam H. 2017. Mobilenets: Efficient convolutional neural networks for mobile vision applications. Arxiv:1704.04861.

Ji SY, Jun HJ. 2020. Deep learning model for form recognition and structural member classification of east Asian traditional buildings. Sustainability 12(13): 5292. DOI: 10.3390/su12135292.

Kang SH, Cho JH, Lee SH. 2014. Identification of butterflies based on their shapes when viewed from different angles using an artificial neural network. J Asia Pac Entomol 17(2): 143-149. DOI: 10.1016/j.aspen.2013.12.004.

Kasinathan T, Singaraju D, Uyyala SR. 2020. Insect classification and detection in field crops using modern machine learning techniques. Inform Process Agric. DOI: 10.1016/j.inpa.2020.09.006.

Kaya Y, Kayci L. 2014. Application of artificial neural network for automatic detection of butterfly species using color and texture features. Vis Comput 30: 71-79. DOI: 10.1007/s00371-013-0782-8.

Larios N, Lin J, Zhang M, Lytle D, Moldenke A, Shapiro L, Dietterich T. 2011. Stacked spatial-pyramid kernel: An object-class recognition method to combine scores from random trees. IEEE Workshop on Applications of Computer Vision (WACV) 329-335. DOI: 10.1.1.363.9133.

Lim J, Kim KM, Jin R. 2019. Tree species classification using Hyperion and sentinel-2 data with machine learning in South Korea and China. ISPRS Int J Geoinf 8(3): 150-177. DOI: 10.3390/ijgi8030150.

Miranda JL, Gerardo BD, Tanguilig Iii BT. 2014. Pest detection and extraction using image processing techniques. Int J Comput Commun Eng 3(3): 189-192. DOI: 10.7763/IJCCE.2014.V3.317.

Puig E, Gonzalez F, Hamilton G, Grundy P. 2015. Assessment of crop insect damage using unmanned aerial systems: A machine learning approach. Proc. 21st Int. Congr. Modelling Simulation, Gold Coast, Australia, 29 November - 4 December 2015. DOI: 10.36334/modsim.2015.f12.puig.

Silva DF, De Souza VM, Batista GE, Keogh E, Ellis DP. 2013. Applying machine learning and audio analysis techniques to insect recognition in intelligent traps. 12th International Conference on Machine Learning and Applications, 99-104 2013. DOI: 10.1109/icmla.2013.24.

Toivonen T, Jormanainen I, Kahila J, Tedre M, Valtonen T, Vartiainen H. 2020. Co-designing machine learning apps in k–12 with primary school children. IEEE 20th International Conference on Advanced Learning Technologies (ICALT), 308-310. DOI: 10.1109/ICALT49669.2020.00099.

Tuda M, Luna-Maldonado AI. 2020. Image-based insect species and gender classification by trained supervised machine learning algorithms. Ecol Inform 60: 101135. DOI: 10.1016/j.ecoinf.2020.101135.

Wang D, Wan B, Qiu P, Su Y, Guo Q, Wu X. 2018. Artificial mangrove species mapping using pléiades-1: An evaluation of pixel-based and object-based classifications with selected machine learning algorithms. Remote Sens 10: 294-315. DOI: 10.3390/rs10020294.

Yang HP, Ma CS, Wen H, Zhan QB, Wang XL. 2015. A tool for developing an automatic insect identification system based on wing outlines. Sci Rep 5: 1-11. DOI: 10.1038/srep12786.

Yu WJ, Jang JH, Ahn JM, Park DR, Yoo IH, Bae YK, Kim WY. 2019. Design of artificial intelligence education program based on design-based research. Int J Adv Smart Converg 8: 113-120. DOI: 10.7236/IJASC.2019.8.4.113.

NUSANTARA BIOSCIENCE
Vol. 13, No. 1, pp. 73-84
May 2021

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n130111

Review: Bioaccumulation of heavy metals in fish and other aquatic organisms from Karachi Coast, Pakistan

RAMZY A. YOUSIF[1,♥], MUHAMMAD IQBAL CHOUDHARY[2], SHAKEL AHMED[2], QURATULAN AHMED[3]

[1]Department of Fisheries and Wildlife Science, Sudan University of Science and Technology. P.O.BOX 204, Khartoum, Sudan. Tel./fax.: +249-912-802311, ♥email: ramzy@sustech.edu; ramzy173@gmail.com
[2]H.E.J. Research Institute of Chemistry, International Centre for Chemical and Biological Sciences, University of Karachi. Karachi 75270, Pakistan
[3]The Marine Reference Collection and Resources Centre, University of Karachi. Karachi 75270, Pakistan

Manuscript received: 25 December 2020. Revision accepted: 10 February 2021.

Abstract. *Yousif RA, Choudhary MI, Ahmed S, Ahmed Q. 2021. Review: Bioaccumulation of heavy metals in fish and other aquatic organisms from Karachi Coast, Pakistan. Nusantara Bioscience 13: 73-84.* Heavy metals are being utilized in a variety of ways in industries, agriculture, food processing and household in many forms. Metals are unique environmental and industrial pollutants in the sense that they are neither created nor destroyed by human beings but are only transported and transformed into various products. The present study deals with the findings of various investigators on the effect of heavy metals on fish and other aquatic organisms on Karachi coasts of Pakistan. The polluted areas (Rivers and Karachi coasts) receiving effluents from industrial, agricultural, municipal and domestic wastes. The order of abundance of the metals were as fellow; Fe > Zn > Cu > Mn > Cd > Pb > Cr > Ni > Hg > As. Most studies showed that essential metals (Fe, Zn, Cu, and Mn) in aquatic organisms are much high, but the quantities of non-essential metals are found to be less. This review has shown that fish and other aquatic organisms are used as bio-monitoring species in heavy metal pollution. It is suggested that such investigations should be continuous in terms of both human health and determination of metal pollution in aquatic environment.

Keywords: Aquatic organisms, fish, heavy metals, Karachi Coast

INTRODUCTION

Heavy metals are essential components of aquatic environment, usually found in really low concentrations. The many parts of the heavy metals released into the earth discover their way into the freshwater and marine environment as by many ways as input of direct air deposition, climatic, and disintegration due to rainwater. The levels of heavy metals are too high, in the area where domestic activities, mining activities, mechanical and cultivating activities are across the natural areas (Langston 1990; Bryan and Langston 1992; Sulieman and Suliman 2019).

Heavy metals accumulated in the fish and other aquatic organisms in two ways direct and indirect accumulation, consumption of contaminated water and food through the digestive system considered as direct exposure or indirectly through the permeable membranes such as skin and gills. The level of heavy metals concentration in fish and other aquatic organisms' organs indicate their levels also in their surrounding environment. The accumulation of heavy metals in aquatic organism organs sometimes can exceed the environmental levels. The toxic effect usually occurs when the rate of uptake is exceeding the mechanism of metabolism, storage, and detoxification (Ali et al. 2011; Baki et al. 2018; Rajeshkumar and Li 2018).

Contamination of the freshwater and marine environment by heavy metals has been reported by many authors such as Rashed (2001), Rahman et al. (2012), Yousif et al. (2016) and Rajeshkumar and Li (2018), have reported that zinc, chromium, mercury, lead, copper, cadmium and arsenic are the important metals which contaminate the water and harmful to aquatic organisms. Many marine organisms (fish, shrimp, and crab) at the highest point of the natural feeding ground items and aggregates high levels of metals from the water and sediment (Lambert et al. 2000; Tüzen 2003; Arulkumar et al. 2017; Daellenbach et al. 2017; Narsimha and Wang 2018). Heavy metals accumulated in aquatic organisms and their effect can move to the human after using contaminated fish and other aquatic organisms and the consequences can deteriorate the human health (Raja et al. 2009; Alinnor and Obiji 2010; Abarshi et al. 2017).

The levels of dangerous contaminants in aquatic organisms are a critical factor in view of their potential effects on the organisms themselves and the health status of humans that utilize them. Health organizations and institutions, for example, Food and Drug Administration (FDA), have started late raised stresses over the security of fisheries obtained from business sources (Burger et al. 2004).

THE MAIN SOURCES OF HEAVY METALS

Contaminations of heavy metals in water also lead to changes in the chemical components of the aquatic environment, usually influences the behavioral, physiological, and bloodstream patterns, cell structures ionic balance (Oikari and Soivio 1976), liver function, and carbohydrate metabolism (Oikari and Soivio 1976; Oikari and Nakari 1982) of fishes. Earlier studies, showed that anthropogenic activities and domestic effluent constitute huge sources of heavy metal which contribute to the steadily increasing metallic pollutant in aquatic environment in most part of the world (Jibiri and Adewuyi 2008; Ates et al. 2015).

Recent development and advancements in the agricultural sector, industrialization, and urbanization have contributed substantially to elevated heavy metal pollution in freshwater and marine environments. Anthropogenic activities such as mining and smelting (Chen et al. 2015), combustion of fossil fuel refining (Muradoglu et al. 2015), discharge and disposal of domestic and municipal wastes (Khan et al. 2016), using pesticides in agricultural sector (Ogunlade and Agbeniyi 2011), sewage irrigation in some countries (Sun et al. 2013), fertilizer and urea application (Atafar et al. 2010), dust (Chen et al. 2011) contribute to spread the levels and concentrations of dangerous heavy metals in the aquatic environments. The major sources of heavy metals are summarized in Table 1.

Generally, metals can be categorized as biologically essential and nonessential. The nonessential metals (e.g., lead (Pb), mercury (Hg), cadmium (Cd), aluminum (Al), and tin (Sn)) no study has proven their biological function (also called xenobiotics elements or foreign elements), and their effects and toxicity rise with increasing the levels and concentration of these metals, on the other hand essential metals (e.g., zinc (Zn), iron (Fe) copper (Cu), cobalt (Co), nickel (Ni), chromium (Cr) and molybdenum (Mo)), have a known their biological role, and their effects and toxicity occur either at metabolic deficiencies or at high levels and concentrations of these metals (Sfakianakis et al. 2015). The deficiency of an essential metal can lead to adverse health effects, whereas the high levels of essential elements can also lead to negative effects which are equivalent to or worse than those effects caused by non-essential metals (Kennedy 2011; Sfakianakis et al. 2015). The most commonly investigated and found heavy metals in fish and other aquatic organisms in many studies are Zn, Cu, Pb, Cr, Cd, Ni, Hg, Co, Sn, and Mo. Amongst them, the most frequently studied, with respect to fish and aquatic organisms' deformities, include Zn, Pb, Cu, Cr, Cd, and Hg.

ACCUMULATION OF HEAVY METALS AND THE ECOLOGICAL STATUS OF AQUATIC ORGANISMS

The rapid development of industry and agriculture has resulted in an increase in the pollution of coastal areas with heavy metals, which have been identified as a significant environmental hazard for invertebrates, fish, and humans (Yousif et al. 2016; Khan and Strand 2018) Significant quantities of heavy metals in waster water are discharged into aquatic environments. These metals can be strongly accumulated and biomagnified along water, sediment, and aquatic food chains, thus resulting in sublethal effects or death in local fish populations and other aquatic animals (Yi and Zhangc 2012). Heavy metals like copper and zinc are essential for fish metabolism, while others such as mercury, cadmium, and lead have no known role in biological systems (Yi and Zhangc 2012; Ates et al. 2015). Therefore, it is important to better understand the relationships between ecological status of aquatic organisms and the concentrations of both essential and non-essential metals. Heavy metal pollution of water and sediment in the Karachi Coast has attracted much attention from researchers (Ahmed et al. 2017b).

HEAVY METAL AND HUMAN HEALTH

Consumption of contaminated food is the main source of exposure of humans to the risks of heavy metals (Liu et al. 2010). The presence of heavy metal in commercial fish can pose potential health risks to humans (Cid et al. 2001; Castro-gonzález and Méndez-armenta 2008; Saeedi et al. 2012; Ullah et al. 2017). Hence, it is important to know the level and concentration of heavy metal contents in aquatic organisms in order to ensure that it does not expose any hazard to the human and maintain concentration under permissible level (Sivaperumal et al. 2007; Uysal et al. 2008; Palaniappan and Karthikeyan 2009; Dehghani et al. 2017; Pal et al. 2018). Heavy metal pollution is increasingly recognized as a serious, environmental issue by environmentalists, high levels of toxicity, persistence, and potential for accumulation inside human body pose a serious health threat to the residents of urban areas (Karim et al. 2015; Mohmad et al. 2015; Hwang et al. 2016; Gope et al. 2017; Khan and Strand 2018; You et al. 2018; Liu et al. 2019; Men et al. 2019; Tian et al. 2019).

Many organizations and institutions such as the Food and Agriculture Organization (FAO), World Health Organization (WHO) and European Union (EU) from different countries have been established about the maximum permitted concentration of heavy metals in foodstuffs including fish and other seafood (Chary et al. 2008; Xue et al. 2012). For example, European Union (2006) reported that the maximum tolerable limit (MTL) of lead (Pb) in the edible tissues of fish is 0.3 mg/kg where Cd and Hg were about 0.05-0.30 and 0.5-1.00 mg/kg wet weight respectively depends on the type of fish. Heavy metals such as Pb, Cd and Hg are categorized as non-essential elements and they are very toxic and harmful to individuals and aquatic organisms, even at small levels (Thomas et al. 2009; Zheng et al. 2011; Bourliva et al. 2018). While, Zn, Mn, Cu, and Ni are essential elements due to their important function in biological systems (Stern et al. 2007; Fernandes et al. 2008). The dose-response curve for essential metals is U-shaped due to those metals that have both deficiency and copper excess which produce adverse health (Stern et al. 2007).

Table 1. Sources of heavy metal contaminations in the aquatic environment (Lone et al. 2008; Changfeng et al. 2019)

Heavy metals	Sources
Zn	Electroplating industry, smelting and refining, mining, biosolids
Fe	Iron alloys are processed to containers, cars, laundry machines, bridges, buildings, and also the other sources of iron as pharmaceutics, chemicals, iron fertilizers, and pesticides.
Cu	Electroplating industry, mining, biosolids, smelting, and refining
Pb	Mining and smelting of metalliferous ores, burning of leaded gasoline, municipal sewage, industrial wastes enriched in Pb, paints
Cd	Geogenic sources, anthropogenic activities, metal smelting and refining, fossil fuel burning, application of phosphate fertilizers, sewage sludge.
Hg	Volcano eruptions, forest fire, emissions from industries producing caustic soda, coal, peat, and wood burning
As	Semiconductors, wood preservatives, mining and smelting, coal power plants, herbicides, volcanoes, petroleum refining, animal feed additives
Cr	Electroplating industry, sludge, solid waste, tanneries
Mn	Municipal wastewater discharges, sewage sludge, mining and mineral processing, emissions from alloy, steel, and iron production, combustion of fossil fuels and to a much lesser extent.
Ni	Volcanic eruptions, landfill, forest fire, bubble bursting and gas exchange in ocean, weathering of soils and geological materials, industrial effluents, kitchen appliances, surgical instruments, steel alloys, automobile batteries

Many organizations and institutions such as the Food and Agriculture Organization (FAO), World Health Organization (WHO) and European Union (EU) from different countries have been established about the maximum permitted concentration of heavy metals in foodstuffs including fish and other seafood (Chary et al. 2008; Xue et al. 2012). For example, European Union (2006) reported that the maximum tolerable limit (MTL) of lead (Pb) in the edible tissues of fish is 0.3 mg/kg where Cd and Hg were about 0.05-0.30 and 0.5-1.00 mg/kg wet weight respectively depends on the type of fish. Heavy metals such as Pb, Cd, and Hg are categorized as non-essential elements and they are very toxic and harmful to individuals and aquatic organisms, even at small levels (Thomas et al. 2009; Zheng et al. 2011; Bourliva et al. 2018). While, Zn, Mn, Cu, and Ni are essential elements due to their important function in biological systems (Stern et al. 2007; Fernandes et al. 2008). The dose-response curve for essential metals is U-shaped due to those metals that have both deficiency and copper excess which produce adverse health (Stern et al. 2007).

Zn is a ubiquitous trace element and one of the essential elements that important to humans and plants. Zn is known as a cofactor to more than 300 enzymes that involved in RNA and DNA metabolism, and it is also important in the structural stabilization of a large amount of proteins (Song et al. 2010; Chasapis et al. 2012). When exceeding amounts are present, Zn becomes toxic (Krishna et al. 2014; Kastury et al. 2017) but a deficiency of Zn can lead to several disorders (Scherz and Kirchhoff 2006). such as results in poor pregnancy outcomes (King 2000; Uriu-adams and Keen 2010) and development of chronic diseases, including cardiovascular disease (Messner et al. 2009; Afridi et al. 2011) and also cause cancer (Kazi et al. 2010).

Fe is essential element for every living cell and necessary for the synthesis of many enzymes, myoglobin, and hemoglobin in the blood. The result deficiency of iron can lead to weakness, susceptibility, and inability to concentrate and other symptoms (Akoto et al. 2014). Anderson and Fitzgerald (2010) reported that one of the most common nutrient deficiencies in the world is Fe deficiency in anemia such as malaria. Anaemia disease gives poor performance in circulatory transport and also reduces oxygen supply to muscle, less efficiency due to the decreasing of myoglobin content and impairing endurance capacity (Erdman et al. 2012).

Cu is an essential metal of enzymes and necessary for hemoglobin synthesis (Sivaperumal et al. 2007). Impaired delivery of Cu can result in decreased cuproenzyme activity, the skeletal and vascular systems Failla et al. (2001), and also cause anemia, neutropenia, and osteoporosis (Angelova et al. 2011). Impaired metabolism of Cu could cause two genetic diseases which are Mense disease and Wilson disease. Accumulation of Cu can expose to the Mense disease which is a fatal disorder (Gu et al. 2002; Ameh and Sayes 2019). Wilson disease also could occur due to Cu accumulates in the brain and eyes in the form of Kayaer-Fleischer ring (Sarkar 1999; Attri et al. 2006). Excessive intake of Cu also could cause kidney damage and even death (U.S. Department of Health and Human Services 2004).

Mn has a biological significance due to its ability to prevent heart attack, stroke, and cardiac arrest, which is also an element of low toxicity. Deficiency of manganese can lead to poor growth performance, congenital malformations in offspring, and low efficiency of the reproductive system (Goldhaber 2003). However, it's become dangerous and toxic at high concentrations and usually may lead to neurologic and psychologic disorders (Perl and Olanow 2007; Saha and Zaman 2013). Normally, nickel (Ni) is an essential metal and occurs at very low levels in the environment. However, a deficiency of Ni in humans has not yet been reported and documented (Barceloux 1999). Ni is known to be carcinogenic (Salnikow and Kasprzak, 2005). Moreover, fibrosis, tumors, lung inflammation, and emphysema occur also caused by Ni (Forti et al. 2011).

Cr is an essential trace element in some aquatic animals and humans. Cr may reduce body fat and also improve lean body mass. However, their effects are small compared to those of a well-balanced diet and exercise (Roussel et al. 2007; Tulasi and Rao 2014). But it could have an undesirable fatal effect in excess amount. Lack of Cr can affect the growth and disturbances in glucose, lipid, and protein metabolism (Akoto et al. 2014). According to Stipanuk and Caudill (2012), they found that 12 out of 15

studies showed a positive effect on the relationship between Cr and impaired glucose tolerance based on a meta-analysis.

Besides, Hg is a non-essential element. The levels of Hg increase due to the increases in fish size (Burger and Gochfeld 2011). Toxicity of Hg can damage the organ in fish (Sary and Mohammadi 2011; Krishna et al. 2014; Abu-Dieyeh et al. 2018). While, in humans, Hg can cause the development of fetus destroyed due to their toxicity and also considered as a carcinogen (Ikem and Egilla 2008). While, Vettori et al. (2003) studied that neuronal loss in the cerebellum granule layer and damage of discrete visual cortex area occurs in adult brain due to Hg poisoning. Emami-Khansari et al. (2005) also stated that Hg is a human toxicant and become primary source of human by eating fish.

Food consumption is the main source of exposure cadmium (Cd) in the human body. Cd is known as an endocrine disturbing substance and it is well documented that Cd can cause to develop breast cancer and prostate cancer in humans (Saha and Zaman 2013). Cd also causes damage in kidney, hypertension, tumors, poor reproductive performance, and hepatic dysfunction (Rahman and Islam 2010; Al-Busaidi et al. 2011; Hao et al. 2013).

In addition, lead (Pb) is a naturally-occurring and industrially-produced element that is very toxic to the human, especially children (Koyashiki et al. 2010; Kastury et al. 2019). Children are the most vulnerable to Pb because having less effective renal excretion and greater absorption of gastrointestinal. The fetal brain presents a greater sensitivity to the toxic effects of Pb compared to the mature brain (Schnaas et al. 2006). Umar et al. (2001) stated that symptoms of intestinal cramps, anemic condition and fatigue caused by poisoning of Pb. Lead also can cause nephrotoxicity and neurotoxicity (García-lestón et al. 2010).

Nowadays, Arsenic (As) is widely spread in the environment due to both natural and anthropogenic processes (Rahman et al. 2012). As is a carcinogen and potent toxicant. As also has potential to destroy communities of ecological systems (Sadiq et al. 2003). Toxicity of as depends on the speciation (Devesa et al. 2008) and trivalent as (III) has the greatest toxicity. According to ATSDR (2000), mono and dimethyl arsenic have low toxicity.

STUDIES ON HEAVY METALS IN FISH AND OTHER AQUATIC ORGANISMS IN KARACHI COAST

Pakistan has geologically and ecologically diverse coastline dissected by harbors, estuaries, bays, and creeks exhibiting wide characteristics in the marine species. Unfortunately, the littoral state of Pakistan is facing many environmental issues as increasing pollution and human-induced environmental changes particularly fishing, coastal aquaculture, waste disposal, industrial activity, agriculture, domestic effluents, salt making, unplanned tourism, etc. Contaminated ecosystem destroys the life of aquatic animals and decreases the market value of seafood products and increase in bacterial diseases (Kamal et al. 2015b; Shahab et al. 2016; Ahmed et al. 2017a; Chandio et al. 2018; Ahmed et al. 2019).

Karachi coast is a very important coast for its dimensions and economic activity. There are over 11000 industrial units (CDGK 2012) present, more than 2000 units in Federal-B-Area, 2571 units in Korangi zone, 2000 units in North Karachi, 1200 units in Landhi zone, and 4000 units in Mangopir zone in Karachi (Aziz and Khan 2014; Ahmed et al. 2018c; Mujeeb et al. 2020) Figure 1. By the Karachi coastline ever-growing pollution level, which is linked to the increase of the shipping industry through the Karachi port, is severely contaminating the mangrove, forests, and marine life (Ahmed et al. 2018c; Ali et al. 2019). The dumping of wastes on the coast provides a major source of heavy metal input (Khattak et al. 2012; Mukhtar and Hannan 2012; Chaudhary et al. 2013; Ahmed et al. 2018c). The important sources of heavy metals pollution are industrial activities and dumping of land-based wastes into the river and coasts of the sea. Especially in Pakistan and other countries such as India and Bangladesh most industries are converged on the riverbanks of big cities (Khan et al. 2011; Hasan et al. 2013; Jilani 2015; Hossain and Islam 2019). Not only these countries but all the coastal countries are exposed to heavy metal pollution. In the coastal areas, heavy metal contamination is found in seawater, sediment and aquatic organisms, causing a health risk (Kazmi and Zubair 2014; Elahi et al. 2015; Devault et al. 2017; Ahmed et al. 2018c).

The investigation of heavy metals (Zn, Fe, Cu, Cd, Hg, As, Cr, Ma and Ni) in different aquatic organisms on Karachi coast has been reported in numerous earlier studies such as *Thunnus* spp. (Yousuf and Ahmed 2010; Ahmed et al. 2012; Ahmed et al. 2015a), *Pongamia pinnata* (Shafiq et al. 2012), *Fenneropenaeus penicillatus* (Kamal et al. 2015a), *Rastrelliger* kanagurta (Yousuf and Ahmed 2011; Ahmed et al. 2014a; Ahmed and Bat 2015; Ahmed et al. 2016b), *Holothurians* spp. (Ahmed et al. 2017b; Ahmed et al. 2018b), *Ohshimella ehrenbergii* and *Stolus buccalis* (Ahmed et al. 2019) (Table 2, Figure 2). Heavy metals are being utilized in a variety of ways in industries, agriculture, food processing and household in many forms. The present study deals with the findings of various investigators on the effect of heavy metals on fish and other aquatic organisms in Karachi. The polluted areas (rivers and Karachi coast) receive effluents from industrial, agricultural, municipal and domestic sources (Ali et al. 2014; Iftikhar et al. 2018). The order of abundance of the metals were as fellow; Fe > Zn > Cu > Mn > Cd > Pb > Cr > Ni > Hg > As. Pb is a neurotoxic metal that causes many behavioral defects in biotic samples, as a result of which decrease in survival growth rates and metabolism occur. The main source of Pb in the present geographical locale could be the contamination from Ibrahim Hyderi coast. The discharge of industrial waste of Korangi industrial trading estate (KITE) and Gizri Creek causes the increase Pb concentration along with the littoral states of Ibrahim Hyderi (Khwaja et al. 2012; Kamal et al. 2015a).

Figure 1. Map of the study area in Karachi Coast, Pakistan

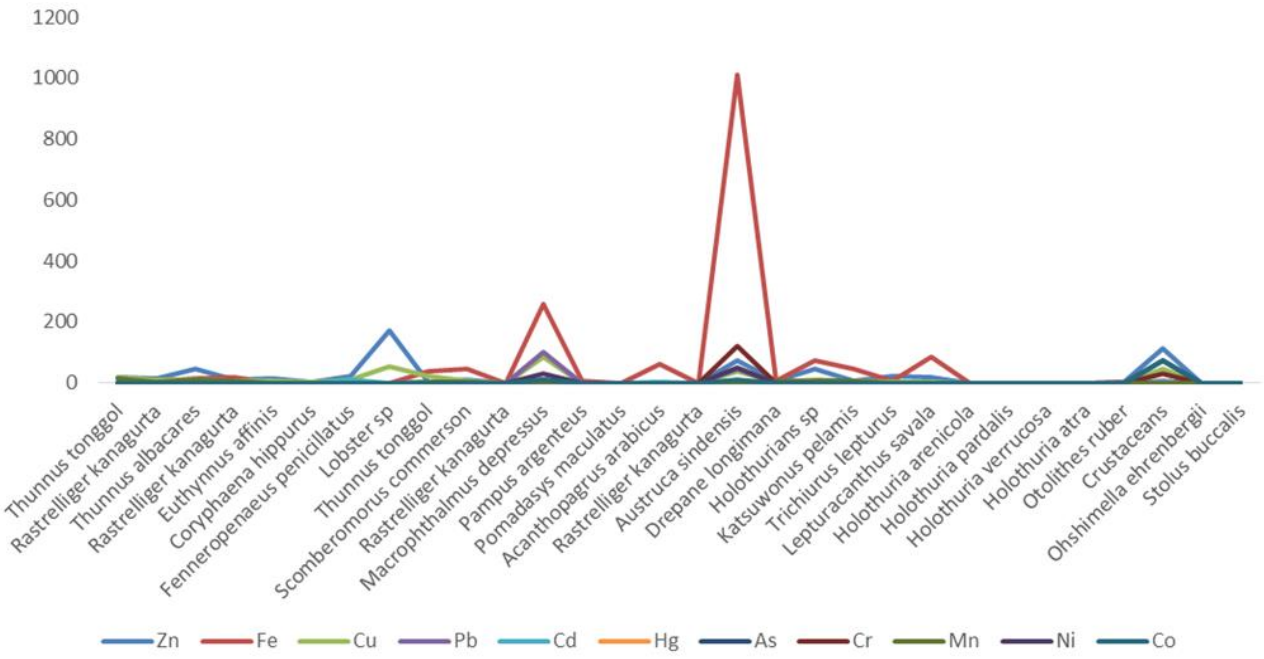


Figure 2. Accumulation of heavy metals (mg/kg) in fish and other aquatic organisms, Karachi Coast, Pakistan

Heavy metal loaded aquatic systems may affect the food chain of inhabitant fish species. It is reported by many researchers that heavy metal pollution in aquatic ecosystems is often more reflected by high metal levels in sediments, macrophytes, and benthic animals than by elevated concentrations in water (Van Hassel et al. 1980; Ashraf et al. 2019). Heavy metal accumulation and its effects on fish are very much complex to elucidate because of dynamic nature of aquatic ecosystems.

Aquatic animals mainly the phyla crustacean species are the bio-indicators of toxic materials because invertebrates have more tendencies to accumulate contaminants as compare to fishes (Balfour et al. 2012; Kamal et al. 2015b; Ahmed et al. 2017a). Hazardous material from the surrounding continuously enter in fresh and marine environment and deposit in biota from where it subsequently transferred in to human through the food chain and when the concentration of these substances reaches to a certain level, it becomes toxic (Gokoglu et al. 2008; Copat et al. 2013; Goretti et al. 2016).

Table 2. Levels of heavy metal in edible tissue of fish and other aquatic organisms from Karachi Coast, Pakistan

Species	Determination techniques	Unit	Metals											References
			Zn	Fe	Cu	Pb	Cd	Hg	As	Cr	Mn	Ni	Co	
Thunnus tonggol	AAS	µg g^{-1}	0.43±0.28	0.98±0.49	8.27±5.79						0.05±0.02			Yousuf and Ahmed 2010
			17.47±7.56	1.81±0.712	10.29±3.33						12.57±7.86			
Rastrelliger kanagurta	AAS	µg g^{-1}	4.53-12.71	5.89-12.74	1.94 -9.81						0.17-3.67			Yousuf and Ahmed 2011
Thunnus albacares	AAS	µg g^{-1}	12.64-44.50	13.36-2.78	1.68-9.82	0.21-0.80	0.02-0.75				2.10-9.98			Ahmed et al. 2012
Rastrelliger kanagurta	AAS	µg g^{-1}	9.41±3.14	18.92±13.12	8.21±3.37						6.15±4.44			Ahmed et al. 2014a
			2.96±1.22	56.17±24.23	2.03±2.23						1.42±1.20			
Euthynnus affinis	AAS	µg g^{-1}	16.33± 2.26		6.63± 1.65	0.54± 0.15	0.50 ±0.17							Ahmed et al. 2014b
			6.56± 1.06		2.36± 1.78	0.06± 0.05	0.14 ±0.12							
Coryphaena hippurus	AAS	µg g^{-1}	4.13		2.92		0.18					0.40		Ahmed and Benzer 2015
Fenneropenaeus penicillatus	AAS	µg g^{-1}	6.83-23.83		4.94-10.08	0.05-0.07	3.57-10.79							Kamal et al. 2015a
Lobster sp	AAS	µg g^{-1}	7.96-173.61		4.65-55.56									Kamal et al. 2015b
Thunnus tonggol	AAS, Aanalyst 700	µg g^{-1}	3.89±2.23	36.43±11.41	23.35±11.47	0.27±0.12	0.71±0.3			0.35±0.20	1.29±1.08	0.35±0.12		Ahmed et al. 2015a
Scomberomorus commerson	AAS	µg g^{-1}	3.17-9.43	23.71-44.40	2.78-6.83	0.14-0.57	0.19-0.68			0.14-0.51	1.30-2.20			Ahmed et al. 2015b
Rastrelliger kanagurta	AAS	mg kg^{-1}						0.01-0.09						Ahmed and Bat 2015
								0.042±0.023						
Macrophthalmus depressus	AAS	mg kg^{-1}	88.38	259.9	86.5	102.64	2.04			11.94		31.17	9.72	Siddiqui and Saher 2015
Pampus argenteus	AAS	mg kg^{-1}		4.952	0.189	0.569	0.041							Yasmeen et al. 2016
Pomadasys maculatus	AAS	mg/kg				0.54 ± 0.05	0.59 ± 0.05							Ahmed and Bat 2016
Acanthopagrus arabicus	AAS	mg/kg		20.16-63.52		0.1-0.52	0.23-0.88							Ahmed et al. 2016a
Rastrelliger kanagurta	AAS	µg g^{-1}				0.32±0.26	0.31±0.29			0.37±0.26				Ahmed et al. 2016b
Austruca sindensis	AAS	µg g^{-1}	71.86	1009.9	36.33	44.08	1.39			121.6		50.89	11.52	Saher and Siddiqui 2017
Drepane longimana	AAS Aanalyst 700	mg/kg	7	5.6	3.5	0.007	0.07				2-5			Ahmed and Bat 2017
Holothurians sp	AAS Aanalyst 700	µg g^{-1}	11-46	14-73	0.43-8.93	0.52-3.02	0.11-2.67				0.76-7.12			Ahmed et al. 2017b
Katsuwonus pelamis	AAS	µg g^{-1}	2±1	16±6	3±1						4±1			Ahmed et al. 2017c
			7±2	46±17	7±2						6±2			
Trichiurus lepturus	AAS	µg g^{-1}	20.34±8.49	7.72±47.84	2.23±1.16	0.20±0.16	0.42±0.19				0.57±0.36			Ahmed et al. 2018a
Lepturacanthus savala	AAS	µg g^{-1}	16.63±9.25	85.11±57.64	2.53±1.90	0.23±0.18	0.47±0.20				0.47±0.27			Ahmed et al. 2018a
Holothuria arenicola	AAS	mg/kg						0.018						Ahmed et al. 2018b
Holothuria pardalis	AAS	mg/kg						0.026						Ahmed et al. 2018b
Holothuria verrucosa	AAS	mg/kg						0.024						Ahmed et al. 2018b
Holothuria atra	AAS	mg/kg						0.036						Ahmed et al. 2018b
Otolithes ruber	AAS	µg g^{-1}	3.34±1.19	4.23±1.38	0.33±1.14	0.02±0.01	0.46±0.06				0.28±0.18			Baloch et al. 2018
Crustaceans	AAS	mg/kg	113.71		45.92	4.78	0.66			30.89		72.71	72.8	Saher and Kanwal 2018
Ohshimella ehrenbergii	AAS Aanalyst 700	mg/kg						0.0176						Ahmed et al. 2019
Stolus buccalis	AAS Aanalyst 700	mg/kg						0.0155						Ahmed et al. 2019

International limits											
WHO	40	100	30	2	0.5	-	-	-	-	-	WHO 1989
FAO	30	-	30	0.5-6	1	-	-	5	-	-	FAO 1983
USA	75	11	6	1	1	-	-	-	-	6	Cohen et al. 2001
European Community	-	-	-	0.2	0.05	-	-	-	-	-	EC 2005
England	50	-	20	2	0.2	-	-	-	-	-	MAFF 2000
EU lmits	-	-	10	0.1	0.1	-	-	-	-	-	EU 2001

Previous investigations have shown declined condition of aquatic environment on Karachi coast, due to heavy metals contamination which is one of the most critical environmental issues in Pakistan and worldwide (Shaheen et al. 2016). The main source of Cu and Zn metals in the present geographical location could be the effluents of Bin Qasim thermal power plants, seaport activities, industrial effluents of SITE (Sindh Industrial Trade Estate) through Layari River, and unloading of raw materials for Pakistan steel mill; which is further fractionated into water, seaweed and sediments. Industrial effluents coming through Malir River, sewage water and oil refinery situated in the coastal region are the other sources of Cu and Zn contamination (Kamal et al. 2015a; Shahid et al. 2016).

Major source of copper contamination in marine organisms is via food chain rather seawater. Thus increasing ambient pollution levels in water do not directly affects the marine life. Copper is considered highly toxic metal after mercury and silver for marine life because of the existence of a number of detoxifying and storage systems for Cu (Mitra et al. 2012).

Cadmium is mainly concerned pollutant because it is very much toxic metal to aquatic organisms. Cadmium is absorbed in excess by human being through seafood and tends to accumulate mainly in liver and kidneys (Kamal et al. 2015; Ahmed et al. 2018a). The main source of cadmium contamination along the coastal areas is electroplating and industrial waste because it is an important metal with industrial applications.

This review was carried out to provide information on heavy metal concentrations in fish and other aquatic organisms on Karachi coast, Pakistan. The findings of various investigators on the effect of heavy metals on fish and other aquatic organism in Karachi were as follow; Fe > Zn > Cu > Mn > Cd > Pb > Cr > Ni > Hg > As. Fish and other aquatic organisms are used as a bio-indicator to evaluate the health of aquatic ecosystems since heavy metals accumulate in food, that it would be useful to carry out in detailed, extensive observations to monitor this situation in the future in (Rivers and Karachi coast) especially around the industrial, agricultural, municipal and domestic and polluted areas and their impact on the environment.

ACKNOWLEDGEMENTS

The authors are thankful to the Director International Center for Chemical and Biological Sciences, University of Karachi, Pakistan for providing necessary laboratory facilities, and also our thanks extend to Sudan University of Science & Technology and Ministry of Higher Education & Scientific Research-Sudan. We also gratefully acknowledge the financial assistance from TWAS World Academy of Sciences, Trieste, Italy (TWAS-ICCBS Post-Doctoral Fellowship No. RF 3240305609) awarded to Dr. Ramzy A. Yousif.

REFERENCES

Abarshi MM, Dantala EO, Mada SB. 2017. Bioaccumulation of heavy metals in some tissues of croaker fish from oil spilled rivers of Niger Delta region, Nigeria. Asian Pac J Trop Biomed 7: 563-568.

Abu-Dieyeh MH, Usman K, Alduroobi H, Al-Ghouti M. 2018. Mercury toxicity: The importance of microbial diversity for improved environmental remediation. In: Donati ER (eds). Heavy Metals in the Environment. CRC Press, Boca Raton, Florida.

Afridi HI, Kazi TG, Kazi N, Kandhro GA, Baig JA, Jamali MK, Shah AQ. 2011. Interactions between cadmium and zinc in the biological samples of Pakistani smokers and nonsmokers cardiovascular disease patients. Biol Trace Elem Res 139: 257-268. DOI: 10.1007/s12011-009-8607-3

Agency for Toxic Substances and Disease Registry. 2000. Toxicological Profile for Arsenic. Department of Health and Human Services, Public Health Service, Atlanta, US.

Ahmed Q, Bat L, Mohammad Ali Q. 2012. Heavy metal levels in *Thunnus albacares* (Bonnaterre, 1788) from Karachi fish harbour, Pakistan. Pak J Mar Sci 21(1 and 2): 13-21.

Ahmed Q, Yousuf F, Sarfraz M, Abu Bakar NK, Balkhour MA, Ashraf MA. 2014a. Seasonal elemental variations of Fe, Mn, Cu and Zn and conservational management *Rastrelliger kanagurta* fish from Karachi fish harbour, Pakistan. J Food Agric Environ 12 (3&4): 405-414.

Ahmed Q, Yousuf F, Sarfraz M, Ali QM, Balkhourd M, Safie SZ, Ashraf MA. 2014b. *Euthynnus affinis* (little tuna): Fishery, bionomics, seasonal elemental variations, health risk assessment and conservational management. Front Life Sci 8: 71-96. DOI: 10.1080/21553769.2014.961617

Ahmed Q, Benzer S. 2015. Heavy metal concentration in dolphinfish (*Coryphaena* hippurus Linnaeus, 1758) from Karachi Harbour, Pakistan. IJFAS 2 (5): 26-29.

Ahmed Q, Bat L. 2015. Mercury (Hg) levels in Indian mackerel *Rastrelliger kanagurta* (Scombridae) from Karachi fish harbour and its risk assessment. J Fish Sci 9 (1): 356-360. DOI: 10.3153/jfscom.201444

Ahmed Q, Bat L, Yousuf F. 2015. Accumulation of heavy metals in tissues of long tail tuna from Karachi Fish Harbour, Pakistan. Aquat Sci Technol 3 (1): 103-115. DOI: 10.5296/ast.v3i1.6814

Ahmed Q, Bat L, Yousuf F, Ali QM, Nazim K. 2015b. Accumulation of heavy metals (Fe, Mn, Cu, Zn, Ni, Pb, Cd and Cr) in Tissues of narrow-barred *Spanish mackerel* (Family-Scombridae) fish marketed by Karachi Fish Harbor. Open Biol Sci J 1: 20-28. DOI: 10.2174/2352633501501010020

Ahmed Q, Bat L. 2016. Levels and health risk assessments of Cd and Pb in *Pomadasys maculatus* marketed by Karachi Fish Harbor, Pakistan. Ilmu Kelautan 21 (2): 53-58. DOI: 10.14710/ik.ijms.21.2.53-58

Ahmed Q, Bat L, Yousuf F, Arıcı E. 2016. Heavy metals in *Acanthopagrus arabicus* Iwatsuki, 2013 from Karachi Coasts, Pakistan and potential risk of human health. Int J Fish Aquat Stud 4 (1): 203-208

Ahmed Q, Benzer S, Yousuf F. 2016b. Distribution of heavy metals in different tissues of Indian mackerel from Karachi fish harbour, Karachi, Pakistan. Indian J Anim Res 50 (5): 759-763. DOI: 10.18805/ijar.11423

Ahmed Q, Bat L. 2017. Heavy metal levels in different sizes and tissues of *Drepane longimana* (Bloch & Schneider, 1801) from Arabian Sea. J Coast Life Med 5 (12): 505-509. DOI: 10.12980/jclm.5.2017J7-148

Ahmed Q, Ali QM, Bat L, Ghory FS. 2017a. Concentration of heavy metals in red, brown and green algae collected from the Karachi Coast of Pakistan. Pak J Mar Sci 26 (1&2): 57-65.

Ahmed Q, Ali QM, Bat L. 2017b. Assessment of heavy metals concentration in holothurians, sediments and water samples from coastal areas of Pakistan (Northern Arabian Sea). J Coast Life Med 5 (5): 191-201. DOI: 10.12980/jclm.5.2017J7-56

Ahmed Q, Bat L, Yousuf F. 2017c. Contamination of Cu, Zn, Fe and Mn in *Katsuwonus pelamis* (Linnaeus, 1758) from Karachi fish harbor and potential risks to human health. Int J Mar Sci 7 (9): 76-87. DOI: 10.5296/ast.v3i1.681410.5376/ijms.2017.07.0009

Ahmed Q, Benzer S, Ali QM. 2018a. Heavy metal concentration in Largehead Hairtail (*Trichiurus lepturus* Linneaus, 1758) and Savalai Hairtail (*Lepturacanthus savala* (Cuvier, 1829)) obtained from Karachi Fish Harbour, Pakistan. Bull Environ Contam Toxicol 101 (4): 467-472. DOI: 10.1007/s00128-018-2418-1

Ahmed Q, Bat L, Ali QM. 2018b. Analysis of mercury (Hg) in four Holothurians species (Phylum Echinodermata) from Karachi Coast Northern Arabian Sea. Aquat Res 1 (2): 55-63. DOI: 10.3153/AR18007

Ahmed Q, Bat L, Öztekin A, Mohammad Ali Q. 2018c. A review on studies of heavy metal determination in Mackerel and Tuna (Family-Scombridae) fishes. J Anatolian Env Anim Sci 3 (3): 104-120.

Ahmed Q, Bat L, Ali MQ. 2019. Determination of mercury (Hg) in two Sea Cucumber species *Ohshimella ehrenbergii* (Selenka, 1868) and *Stolus buccalis* (Stimpson, 1855) from The Karachi Coast. Pak J Mar Sci 28 (1): 55-62.

Akoto O, Bismark Eshun F, Darko G, Adei E. 2014. Concentrations and health risk assessments of heavy metals in fish from the Fosu Lagoon. Int J Environ Res 8 (2): 403-410.

Al-Busaidi M, Yesudhason P, Al-Mughairi S, Al-Rahbi WA, Al-Harthy K, Al-Mazrooei N. 2011. Chemosphere toxic metals in commercial marine fish in oman with reference to national and international standards. Chemosphere 85 (1): 67-73. DOI: 10.1016/j.chemosphere.2011.05.057

Ali M, Adnan M, Noman S, Baqueri S. 2014. Estimation of tract congestion cost-A case study of a major arterial in Karachi. Proc Eng 77: 37-44.

Ali AA, Elazein EM, Alian MA. 2011. Investigation of heavy metals pollution in water, sediment and fish at Red Sea-Jeddah Coast-KSA at two different locations. J Appl Environ Biol Sci 1: 630-637.

Ali N, Khan MU, Khoso SA. 2019. Identification of biological threats zones along the coastline of Karachi. Int J Environ Sci Tech 16 (7): 3557-3564. DOI: 10.1007/s13762-018-1884-7

Alinnor I, Obiji I. 2010. Assessment of trace metal composition in fish samples from Nworie River. Pak J Nutr 9 (1):81-85. DOI: 10.3923/pjn.2010.81.85

Ameh T, Sayes C.M. 2019. The potential exposure and hazards of copper nanoparticles: A review. Environ Toxicol Pharmacol 71: 103220. DOI: 10.1016/j.etap.2019.103220

Anderson J, Fitzgerald C. 2010. Iron: An Essential Nutrient Review. [Online]. From: http://www.ext.colostate.edu/pubs/foodnut/09356.html

Angelova M, Asenova S, Nedkova V, Koleva-Kolarova R. 2011. Copper in the human organism. Trakia J Sci 9 (1): 88-98.

Arulkumar A, Paramasivam S, Rajaram R. 2017. Toxic heavy metals in commercially important food fishes collected from Palk Bay, Southeastern India. Mar Pollut Bull 119 (1): 454-459. DOI: 10.1016/j.marpolbul.2017.03.045

Ashraf S, Ali Q, Zahir ZA, Ashraf S, Asghar HN. 2019. Phytoremediation: Environmentally sustainable way for reclamation of heavy metal polluted soils. Ecotoxicol Environ Saf 174: 714-727. DOI: 10.1016/j.ecoenv.2019.02.068

Atafar Z, Mesdaghinia A, Nouri J, Homaee M, Yunesian M, Ahmadimoghaddam M, Mahvi AH. 2010. Effect of fertilizer application on soil heavy metal concentration. Environ Monit Assess 160 (1-4): 83-89. DOI: 10.1007/s10661–008–0659–x

Ates A, Türkmen M, Tepe Y. 2015. Assessment of heavy metals in fourteen marine fish species of four Turkish seas. Indian J Mar Sci 44 (1): 49-55.

Attri S, Sharma N, Jahagirdar S, Thapa BR, Prasad R. 2006. Erythrocyte metabolism and antioxidant status of patients with Wilson disease with hemolytic anemia. Pediatr Res 59 (4): 593-597. DOI: 10.1203/01.pdr.0000203098.77573.39

Aziz I, Khan F. 2014. Distribution, ecology & ecophysiology of mangroves in Pakistan. In: Khan MA, Böer B, Öztürk M, Al Abdessalaam TZ, Clüsener-Godt M, Gul B. Sabkha Ecosystems Vol. IV: Cash Crop Halophyte & Biodiversity Conservation. Springer, Berlin, Germany.

Baki MA, Hossain MM, Akter J, Quraishi SB, Shojib MFH, Ullah AKMA, Khan MF. 2018. Concentration of heavy metals in seafood (fishes, shrimp, lobster and crabs) and human health assessment in Saint Martin Island, Bangladesh. Ecotoxicol Environ Saf 159: 153-163.

Balfour S, Badrie N, Yen IC, Chatergoon L. 2012. Seasonal influence and heavy metal analysis in marine shrimp (*Penaeus* spp.) sold in Trinidad, Westindies. J Food Res 1 (1): 193-199. DOI: 10.5539/jfr.v1n1p193

Baloch AB, Bat L, Ali QM, Ahmed Q. 2018. Concentration of heavy metals (Fe, Zn, Mn, Cu, Pb and Cd) in *Otolithes ruber* (Bloch & Schneider, 1801) from damb fish landing centre at Sonmiani, Makran Coast, Pakistan. Pak J Mar Sci 27 (2): 111-119.

Barceloux DG. 1999. Nickel. Clin Toxicol 37: 239. DOI: 10.1081/CLT-100102423

Bourliva A, Kantiranis N, Papadopoulou L, Aidona E, Christophoridis C, Kollias P, Evgenakis M, Fytianos K. 2018. Seasonal and spatial variations of magnetic susceptibility and potentially toxic elements (PTEs) in road dust of Thessaloniki city, Greece: A one-year monitoring period. Sci Total Environ 639: 417-427. DOI: 10.1016/j.scitotenv.2018.05.170

Bryan G, Langston W. 1992. Bioavailability, accumulation and effects of heavy metals in sediments with special reference to United Kingdom estuaries: A review. Environ Pollut 76 (2): 89-131. DOI: 10.1016/0269-7491(92)90099-V

Burger J, Stern AH, Dixon C, Jeitner C, Shukla S, Burke S, Gochfeld M. 2004. Fish availability in supermarkets and fish markets in New Jersey. Sci Total Environ 333 (1-3): 89-97. DOI: 10.1016/j.scitotenv.2004.05.016

Burger J, Gochfeld M. 2011. Mercury and selenium levels in 19 species of saltwater fish from New Jersey as a function of species, size, and season. Sci Total Environ 409: 1418-1429. DOI: 10.1016/j.scitotenv.2010.12.034

Castro-gonzález MI, Méndez-armenta M. 2008. Heavy metals: Implications associated to fish consumption. Environ Toxicol Pharmacol 26: 263-271. DOI: 10.1016/j.etap.2008.06.001

CDGK. 2012. City District Government of Karachi. www.karachicity.gov.pk.

Chandio AA, Jiang Y, Rehman A. 2018. Credit margin of investment in the agricultural sector and credit fungibility: The case of smallholders of district Shikarpur, Sindh, Pakistan. Financ Innov 4: 27. DOI: 10.1186/s40854-018-0109-x

Changfeng L, Kehai Z, Wenqiang Q, Changjiu T, Miao Q, Xiaoming Y, Wenbing H. 2019. A review on heavy metals contamination in soil: Effects, sources, and remediation techniques. Soil Sediment Contam: An Int J 28: 380-394. DOI: 10.1080/15320383.2019.1592108

Chary NS, Kamala CT, Raj DSS. 2008. Assessing risk of heavy metals from consuming food grown on sewage irrigated soils and food chain transfer. Ecotoxicol Environ Saf 69 (3): 513-524. DOI: 10.1016/j.ecoenv.2007.04.013

Chasapis CT, Loutsidou A, Spiliopoulou CA, Stefanidou ME. 2012. Zinc and human health: An update. Arch Toxicol 86: 521-534. DOI: 10.1007/s00204-011-0775-1

Chaudhary MZ, Ahmad N, Mashiatullah A, Ahmad N, Ghaffar A. 2013. Geochemical assessment of metal concentrations in sediment core of Korangi Creek along Karachi Coast. Pak Environ Monit Assess 185: 6677-6691. DOI: 10.1007/s10661-012-3056-4

Chen ZF, Zhao Y, Fan LD, Xing LT, Yang YJ. 2015. Cadmium (Cd) localization in tissues of cotton (*Gossypium hirsutum* L.) and its phytoremediation potential for Cd-contaminated soils. Bull Environ Contam Toxicol 95: 784-789. DOI: 10.1007/s00128–015–1662–x

Chen J, Wang W, Liu H, Ren L. 2011. Determination of road dust loading and chemical characteristics using resuspension. Environ Monit Assess 184: 1693-1709.

Cid BP, Boia C, Pombo L, Rebelo E. 2001. Determination of trace metals in fish species of the Ria De Aveiro (Portugal) by electrothermal atomic absorption spectrometry. Food Chem 75 (1): 93-100. DOI: 10.1016/S0308-8146(01)00184-4

Cohen T, Que Hee SS, Ambrose RF. 2001. Trace metals in fish and invertebrates of three California coastal Wetlands. Mar Pollut Bull 42: 224-232. DOI: 10.1016/S0025-326X(00)00146-6

Copat C, Arena G, Fiore M, Ledda C, Fallico R, Sciacca S, Ferrante M. 2013. Heavy metals concentrations in fish and shellfish from eastern Mediterranean Sea: Consumption advisories. Food Chem Toxicol 53: 33-37.

Daellenbach K, Stefenelli G, Bozzetti C, Vlachou A, Fermo P, Gonzalez R, Piazzalunga A, Colombi C, Canonaco F, Hueglin C. 2017. Long-term chemical analysis and organic aerosol source apportionment at 9 sites in Central Europe: Source identification and uncertainty assessment. Atmos Chem Phys Discus 17: 13265-13282.
DOI: 10.5194/acp-17-13265-2017.

Dehghani S, Moore F, Keshavarzi B, Hale B. 2017. Health risk implications of potentially toxic metals in street dust and surface soil of Tehran, Iran. Ecotoxicol Environ Saf 136: 92-103.

Devault DA, Beilvert B, Winterton P. 2017. Shipbreaking or scuttling? A review of environmental, economic and forensic issues for decision support. Environ Sci Pollut Res 24: 25741-25774.

Devesa V, Velez D, Montoro R. 2008. Effect of thermal treatments on arsenic species contents in food. Food Chem Toxicol 46: 1-8. DOI: 10.1016/j.fct.2007.08.021

Elahi N, Ahmed Q, Bat L, Yousuf F. 2015. Physiochemical parameters and seasonal variation of coastal water from Balochistan Coast, Pakistan. J Coast Life Med 3 (3): 199-203. DOI: 10.12980/JCLM.3.201514J94

Emami-Khansari F, Ghazi-Khansari M, Abdollahi M. 2005. Heavy Metals content of canned tuna fish. Food Chem 93: 293-296. DOI: 10.1016/j.foodchem.2004.09.025

Erdman JW, Macdonald IA, Zeisel SH. 2012. Present Knowledge in Nutrition. 10th edition. A John Wiley & Sons, Ltd., Publication, New Jersey.

European Community EC. 2005. Commission Regulation No 78/2005 (pp. L16/43–L16/45). Official Journal of the European Union. 20.1.2005.

European Union EU. 2001. Commission Regulation as regards heavy metals, Directive, 2001/22/EC, No: 466.

European Union EU. 2006. Setting Maximum Levels for Certain Contaminants in Foodstuffs, Commission Regulation (EC) No 1881/2006; OJ L 364, 20.12.2006. 5.

Failla ML, Johnson MA, Prohaska JR. 2001. Copper. In: Present Knowledge in Nutrition. Life Sciences Institute Press, Washington D.C.

FAO. 1983. Compilation of legal limits for hazardous substances in fish and fishery products. FAO Fish Circ 764: 5-100.

Fernandes C, Fontaínhas-Fernandes A, Cabral D, Salgado MA. 2008. Heavy metals in water, sediment and tissues of liza saliens from Esmoriz-Paramos Lagoon, Portugal. Environ Monit Assess 136 (1-3): 267-275. DOI: 10.1007/s10661-007-9682-6

Forti E, Salovaara S, Cetin Y, Bulgheroni A, Tessadri R, Jennings P, Prieto P. 2011. In vitro evaluation of the toxicity induced by nickel soluble and particulate forms in human airway epithelial cells. Toxicol in Vitro 25 (2): 454-461. DOI: 10.1016/j.tiv.2010.11.013

García-lestón J, Mendez J, Pásaro E, Laffon B. 2010. Author's personal copy genotoxic effects of lead: An updated review. Environ Int 36: 623-636. DOI: 10.1016/j.envint.2010.04.011

Gokoglu N, Yerlikaya P, Gokoglu M. 2008. Mini-Review: Trace elements in edible tissues of three shrimp species (*Penaeus semisulcatus, Parapenaeus longirostris*, and *Paleomon serratus*). J Sci Food Agric 88: 175-178. DOI: 10.1002/jsfa.3086

Goldhaber SB. 2003. Trace element risk assessment: Essentiality vs toxicity. Regul Toxicol Pharmacol 38: 232-242. DOI: 10.1016/S02732300(02)00020-X

Gope M, Masto R, George J, Hoque R, Balachandran S. 2017. Bioavailability and health risk of some potentially toxic elements (Cd, Cu, Pb and Zn) in street dust of Asansol, India. Ecotoxicol Environ Saf 138: 231-241.

Goretti E, Pallottini M, Ricciarini MI, Selvaggi R, Cappelletti D. 2016. Heavy metals bioaccumulation in selected tissues of red swamp crayfish: An easy tool for monitoring environmental contamination levels. Sci Total Environ 559: 339-346.

Gu Y, Kodama H, Sato E, Mochizuki D, Yanagawa Y, Takayanagi M, Lee C. 2002. Prenatal diagnosis of menkes disease by genetic analysis and copper measurement. Brain Develop 24: 715-718. DOI: 10.1016/S0387-7604(02)00093-1

Hao Y, Chen L, Zhang X, Zhang D, Zhang X, Yu Y, Fu J. 2013. Trace elements in fish from Taihu Lake, China: Levels, associated risks, and trophic transfer. Ecotoxicol Environ Saf 90: 89-97. DOI: 10.1016/j.ecoenv.2012.12.012

Hasan AB, Kabir S, Reza AS, Zaman MN, Ahsan A, Rashid M. 2013. Enrichment factor and geo-accumulation index of trace metals in sediments of the ship breaking area of Sitakund Upazilla (Bhatiary-Kumira), Chittagong, Bangladesh. J Geo Expl 125: 130-137.

Hossain M, Islam M. 2019. Ship breaking activities and its impact on the coastal zone of Chittagong. http://ypsa.org/publications/Impact.pdf

Hwang HM, Fiala M, Park D, Wade T. 2016. Review of pollutants in urban road dust and stormwater runo Part 1. Heavy metals released from vehicles. Int J Urban Sci 20: 334-360.

Iftikhar M, Alam K, Sorooshian A, Syed WA, Bibi S, Bibi H. 2018. Contrasting aerosol optical and radiative properties between dust and urban haze episodes in megacities of Pakistan. Atmos Environ 173: 157-172.

Ikem A, Egilla J. 2008. Trace Element Content of Fish Feed and Bluegill Sunfish (*Lepomis macrochirus*) from aquaculture and wild source in Missouri. Food Chem 110 (2): 301-309. DOI: 10.1016/j.foodchem.2008.02.003

Jibiri N, Adewuyi G. 2008. Radionuclide contents and physicochemical characterization of solid waste and effluent samples of some selected industries in the city of Lagos, Nigeria. Radioprot 43 (2): 203-212. DOI: 10.1051/radiopro:2007053

Jilani S. 2015. Metal pollution and its distribution pattern in the road dust of Karachi. J Biodivers Environ Sci 6: 215-224.

Kamal T, Tanoli MAK, Mumtaz M, Ali N, Ayub S. 2015a. Bioconcentration potential studies of heavy metals in *Fenneropenaeus penicillatus* (Jaira or Red Tail Shrimp) along the Littoral States of Karachi City. J Basic Appl Sci 11: 611-618. DOI: 10.6000/1927-5129.2015.11.82

Kamal T, Tanoli MAK, Mumtaz M, Ali N, Ayub S. 2015b. Analysis of essential elements in commercially important lobster species collected from coastal areas of Karachi City, Pakistan. J Pharm Nutr Sci 5: 103-113. DOI: 10.6000/1927-5951.2015.05.02.2

Karim Z, Qureshi BA, Mumtaz M. 2015. Geochemical baseline determination and pollution assessment of heavy metals in urban soils of Karachi, Pakistan. Ecol Indic 48: 358-364.

Kastury F, Smith E, Juhasz AL. 2017. A critical review of approaches and limitations of inhalation bioavailability and bioaccessibility of metal(loid)s from ambient particulate matter or dust. Sci Total Environ 574: 1054-1074.

Kastury F, Smith E, Doelsch E, Lombi E, Donnelley M, Cmielewski P, Parsons D, Scheckel K, Paterson D, de Jonge M. 2019. In vitro, in vivo, and spectroscopic assessment of lead exposure reduction via ingestion and inhalation pathways using phosphate and iron amendments. Environ Sci Technol 53 (17): 10329-10341. DOI: 10.1021/acs.est.9b02448

Kazi TG, Wadhwa SK, Afridi HI, Kazi N, Kandhro GA, Baig JA, Arain MB. 2010. Interaction of Cadmium and Zinc in biological samples of smokers and chewing tobacco female mouth cancer patients. J Hazard Mater 176: 985-991. DOI: 10.1016/j.jhazmat.2009.11.139

Kazmi J, Zubair S. 2014. Estimation of vehicle damage cost involved in road trac accidents in Karachi, Pakistan: A geospatial perspective. Proc Eng 77: 70-78.

Kennedy CJ. 2011. The toxicology of metals in fishes. In: Farrell AP (eds.) Encyclopedia of Fish Physiology: From Genome to Environment. Academic Press, San Diego, Calif, USA.

Khan R, Strand M. 2018. Road dust and its effect on human health: A literature review. Epidemiol Health 40: e2018013. DOI: 10.4178/epih.e2018013.

Khan I, Ghani A, Rehman AU, Awan SA, Noreen A, Khalid I. 2016. Comparative analysis of heavy metal profile of *Brassica campestris* (L.) and *Raphanus sativus* (L.) irrigated with municipal wastewater of Sargodha city. J Clin Toxicol 6: 1-4. DOI: 10.4172/2161-0495.1000307

Khan MN, Wasim AA, Sarwar A, Rasheed, MF. 2011. Assessment of heavy metal toxicants in the roadside soil along the N-5, National Highway, Pakistan. Environ Monit Assess 182: 587-595.

Khattak MI, Khattak MI, Mohibullah M. 2012. Study of heavy metal pollution in mangrove sediments reference to marine environment along the coastal areas of Pakistan. Pak J Bot 44 (1): 373-378.

Khwaja H, Fatmi Z, Malashock D, Aminov Z, Kazi A, Siddique A, Zeb J, Carpenter D. 2012. Effect of air pollution on daily morbidity in Karachi, Pakistan. J Local Glob Health Sci 3: 1-13.

King JC. 2000. Determinants of maternal zinc status during pregnancy. Am J Clin Nutr 71: 1334-1343. DOI: 10.1093/ajcn/71.5.1334s

Koyashiki GAK, Paoliello MMB, Tchounwou PB. 2010. Lead levels in human milk and children's health risk: A systematic review. Rev Environ Health 25 (3): 243-253.

Krishna P, Jyothirmayi V, Madhusudhana Rao K. 2014. Human health risk assessment of heavy metal accumulation through fish consumption, from Machilipatnam Coast, Andhra Pradesh, India. Int Res J Public Environ Health 1 (5): 121-125.

Lambert M, Leven B, Green R. 2000. New methods of cleaning up heavy metal in soils and water. Environmental Science and Technology Briefs for Citizens, Kansas State University, Manhattan, New York.

Langston W. 1990. Toxic effects of metals and incidence of marine ecosystems. In: Furness RW, Rainbow PS (eds.) Heavy Metals in the Marine Environment. CRC Press, New York.

Liu P, Wang C, Song X, Wu Y. 2010. Dietary intake of lead and Cadmium by children and adults- result calculated from dietary recall and available lead/cadmium level in food in comparison to result from food duplicate diet method. Int J Hyg Environ Health 213: 450-457. DOI: 10.1016/j.ijheh.2010.07.002

Liu E, Wang X, Liu H, Liang M, Zhu, Y, Li Z. 2019. Chemical speciation, pollution and ecological risk of toxic metals in readily washed o road dust in a megacity (Nanjing), China. Ecotoxicol Environ Saf 173: 381-392.

Lone MI, He ZL, Stoffella PJ, Yang XE. 2008. Phytoremediation of heavy metal polluted soils and water: Progress and perspectives. J Zhejiang Univ Sci B 9: 210-220. DOI: 10.1631/jzus.B0710633

MAFF (Ministry of Agriculture, Fisheries and Food). 2000. Monitoring and surveillance of non-radioactive contaminants in the aquatic environment and activities regulating the disposal of wastes at sea, 1997. In: Aquatic Environment Monitoring Report No. 52. Center for Environment, Fisheries and Aquaculture Science, Lowestoft, UK.

Men C, Liu R, Wang Q, Guo L, Miao Y, Shen Z. 2019. Uncertainty analysis in source apportionment of heavy metals in road dust based on positive matrix factorization model and geographic information system. Sci Total Environ 652: 27-39.

Messner B, Knoflach M, Seubert A, Ritsch A, Pfaller K, Henderson B, Bernhard D. 2009. Cadmium is a novel and independent risk factor for early atherosclerosis mechanisms and in vivo relevance. Arterioscler Thromb Vasc Biol 29: 1392-1398. DOI: 10.1161/ATVBAHA.109.190082

Mitra A, Barua P, Zaman S, Banerjee K. 2012. Analysis of trace metals in commercially important crustaceans collected from UNESCO protected world heritage site of Indian Sundarbans. Turkish J Fish Aquat Sci 12: 53-66. DOI: 10.4194/1303-2712-v12_1_07

Mohmad J, Eqani S, Fasola M, Alamdar A, Mustafa I, Ali N, Liangpo L, Peng S, Shen H. 2015. Human exposure to toxic metals via contaminated dust: Bio-accumulation trends and their potential risk estimation. Chemosphere 132: 142-151.

Mujeeb A, Aziza I, Ahmed MZ, Sofia KA, Shafiq S. 2020. Comparative assessment of heavy metal accumulation and bio-indication in coastal dune halophytes. Ecotoxicol Environ Saf 195: 110486. DOI: 10.1016/j.ecoenv.2020.110486

Mukhtar I, Hannan A. 2012. Constrains on mangrove forests and conservation projects in Pakistan. J Coast Conserv 16: 51-62. DOI: 10.1007/s11852-011-0168-x

Muradoglu F, Gundogdu M, Ercisli S, Encu T, Balta F, Jaafar HZE, Zia-Ul-Haq M. 2015. Cadmium toxicity affects chlorophyll a and b content, antioxidant enzyme activities and mineral nutrient accumulation in strawberry. Biol Res 48: 1-7. DOI: 10.1186/S40659–015–0001–3

Narsimha A, Wang H. 2018. Distribution, contamination, and health risk assessment of heavy metals in surface soils from northern Telangana, India. Arab J Geosci 11 (21): 684. DOI: 10.1007/s12517-018-4028-y

Oikari A, Nakari T. 1982. Kraft pulp mill effluent components cause liver dysfunction in trout. Bull Environ Contam Toxicol 28 (3): 266-270. DOI: 10.1007/BF01608505

Oikari A, Soivio A. 1976. Physiological Condition of Fish Exposed to Water Containing Pulp and Paper Industry Wastes and Sewage. FAO, Rome, Italy.

Ogunlade MO, Agbeniyi SO. 2011. Impact of pesticides use on heavy metals pollution in cocoa soils of Cross-River State, Nigeria. Afr J Agri Res 6: 3725-3728.

Pal J, Shukla BN, Maurya AK, Verma HO, Pandey G, Amitha A. 2018. A review on role of fish in human nutrition with special emphasis to essential fatty acid. Int J Fish Aquat Stud 6 (2): 427-430.

Palaniappan PR, Karthikeyan S. 2009. Bioaccumulation and depuration of chromium in the selected organs and whole body tissues of freshwater fish cirrhinus mrigala individually and in binary solutions with nickel. J Environ Sci 21 (2): 229-236. DOI: 10.1016/S1001-0742(08)62256-1

Perl DP, Olanow CW. 2007. The neuropathology of manganese-induced parkinsonism. J Neuropathol Exp Neurol 66: 675-682. DOI: 10.1097/nen.0b013e31812503cf

Rahman M, Islam M. 2010. Adsorption of Cd (II) ions from synthetic waste water using maple sawdust adsorption of Cd (II) ions from synthetic waste water using maple sawdust. Energy Sour Part A 32: 222-231. DOI: 10.1080/15567030802459297

Rahman MS, Molla AH, Saha N, Rahman A. 2012. Study on heavy metals levels and its risk assessment in some edible fishes from Bangshi River, Savar, Dhaka, Bangladesh. Food Chem 134 (4): 1847-1854. DOI: 10.1016/j.foodchem.2012.03.099

Raja P, Veerasingam S, Suresh G, Marichamy G, Venkatachalapathy R. 2009. Heavy metals concentration in four commercially valuable marine edible fish species from Parangipettai Coast, South East Coast of India. Int J Anim Vet Adv 1 (1): 10-14.

Rajeshkumar S, Li X. 2018. Bioaccumulation of heavy metals in fish species from the Meiliang Bay, Taihu Lake, China. Toxicol Rep 5: 288-295. DOI: 10.1016/j.toxrep.2018.01.007

Rashed M. 2001. Monitoring of environmental heavy metals in fish from Nasser Lake. Environ Int 27 (1): 27-33. DOI: 10.1016/S0160-4120(01)00050-2

Roussel A, Andriollo-sanchez M, Ferry M, Bryden NA, Anderson RA. 2007. Food chromium content, dietary chromium intake and related biological variables in french free-living elderly. British J Nutr 98: 326-331. DOI: 10.1017/S000711450770168X

Sadiq R, Husain T, Bose N, Veitch B. 2003. Distribution of heavy metals in sediment pore water due to offshore discharges: An ecological risk assessment. Environ Model Softw 18: 451-461. DOI: 10.1016/S1364-8152(03)00010-0

Saeedi M, Li LY, Salmanzadeh M. 2012. Heavy metals and polycyclic aromatic hydrocarbons: Pollution and ecological risk assessment in street dust of Tehran. J Hazard Mater 227: 9-17.

Saha N, Zaman MR. 2013. Evaluation of possible health risks of heavy metals by consumption of foodstuffs available in the central market of Rajshahi City, Bangladesh. Environ Monit Assess 185: 3867-3878. DOI: 10.1007/s10661-012-2835-2

Saher NU, Siddiqui AS. 2017. Evaluation of heavy metals contamination in mangrove sediments and their allied fiddler crab species (*Austruca sindensis* (Alcock, 1900) from Hawks Bay, Karachi, Pakistan. Int J Biol Biotech 14 (3): 411-417.

Saher NU, Kanwal N. 2018. Some biomonitoring studies of heavy metals in commercial species of crustacean along Karachi coast, Pakistan. Int J Biol Biotech 15 (2): 269-275.

Salnikow K, Kasprzak KS. 2005. Ascorbate depletion: A critical step in nickel carcinogenesis? Environ Health Perspect 113 (5): 577-584. DOI: 10.1289/ehp.7605

Sarkar B. 1999. Treatment of Wilson and Menkes diseases. Chem Rev 99: 2535-2544.

Sary AA, Mohammadi M. 2011. Human health risk assessment of heavy metals in fish from freshwater. Res J Fish Hydrobiol 6 (4): 404-411.

Scherz H, Kirchhoff E. 2006. Trace elements in foods: Zinc contents of raw foods-a comparison of data originating from different geographical regions of the world. J Food Compos Anal 19: 420-433. DOI: 10.1016/j.jfca.2005.10.004

Schnaas L, Rothenberg SJ, Flores M, Martinez S, Hernandez C, Osorio E, Perroni E. 2006. Reduced intellectual development in children with prenatal lead exposure. Environ Health Perspect 114 (5): 791-797. DOI: 10.1289/ehp.8552

Sfakianakis DG, Renieri E, Kentouri M, Tsatsakis AM. 2015. Effect of heavy metals on fish larvae deformities: A review. Environ Res 137: 246-255. DOI: 10.1016/j.envres.2014.12.014

Shafiq M, Iqbal MZ, Arayne M, Athar M. 2012. Biomonitoring of heavy metal contamination in *Pongamia Pinnata* and *Peltophorum Pterocarpum* growing in the polluted environment of Karachi. J Appl Bot Food Qual 85: 120-125.

Shahab A, Qi S, Rashid A, Hasan F, Sohail MT. 2016. Evaluation of water quality for drinking and agricultural suitability in the lower Indus plain in Pakistan. Pol J Environ Stud 25: 2563-2574.

Shaheen A, Baig HS, Kazmi SU. 2016. Microbial flora isolated from polluted and non-polluted coastal waters of Karachi. Pak J Bot 48: 1703-1708.

Shahid I, Kistler M, Mukhtar A, Ghauri BM, Ramirez-Santa Cruz C, Bauer H, Puxbaum H. 2016. Chemical characterization and mass closure of PM10 and PM2.5 at an urban site in Karachi-Pakistan. Atmos Environ 128: 114-123.

Siddiqui AS, Saher NU. 2015. Heavy metals distribution in sediments and their transfer rate to benthic fauna in mangrove area near hawks bay karachi, Pakistan. Pak J Mar Sci 24 (1&2): 9-17.

Sivaperumal P, Sankar TV, Nair PGV. 2007. Food chemistry heavy metal concentrations in fish, shellfish and fish products from internal markets of India vis-a-vis international standards. Food Chem 102: 612-620. DOI: 10.1016/j.foodchem.2006.05.041

Song Y, Elias V, Loban A, Scrimgeour AG, Ho E. 2010. Marginal zinc deficiency increases oxidative DNA damage in the prostate after chronic exercise. Free Radic Biol Med 48: 82-88. DOI: 10.1016/j.freeradbiomed.2009.10.030

Stern BR, Solioz M, Krewski D, Aggett P, Aw T-C, Baker S, Starr T. 2007. Copper and human health: Biochemistry, genetics, and strategies for modeling dose-response relationships. J Toxicol Environ Health 10: 157-222. DOI: 10.1080/10937400600755911

Stipanuk MH, Caudill MA. 2012. Biochemical, Physiological, and Molecular Aspects of Human Nutrition. 3rd Edition. Elsevier Saunders Publishing, Philadelphia.

Sulieman HMA, Suliman EM. 2019. Appraisal of heavy metal levels in some marine organisms gathered from the Vellar and Uppanar estuaries Southeast Coast of Indian Ocean. J Taibah Univ Sci 13 (1): 338-343. DOI: 10.1080/16583655.2019.1576276

Sun WH, Jiang YX, Li X. 2013. Research of the evaluation on heavy-metal pollution in rice by sewage irrigation. Appl Mech Mater 295-298: 1594-1599.2 DOI: 10.4028/www.scientific.net/AMM.295–298.1594

Thomas LDK, Hodgson S, Nieuwenhuijsen M, Jarup L. 2009. Early kidney damage in a population exposed to cadmium and other heavy metals. Environ Health Perspec 117 (2): 181-184. DOI: 10.1289/ehp.11641

Tian S, Liang T, Kexin L. 2019. Fine road dust contamination in a mining area presents a likely air pollution hotspot and threat to human health. Environ Int 128: 201-209.

Tulasi G, Rao KJ. 2014. Essentiality of chromium for human health and dietary nutrition. J Entomol Zool Stud 2 (1): 107-108.

Tüzen M. 2003. Determination of heavy metals in fish samples of the middle Black Sea (Turkey) by graphite furnace atomic absorption spectrometry. Food Chem 80 (1): 119-123. DOI: 10.1016/S0308-8146(02)00264-9

U.S. Department of Health and Human Services. 2004. Toxicological Profile for Copper (Public Hea., pp. 1–314). Agency for Toxic Substances and Disease Registry.

Ullah AKMA, Maksud MA, Khan SR, Lutfa LN, Quraishi SB. 2017. Dietary intake of heavy metals from eight highly consumed species of cultured fish and possible human health risk implications in Bangladesh. Toxicol Rep 4: 574-579.

Umar A, Umar R, Ahmad MS. 2001. Hydrogeological and hydrochemical frame works of regional aquter system in Kali-Ganga Sub-Basin. India Envir Geol 40 (4-5): 602-611. DOI: 10.1007/s002540000215

Uriu-adams JY, Keen CL. 2010. Zinc and reproduction: Effects of zinc deficiency on prenatal and early postnatal development. Birth Defects Res (Part B) 89: 313-325. DOI: 10.1002/bdrb.20264

Uysal K, Emre Y, Köse E. 2008. The determination of heavy metal accumulation ratios in muscle, skin and gills of some migratory fish species by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) in Beymelek Lagoon (Antalya/Turkey). Microchem J 90 (1): 67-70. DOI: 10.1016/j.microc.2008.03.005

Van Hassel JH, Ney JJ, Garling DL. 1980. Heavy metals in a stream ecosystem at sites near highways. Trans Am Fish Soc 109: 636-643.

Vettori MV, Alinovi R, Belletti S, Goldoni M, Franchini I, Multi A. 2003. In vitro model for the evaluation of the neurotoxicity of methyl mercury. Curr State Knowl Med Lav 94 (2): 183-191.

World Health Organization (WHO). 1989. Heavy Metals Environmental Aspects. Environmental Health Criteria. No. 85, Geneva.

Xue Z, Liu S, Liu Y, Yan YL. 2012. Health risk assessment of heavy metals for edible parts of vegetables grown in sewage-irrigated soils in suburbs of Baoding City, China. Environ Monit Assess 184 (6): 3503-3513. DOI: 10.1007/s10661-011-2204-6

Yasmeen K, Mirza AM, Khan NA, Kausar N, Attaur R, Hanif M. 2016. Trace metals health risk appraisal in fish species of Arabian Sea. SpringerPlus 5: 859. DOI: 10.1186/s40064-016-2436-6

Yi YJ, Zhang S.H. 2012. The relationships between fish heavy metal concentrations and fish size in the upper and middle reach of Yangtze River. The 18th Biennial Conference of International Society for Ecological Modelling. Proc Environ Sci 13: 1699-1707.

You SH, Wang SL, Pan WH, Chan WC, Fan AM, Lin P. 2018. Risk assessment of methylmercury based on internal exposure and fish and seafood consumption estimates in Taiwanese children. Int J Hyg Environ Health 221: 697-703.

Yousif RA, Masyamsir, Dhahiyat, Sunarto, Zahidah. 2016. Assessment the levels of heavy metals and water quality in Cikuda River, Indonesia. Glob J Bio-Sci Biotechnol 5 (2): 240-244.

Yousuf F, Ahmed Q. 2010. Heavy metals (Cu, Fe, Mn, Zn) content in *Thunnus tonggol* (Family-Scombridae) from Karachi coast. Karachi Univ J Sci 38: 5-9.

Yousuf F, Ahmed Q. 2011. Concentration of heavy metals in liver and muscles of fish, Rastrelliger kanagurta, from the coast of Karachi, Pakistan. Int J Biol Biotechnol 8: 543-550.

Zheng G, Tian L, Liang Y, Broberg K, Lei L, Guo W, Nilsson J, Bergdahl IA, Skerfving S, Jin T. 2011. δ-Aminolevulinic acid Dehydratase genotype predicts toxic effects of lead on workers' peripheral nervous system. Neurotoxicol 32: 374-382. DOI: 10.1016/j.neuro.2011.03.006

NUSANTARA BIOSCIENCE
Vol. 13, No. 1, pp. 85-90
May 2021

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n130112

Faunistic study on scorpions and their health impact in Bashagard County, Hormozgan Province, Southern Iran

MEHRAN SHAHI[1], SEYED AGHIL JABERHASHEMI[2], AHMAD ALI HANAFI-BOJD[3], MORTEZA AKBARI[4], JAVAD RAFINEJAD[3,♥]

[1]Department of Medical Entomology and Vector Control, School of Public Health and Infectious & Tropical Diseases Research Center, Hormozgan Health Institute, Hormozgan University of Medical Sciences, Bandar Abbas, Iran.
[2]Department of Diseases Control, Hormozgan University of Medical Sciences, Bashagard, Iran
[3]Department of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Sciences. Tehran, Iran.
[♥]email jrafinejad@yahoo.com
[4]Department of Public Health, School of Health, Ilam University of Medical Sciences. Ilam, Iran

Manuscript received: 14 February 2021. Revision accepted: 12 April 2021.

Abstract. *Shahi M, Jaberhashemi SA, Hanafi-Bojd AA, Akbari M, Rafinejad J. 2021. Faunistic study on scorpions and their health impact in Bashagard County, Hormozgan Province, Southern Iran. Nusantara Bioscience 13: 85-90.* Scorpion sting is a major public health problem in tropical and subtropical countries, endangering thousands of lives annually. About 2300 scorpion sting cases including several deaths are reported from Hormozgan Province annually. This study aimed to determine the fauna of scorpions and epidemiological aspects of scorpion sting in one of the high-risk areas in this province. Scorpions were collected by diurnal searching and night catch using UV light during 2015-2016. Clinical and demographic data of scorpion sting were obtained from Hormozgan Health Center using the checklist of the Center for Disease Control (CDC) archive during the study period. Data analysis was performed using SPSS 21 software**.** A total of 382 scorpions comprising of 9 species belonging to Buthidae (76%) and Hemiscorpiidae (24%) families were collected and identified as *Mesobuthus phillipsi*, *Androctonus crassicauda*, *Hottentotta sistanensis*, *Compsobuthus persicus*, *Hemiscorpius acanthocercus*, *Orthochirus farzanpayi*, *H. acanthocercus*, and *Odontobuthos sp*. The most abundant species in the study area was *M. phillipsi*. During 2015-2016, a total of 1221 scorpion sting cases including four deaths were recorded in Bashagard County, while most of the cases occurring during summer. The climate of the eastern regions of Hormozgan Province provides a suitable habitat for one of the deadliest genera of scorpions, i.e. *Hemiscorpius*. This genus is the main cause of death due to scorpion sting in these regions. It is thus necessary to design appropriate programs for the prevention and treatment of scorpion sting, including health education programs for both community members and healthcare personnel.

Keywords: Faunistic, scorpion sting, *Hemiscorpius*, health, Iran

INTRODUCTION

Scorpion sting is a major public health problem in tropical and subtropical countries, which endangers thousands of lives annually. Factors such as geographic location, lifestyle, socioeconomic status, housing, health care, and the type of scorpion species native to each geographical area have variable effects on the incidence of a scorpion sting (Kassiri et al. 2012). Most of the deadly scorpions are found in North Africa, the Middle East (*Androctonus*, *Buthus*, *Hottentotta*, and *Leiurus*), India (*Mesobuthus*), America (*Tityus*), and Mexico (*Centruroides*) (Prendini and Wheeler 2005). Species diversity and distribution of scorpions are affected by the climatic conditions of each geographical area. Information on the distribution of scorpion species in some areas of Iran is very limited.

Most of the cases of death due to scorpion stings are reported from the southern and southwestern areas of the country (Dehghani et al. 2010; Vazirianzadeh et al. 2013; Mousavi et al. 2015). Khuzestan, Hormozgan, and Kerman provinces are the most important foci of scorpion sting in the southern part of Iran, and several cases of death due to scorpion sting reported in these provinces every year (Radmanesh 1990; Pipelzadeh et al. 2007; Dehghani and Fathi 2012; Mousavi et al. 2015; Dehghani et al. 2016; Shahi et al. 2016). *Androctonus*, *Hottentotta*, *Mesobuthus*, *Odontobuthus*, and *Hemiscorpius* genera are the dangerous scorpions to humans found in the southern part of Iran (Jalali et al. 2010).

Hormozgan Province is among the high-risk zones of scorpion sting. Previous studies found 22 scorpion species in this province (Navidpour et al. 2013; Shahi et al. 2016). *Hemiscorpius* species such as *H. acanthocercus*, *H. enischnochela*, *H. gaillardi*, *H. persicus*, and *H. lepturus* are the most lethal scorpions in this Province (Shahi et al. 2015;2016). Nevertheless, little information is known about the fauna and distribution of scorpion species in some high-risk areas in the south of the country. Annually, about 2300 scorpion sting cases including several deaths are reported in Hormozgan Province. This study was designed to identify the deadliest scorpion species and their distribution in Bashagard County in the Northeast of Hormozgan Province, as well as the epidemiological aspects of scorpion stings in this area.

MATERIALS AND METHODS

Study area

Bashagard County, located in the east of Hormozgan Province, Iran (Figure 1) consists of three rural districts, two cities, and 166 villages. According to the 2016 census in the country, Bashagard County has a population of 40,007 people. The center of this county, Sardasht, is located at 26º 21' N and 57º 54' E. This county is 1480 m above sea level (ASL) and has a land area of 9,209 km^2. It is located in a mountainous area with a dry and hot climate. The average rainfall in this county is about 200 mm per year, and the minimum and maximum temperatures range between 7 to 45 ºC. The study was conducted in five main sites, selected based on geographical direction including north, south, east, west, and the center of Bashagard county. Geographical coordinates of the five main study sites are as follow: Goharan: 26° 36' 13.42" N, 57° 53' 16.67" E, 941.56 m ASL; Gafr: 26° 14' 18.54" N, 58° 12' 40.63" E, 564.44 m ASL; Paramon: 26° 29' 27.38" N, 58° 15' 2.61" E, 842.42 m ASL; Jagdan: 26° 26' 12.35" N, 57° 44' 20.11" E, 908.48 m ASL; Sardasht: 26° 27' 33.06" N, 57° 54' 1.52 E, 716.57 m ASL (Figure 1).

Scorpion samplings

Scorpions were collected from their habitats by diurnal searching and night catch using UV light, between September 2015 and October 2016 (Figure 2). The specimens were preserved in 75% ethanol alcohol and kept in the Medical Entomology laboratory of Bandar Abbas Health School. Geographical coordinates were recorded by a GPS device (Garmin^R). Scorpions were identified using an identification key (Navidpour et al. 2013) in the Medical Entomology laboratory of Bandar Abbas Health School, Hormozgan University of Medical Sciences.

Scorpion sting data

Clinical and demographic data of scorpion sting were obtained using the checklist of the Center for Disease Control (CDC) archive in Hormozgan Health Center during 2015 and 2016. Demographic data included sex, age, geographic location, site of the sting, and patient outcome. Data analysis was performed using SPSS 21 software, and graphs were drawn using Excel**.**

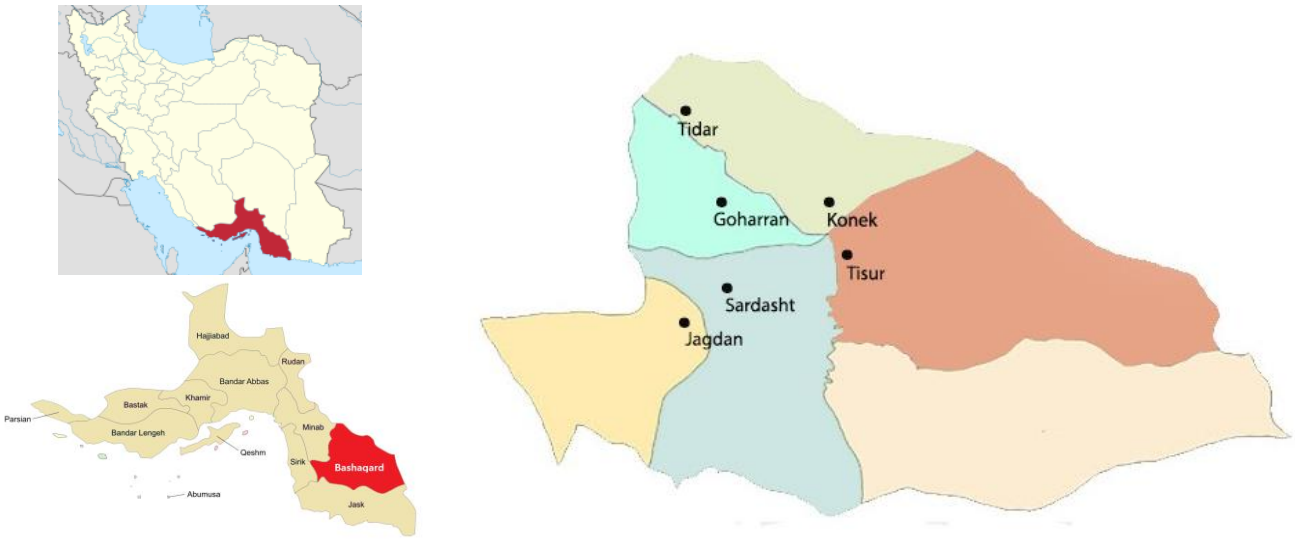


Figure 1. Map of study area in Bashagard County, Hormozgan Province, southern Iran

A **B** **C** **D**

Figure 2. Natural habitats of scorpions in the study area, Bashagard County, Hormozgan Province, southern Iran. A. Compsobuthus, B. Hottentotta, Hemiscorpius, C. Hemiscorpius, Mesbuthus, Orthochirus, D. Odontobuthus

RESULTS AND DISCUSSION

A total of 382 scorpions comprising 9 different species belonging to Buthidae (76%) and Hemiscorpiidae (24%) families were collected and identified. *M. phillipsi* (N=126) was the most abundant species in the study area, and *A. crassicauda* (N=1) and *O. bidentatus* (N=2) were the least abundant species (Figure 3). *Mesobuthus* (N=178) was the most dominant genus. The percentage of male (N=254) and female (N=128) scorpions were 66.5% and 33.5%, respectively. We identified *H. schach* (N=15), *C. persicus* (N=8), *H. acanthocercus* (N=109), and *O. farzanpayi* (N=21) for the first time in Bashagard County. *Mesbuthus phillipsi*, *H. acanthocercus*, and *O. doriae* were the most widely distributed species. *H. acanthicercus* was collected in all sampling sites (Figure 3).

During 2011-2015, a total of 1221 cases of scorpion sting were recorded in the health center of Bashagard County including four deaths. Figure 4 shows scorpion sting cases in different months, with the highest number of cases being recorded in August (Table 1).

About 96.4% of cases occurred in rural areas, whereas only 3.6% occurred in the urban areas (P<0.05). Females (59.6%) were stung more than males (40.4%) during the study period (P<0.05). Table 1 shows the frequency of scorpion stings in different age groups. Most of the scorpion sting cases occurred in the age group 10-24 years (P<0.05).

Figure 5 shows the most common sites of scorpion sting. The foot of patients represented the most common site (38.4%) for scorpion stings (Figure 5).

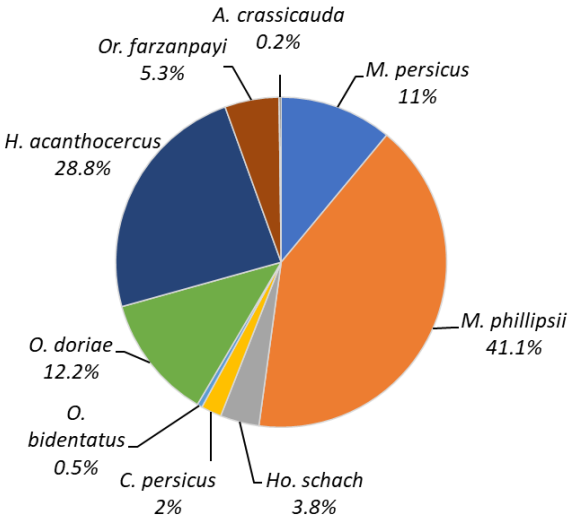


Figure 3. Species composition of scorpions in Bashagard County, Southern Iran, 2016

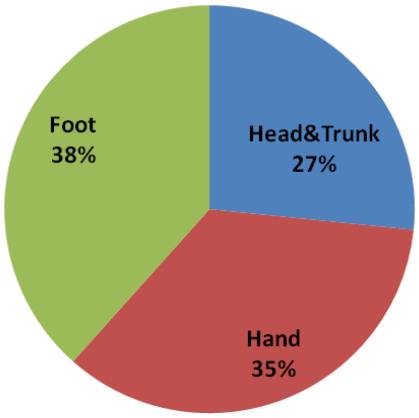


Figure 5. Scorpion sting in different parts of the body, Basaghard county, Southern Iran, 2011-2015

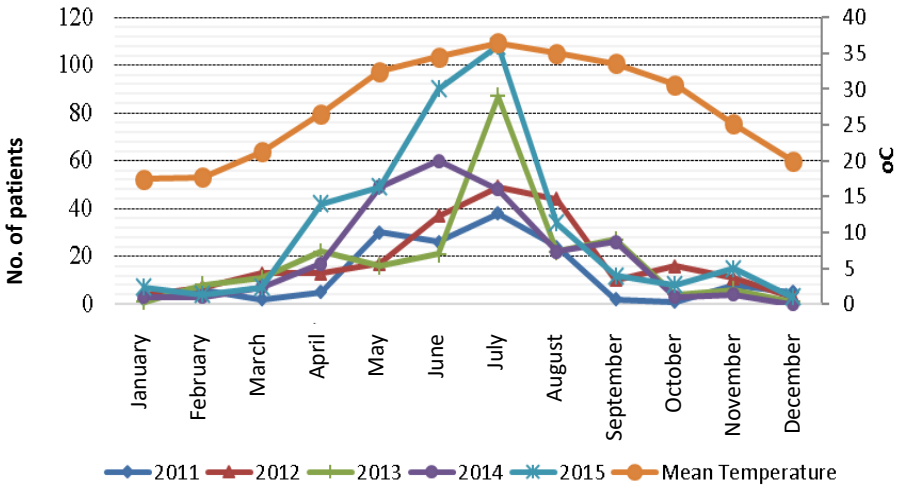


Figure 4. Monthly scorpion sting cases in Bashagard County, Southern Iran, 2011-2015

Table 1. Scorpion sting in different age groups, Bashagard County, Southern Iran, 2011-2015

Year	Age group					Total
	<9	10-24	25-44	45-64	>65	
2011	19	65	32	22	11	149
2012	44	85	53	30	13	225
2013	42	88	63	21	12	226
2014	61	65	70	30	16	242
2015	74	130	107	53	15	379
Total	240	433	325	156	67	1221
Percent	19.60	35.50	26.60	12.80	5.50	100

Discussion

The Southern part of Iran is an important region for scorpion sting. Bashagard County is one of the important foci in this region. In 2015, 329 cases of scorpion stings were reported, resulting in 2 deaths (0.61%). The estimated annual number of scorpion stings in the World is 1.2 million leading to 3250 deaths (0.27%) (Chippaux and Goyffon 2008). This report indicates that the mortality rate of scorpion sting is high in this county. In some of the cases, the scorpion species that stung the victims could not be identified, and some of the deaths might have resulted from the sting of unknown dangerous scorpion species in this county.

Hemiscorpius acanthocercus is one of the most important and deadly scorpion species, which we identified for the first time in the study area. In some other studies, *M. phillipsi* was identified as the most abundant species followed by *H. acanthocercus* in Hormozgan Province (Monod and Lourenco 2005; Shahi et al. 2016). A case of death due to *H. acanthocercus* sting has been reported in Bandar Abbas County (Shahi et al. 2015).

Hemiscorpius lepturus is the deadliest scorpion species in the west and south areas of Iran (Radmanesh 1990; Ahmadizadeh and Razi-Jalali 2006; Shahi et al. 2009; Zare et al. 2011; Mohseni et al. 2013). *Hemiscorpius lepturus* envenomation is the main cause of death in the Khuzestan Province of Iran (Rahmani and Jalali 2012; Vazirianzadeh et al. 2013). *Hemiscorpius* scorpions are widely distributed in the Western and Southern regions of the country (Navidpour 2015; Shahi et al. 2015; Dehghani et al. 2016).

In our study, *M. phillipsi* was the most abundant species. In some studies, conducted in other provinces, *Mesobuthus* genus was reported as the most dominant genus in the study areas (Azizi et al. 1998; Dehghani et al. 2008; Nejati et al. 2014; Dehghani et al. 2016). It seems that the climate of the southern areas in Iran provides suitable habitat for species belonging to this genus.

Odontobuthus, a digger scorpion species, was identified in all sampling sites in our study. Other scorpion genera identified in our study include *Odontobuthus, Orthochirus, Hottentotta, Compsobuthus,* and *Androctunus. Odontobutus*, *Hottentotta,* and *Androctonus* are the deadly scorpions in Iran (Dehghani et al. 2008; Nazari and Hassan 2016).

Hottentotta schach was the largest scorpion observed in our study area. The existence of this scorpion in Fars and Khuzestan provinces has been reported (Kovařík 2007; Navidpour 2012; Vazirianzadeh and Salahshoor 2015). This species has limited distribution in Iran, and we identified it for the first time in Bashagard County in Hormozgan Province.

Based on the diversity of scorpion species identified in the present study, it can be concluded that scorpion fauna is very diverse in Bashagard County. This county is located in mountainous areas. Research studies have shown that mountainous areas have more diverse animal species. The main reason is the existence of many shelters and suitable habitats for animals in these areas. Results of faunistic studies conducted in Iran have shown that mountainous areas have richer fauna compared to plains (Azizi et al. 2001; Haghi et al. 2004; Shahi et al. 2009; Kheirabadi et al. 2014).

About 60% of the scorpion sting victims in our study were women. This result is consistent with that of another study conducted in the country (Kassiri et al. 2012). In epidemiological studies on scorpion stings conducted in Turkey, Brazil, USA, and Australia, it was observed that females are more susceptible to scorpion sting compared with males (Isbister et al. 2003; Pardal et al. 2003; Forrester and Stanley 2004; Al et al. 2009). Another study, however, reported that men are more commonly stung by scorpions compared with women in a study conducted in Kashan (Dehghani et al. 2010). This difference may be due to the type of social activities engaged by women in different study areas. In traditional rural societies of Bashagard County, women generally do the task of gathering firewood for cooking and performing household chores.

In our study, we observed that cases of scorpion sting were more common in the age group 10 to 24 years. The lowest percentage of stings occurred in patients older than 60 years. The results of the studies conducted by Talebian and Doroudgar (2006), Hoseininasab et al. (2009), and Saghafipour et al. (2013) are consistent with that of our studies. The 10-24-year age group includes active members of society. Job activity increases the risk of scorpion sting among people in high-risk areas.

The most common site of scorpion sting was on the feet (38.4%) of patients, followed by hand (35%). This result is similar to that of the studies of Pipelzadeh et al. (2007), Shahbazzadeh et al. (2009), and Kassiri et al. (2013) conducted in Iran.

We found that most of the cases of scorpion sting occurred in August and rural areas. In Baghmalek of Khuzestan, 64.8% of scorpion sting cases were reported in rural regions (Kassiri et al. 2014). Rafizadeh et al. (2013) also reported that 57.7% of scorpion stings occur in rural areas. The climatic conditions coupled with the activities of the rural dwellers increase the risk of scorpion stings in these areas.

Figure 4 shows an increasing trend in the number of scorpion sting cases in Bashagard County during the study period (2011-2015). Other studies in the country have reported a high number of scorpion sting cases during summer (Dehghani and Fathi 2012; Nejati et al. 2014;

Khatony et al. 2015). Our result is in agreement with that of other studies conducted in Ramhormoz and Kashan. In Iran, Turkey, and Morocco, there have been reports on a high number of scorpion sting cases during the hot season of the year (Talebian and Doroudgar 2006; Abourazzak et al. 2009; Uluğ et al. 2012; Karami et al. 2013). Scorpions are active in warm seasons, which correlates with the increased number of scorpion stings cases during summer.

The high number of scorpion sting cases in rural areas may also be due to the large number of people living in the villages. The rural to the urban proportion of the study population was 13 to 1.

In 2015, two cases of death due to scorpion stings were recorded in Bashagard County. The patients presented with severe hematuria, kidney failure, and DIC before death. The victims were aged less than ten years. In a study conducted in Bandar Abbas County in Hormozgan province, *Hemiscorpius* sting caused hemolysis, hematuria, and renal failure in patients before death (Shahi et al. 2015). Owing to the similarity in the symptoms presented by the patients in Bandar Abbas County and our study area, it seems likely that the cause of death due to the sting of an unidentified scorpion in Bashagard County was due to *Hemiscorpius*.

This study showed that the eastern regions of the Hormozgan province in southern Iran provide a suitable habitat for one of the most lethal scorpions species, belonging to the *Hemiscorpius* genus. It seems likely that this genus is the main cause of death due to scorpion sting in these regions. Appropriate public health programs are necessary for the prevention and management of scorpion sting in the study area.

ACKNOWLEDGEMENTS

We are grateful to Abedini, Infectious and Tropical Diseases Research Center, Hormozghan University of Medical Science, Iran. This study was funded by Vice-Chancellor for Research and Technology, Hormozghan University of Medical Sciences, Project No. 94140.

REFERENCES

Abourazzak S, Achour S, El Arqam L, Atmani S, Chaouki S, Semlali I, Soulaymani BR, Bouharrou A, Hida M. 2009. Epidemiological and clinical characteristics of scorpion stings in children in Fez, Morocco. J Venom Anim Toxins incl Trop Dis 15 (2): 255-267. DOI: 10.1590/S1678-91992009000200008.

Ahmadizadeh M, Razi-Jalali M. 2006. The effect of *Hemiscorpius lepturus* scorpion venom on the level of some biochemical parameters in rat. Biochem Cell Arch 1: 147-155.

Al B, Yılmaz DA, Sögut O, Orak M, Üstündağ M, Bokurt S. 2009. Epidemiological, clinical characteristics and outcome of scorpion envenomation in Batman, Turkey: An analysis of 120 cases. JAEM 8 (3): 9-14.

Azizi K, Shahraki G, Omrani M. 2001. Determination of the fauna and sex ratio of scorpions from villages and suburbs of Kohgilouieh and Boirahmad province in 1379. J Armaghan Danesh 6 (21-22): 6-13.

Azizi K, Tirgari S, Sayedi-Rashti M. 1998. Faunistic study of scorpions in Shiraz and investigation on their fecundity. J Armaghan Danesh 3 (9): 10.

Chippaux JP, Goyffon M. 2008. Epidemiology of scorpionism: A global appraisal. Acta Trop 107 (2): 71-79. DOI: 10.1016/j.actatropica.2008.05.021.

Dehghani R, Fathi B. 2012. Scorpion sting in Iran: A review. Toxicon 60 (5): 919-933. DOI: 10.1016/j.toxicon.2012.06.002.

Dehghani R, Haghi FM, Mogaddam MY, Sedaghat M, Hajati H. 2016. Review study of scorpion classification in Iran. J Entomol Zool Stud 4 (5): 440-444.

Dehghani R, Moabed S, Kamyabi F, Haghdoost A, Mashayekhi M, Soltani H. 2008. Scorpions fauna of Kerman province-Iran. J Kerman Univ Medical Sci 15 (2): 172-181.

Dehghani R, Vazirianzadeh B, Nasrabadi MR, Moravvej SA. 2010. Study of scorpionism in Kashan in central of Iran. Pak J Med Sci 26 (4): 955-958.

Forrester MB, Stanley SK. 2004. Epidemiology of scorpion envenomations in Texas. Vet Hum Toxicol 46 (4): 219-221.

Haghi F, Tirghari S, Çhangani F, Mohammadpour R. 2004. A study on the scorpion species of the mountainous areas of Sari township in 2001. J Mazandaran Univ Sci 14 (43): 92-96.

Hoseininasab A, Alidoosti K, Torabinezhad M. 2009. The epidemiologic status of scorpion bite and its associated ris factors in south of Kerman Province. J Med Counc I R. Iran 3 (27): 295-301.

Isbister GK, Volschenk ES, Balit CR, Harvey MS. 2003. Australian scorpion stings: A prospective study of definite stings. Toxicon 41 (7): 877-883. DOI: 10.1016/S0041-0101 (03)00065-5.

Jalali A, Pipelzadeh MH, Sayedian R, Rowan E. 2010. A review of epidemiological, clinical and in vitro physiological studies of envenomation by the scorpion *Hemiscorpius lepturus* (Hemiscorpiidae) in Iran. Toxicon 55 (2-3): 173-179. DOI: 10.1016/j.toxicon.2009.09.012.

Karami K, Vazirianzadeh B, Mashhadi E, Hossienzadeh M, Abbas Moravvej S. 2013. A five year epidemiologic study on scorpion stings in Ramhormoz, South-West of Iran. Pak J Zool 45 (2): 469-474.

Kassiri H, Kassiri A, Sharififard M, Shojaee S, Lotfi M, Kasiri E. 2014. Scorpion envenomation study in Behbahan County, southwest Iran. J Coast Life Med 2 (5): 416-420.

Kassiri H, Mohammadzadeh Mahijan N, Hasanvand Z, Shemshad M, Shemshad K. 2012. Epidemiological survey on scorpion sting envenomation in South-West, Iran. Zahedan J Res Med Sci 14 (8): 80-83.

Kassiri H, Shemshad K, Kassiri A, Shemshad M, Valipor AA, Teimori A. 2013. Epidemiological and climatological factors influencing on scorpion envenoming in Baghmalek County, Iran. Acad J Entomol 6 (2): 47-54.

Khatony A, Abdi A, Fatahpour T, Towhidi F. 2015. The epidemiology of scorpion stings in tropical areas of Kermanshah province, Iran, during 2008 and 2009. J Venom Anim Toxins incl Trop Dis 21: 45-52. DOI: 10.1186/s40409-015-0045-4.

Kheirabadi KP, Pirbalouty VK, Jazayeri A. 2014. Geographical distribution of scorpions in Chaharmahal va Bakhteyari Province. Vet J 27 (2): 44-51. DOI: 10.22092/VJ.2014.100982.

Kovařík F. 2007. A revision of the genus *Hottentotta* Birula, 1908, with descriptions of four new species (Scorpiones, Buthidae). Euscorpius 2007 (58): 1-107. DOI: 10.18590/euscorpius.2007.vol2007.iss58.1.

Mohseni A, Vazirianzadeh B, Hossienzadeh M, Salehcheh M, Moradi A, Moravvej SA. 2013. The roles of some scorpions, *Hemiscorpius lepturus* and An-droctonus crassicauda, in a scorpionism focus in Ramhormorz, southwestern Iran. J Insect Sci 13 (1): 1-12. DOI: 10.1673/031.013.8901.

Monod L, Lourenco WR. 2005. Hemiscorpiidae (Scorpiones) from Iran, with descriptions of two new species and notes on biogeography and phylogenetic relationships. Rev Suisse Zool 112 (4): 869-942. DOI: 10.5962/bhl.part.80331.

Mousavi H, Navidpour S, Rafinejad J. 2015. A review study on distribution and medical importance of Hemiscorpius Peters, 1861 in Iran. J Mazandaran Univ Med Sci 24 (120): 107-124.

Navidpour S, Soleglad ME, Fet V, Kovařík F. 2013. Scorpions of Iran (Arachnida, Scorpiones). Part IX. Hormozgan province, with a description of Odontobuthus tavighiae sp. n. (Buthidae). Euscorpius 2013 (170): 1-29. DOI: 10.18590/euscorpius.2013.vol2013.iss170.1.

Navidpour S. 2012. A review study on Hottentotta Birula, 1908, (Scorpionida: Buthidae) species collected from Iran. Arch Razi Inst 67 (2): 93-100.

Navidpour S. 2015. An annotated checklist of scorpions in south and southwestern parts of Iran. Int J Fauna Biol 2 (3): 9-15.

Nazari M, Hassan R. 2016. Study on distribution of scorpions to provide prevention and interventions in combating scorpionism in Poldokhtar county, Lorestan province, Iran. J Clin Diagn Res Title: JCDR 10 (12): LC05-LC09. DOI: 10.7860/JCDR/2016/22759.8960.

Nejati J, Mozafari E, Saghafipour A, Kiyani M. 2014. Scorpion fauna and epidemiological aspects of scorpionism in southeastern Iran. Asian Pac J Trop Biomed 4: S217-S221. DOI: 10.12980/APJTB.4.2014C1323.

Pardal PPdO, Castro LC, Jennings E, Pardal JSdO, Monteiro MRdCdC. 2003. Epidemiological and clinical aspects of scorpion envenomation in the region of Santarém, Pará, Brazil. Rev Soc Bras Med Trop 36 (3): 349-353. DOI: 10.1590/S0037-86822003000300006.

Pipelzadeh MH, Jalali A, Taraz M, Pourabbas R, Zaremirakabadi A. 2007. An epidemiological and a clinical study on scorpionism by the Iranian scorpion *Hemiscorpius lepturus*. Toxicon 50 (7): 984-992. DOI: 10.1016/j.toxicon.2007.07.018.

Prendini L, Wheeler WC. 2005. Scorpion higher phylogeny and classification, taxonomic anarchy, and standards for peer review in online publishing. Cladistics 21 (5): 446-494. DOI: 10.1111/j.1096-0031.2005.00073.x.

Radmanesh M. 1990. Clinical study of Hemiscorpion lepturus in Iran. J Trop Med Hyg 93 (5): 327-332.

Rafizadeh S, Rafinejad J, Rassi Y. 2013. Epidemiology of scorpionism in Iran during 2009. J Arthropod Borne Dis 7 (1): 66-70.

Rahmani A, Jalali A. 2012. Symptom patterns in adult patients stung by scorpions with emphasis on coagulopathy and hemoglubinuria. J Venom Anim Toxins incl Trop Dis 18 (4): 427-431. DOI: 10.1590/S1678-91992012000400011.

Saghafipour A, Noroozi M, Jooshin MK. 2013. The epidemiologic status of scorpion stings in Qom Province, 2001-2011. J Safe Promot Inj Prev 1 (2): 95-101.

Shahbazzadeh D, Amirkhani A, Djadid ND, Bigdeli S, Akbari A, Ahari H, Amini H, Dehghani R. 2009. Epidemiological and clinical survey of scorpionism in Khuzestan province, Iran (2003). Toxicon 53 (4): 454-459. DOI: 10.1016/j.toxicon.2009.01.002.

Shahi M, Azizi K, Ansarian N. 2009. Study on scorpions fauna in high risk area of Hormozgan province, 2006-7. Hormozgan Medical Journal: HMJ 12 (4): 207-214.

Shahi M, Moosavy SH, Hanafi-Bojd AA, Navidpour S, Zare S, Madani A, Rafinejad J. 2016. Spatial distribution of scorpion sting in a high-risk area of southern Iran. J Med Entomol 53 (5): 1198-1204. DOI: 10.1093/jme/tjw043.

Shahi M, Rafinejad J, Az-Khosravi L, Moosavy SH. 2015. First report of death due to *Hemiscorpius acanthocercus* envenomation in Iran: Case report. Electron Physician 7 (5): 1234.

Talebian A, Doroudgar A. 2006. Epidemiologic study of scorpion sting in patients referring to Kashan medical centers during 1991-2002. Iran J Clin Infect Dis 1 (4): 191-194.

Uluğ M, Yaman Y, Yapici F, Can-Uluğ N. 2012. Scorpion envenomation in children: An analysis of 99 cases. Turk J Pediatr 54 (2): 119-127.

Vazirianzadeh B, Hossienzadeh M, Moravvej S, Vazirianzadeh M, Mosavi S. 2013. An epidemiological study on scorpion stings in Lordegan County, south-west of Iran. Arch Razi Inst 68 (1):71-76.

Vazirianzadeh B, Salahshoor A. 2015. Scorpion sting in Izeh, Iran: an epidemiological study during 2009-2011. J Basic Appl Sci 11: 403-409. DOI: 10.6000/1927-5129.2015.11.57.

Zare MA., Mahmoodi KS, Teimoorzadeh S. 2011. Antivenom injection time related effects of *Hemiscorpius lepturus* scorpion envenomation in rabbits. Arch Razi Inst 66 (2):139-145.

NUSANTARA BIOSCIENCE
Vol. 13, No. 1, pp. 91-99
May 2021

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n130113

Strategy for marine ecotourism development based on natural resource management: Case study in Kotania Bay, Western Seram District, Maluku, Indonesia

HENDERINA LELLOLTERY[1], MAMAN RUMANTA[2,♥], RONY MARSYAL KUNDA[3]

[1]Department of Forestry, Faculty of Agriculture, Universitas Pattimura. Jl. Ir. Putuhena, Poka, Ambon 97233, Maluku, Indonesia
[2] Program of Biology Education, Faculty of Teacher Training and Education, Universitas Terbuka. Jl. Cabe Raya, Pondok Cabe, Pamulang, Tangerang Selatan 15418, Banten, Indonesia. ♥email: mamanr@ecampus.ut.ac.id, ronykunda@gmail.com
[3]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Pattimura. Jl. Ir. Putuhena, Poka, Ambon 97233, Maluku, Indonesia

Manuscript received: 10 February 2021. Revision accepted: 14 April 2021.

Abstract. *Lelloltery H, Rumanta M, Kunda RM. 2021. Strategy for marine ecotourism development based on natural resource management: Case study in Kotania Bay, Western Seram District, Maluku. Nusantara Bioscience 13: 91-99.* The study aimed to identify the potential for marine ecotourism in Kotania Bay, and formulated strategies and priorities for developing marine ecotourism in Kotania Bay. Data collection on ecological potential consisted of mangrove and fauna vegetation using the line transect method, while coastal and marine resources included coral reefs and reef fish using the Line Intercept Transect (LIT) method. Development strategies and priorities used the SWOT and AHP methods. The results showed that the potential of coastal natural resources around the Kotania Bay consisted of 12 mangrove species belonging to7 families with species diversity index 1.76, and dominated by *Rhizophora mucronata* and *Bruguiera cylindrica*. The bird species were 33 species belonging to 23 families, with several endemic species such as *Philemon subcorniculatu*s, *Eulipoa wallacei*, *Birgus latro*, and *Pteropus melanopogon*. Potential marine water resources consisted of 45 coral species belonging to 23 genera and 14 families with 10-12 life-forms. The diversity of reef fish species was quite abundant consisting of 129 species from 60 genera and 22 families. The sand beach on Marsegu island is a tourist destination that is demanded by the tourists. SWOT analysis showed that the potential of coastal and marine natural resources in Kotania Bay is very likely to be developed as marine ecotourism.

Keywords: Ecotourism, Kotania Bay, priority, strategy

INTRODUCTION

Maluku is categorized as an archipelago that has 1,450 islands with high natural resources. Most of these islands are small islands that have abundant coastal natural resource potentials such as beautiful beaches, mangrove ecosystems, coral reefs, fishes, seagrass, and various other marine and coastal biota. The resources are very potential to be developed but vulnerable to over-used. Therefore, the right strategy is needed in the development process so that there is no damage to natural resources and it still provides significant benefits to the community and the environment. One alternative that can be considered is through the use of environmental services based on marine ecotourism (Baiquni 2013).

Ecotourism in coastal and marine areas contributes to the economy of local communities and supports conservation and protection actions of coastal and marine ecosystems (Walters and Samways 2001). Efforts to develop marine ecotourism in small islands of Maluku can be done by considering the very abundant potential of natural resources. Baiquni (2013) stated that small islands can be ecotourism destinations since they provide abundant natural resource potential. The concept of ecotourism can broadly minimize negative impacts on the environment, by providing positive experiences to the community and visitors, then contributing to the benefits of economic empowerment to the community (Das and Chatterjee 2015).

One of the ecotourism destinations in Maluku is the Kotania Bay of the West Seram District. Kotania Bay is located among five islands, i.e. Marsegu, Osi, Burung, Buntal, and Tatumba. Marsegu and Osi islands are quite famous tourist destinations. This is due to the coastal natural resources and potential marine in the form of mangrove ecosystems, white sand beaches, coral reefs, reef fish, seagrasses, and various marine biota. These resources can be a potential object and attraction for tourists. The area around Kotania Bay is designated as a marine conservation area with the status of Marsegu Island Nature Tourism Park (Nature Conservation Agency Indonesia, 2014).

The potential of coastal natural resources from the region is not optimally developed for ecotourism activities. This is caused by various factors such as minimal infrastructure and the construction of conventional thinking about the concept and view of optimal uses of the coastal resources. Facts on the ground show that the limited government support can be seen from the lack of regulations regarding tourism activities. Lack of regulation greatly impacts the development process of ecotourism in Kotania Bay is getting slower. To develop the potential of

these natural resources, a comprehensive analysis is required by developing a regional development strategy that takes into account the characteristics of the area and the conditions of the community. For this reason, comprehensive studies are very important to be carried out to understand development strategies that will become an empirical basis for a policy design of sustainable ecotourism. This study aims to identify the potential of ecotourism in the Bay of Kotania, and to formulate a strategy and priority scale for ecotourism-based development.

MATERIALS AND METHODS

Study area

The study was conducted at Kotania Bay areas in Marsegu and Osi Islands, Western Seram District, Maluku for six months from June to December 2019. The locations of sampling sites are shown in Figure 1.

Data collection

Data collection consisted of primary and secondary data. Primary data collection was done through field observations and interviews, secondary data was collected through literature studies including data on marine conditions, the physical condition of the region, regional statistical data, and other supporting data obtained from books, journals, and reports related to the study. Data collection by interview method was carried out for all stakeholders involved including surrounding communities, area managers, and local governments. The interview method was conducted in a structured manner with a questionnaire. Interviews were conducted with people directly involved in ecotourism activities in two sample villages, i.e. Pelita Jaya and Osi Island hamlet. The number of respondents interviewed was 30 peoples for each village.

Data collection of coral reefs and fish used the line intercept transect (LIT) method (English et al. 1997) with observations at six stations. Coral benthic was done by recording all components along the 50 meters coastline. A description of the morphology of coral communities was carried out in the life-form category. Mangrove and other fauna data collection used the line transect method, by making four transects with a length of 500 meters each transect. Potential flora and fauna data, observed in the same path included species, number of species, species diversity, and status of species.

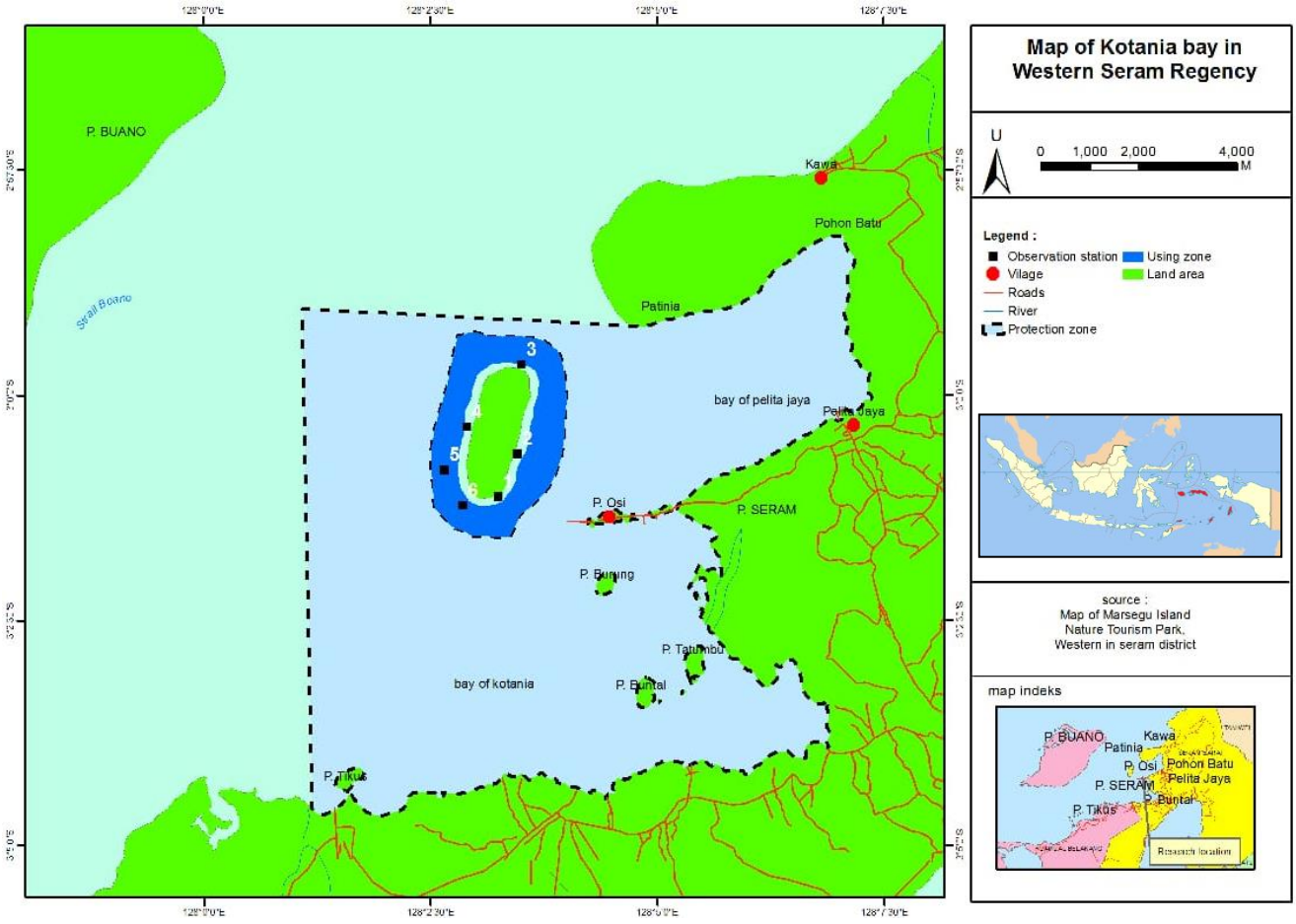


Figure 1. The locations of sampling sites in Kotania Bay, Maluku, Indonesia

Observations of the coastal characteristics were carried out on several parameters related to the suitability of coastal tourism following Yulianda (2007) which was modified for the study including the types of beach, coastal land cover, water bottom materials, dangerous biota (carried out by visual observation), and snorkeling around the coast. Measuring the width of the beach was using roll meters, while the availability of freshwater was measured based on the distance of the source of clean water to the location where ecotourism activities are carried out.

Data analysis

SWOT analysis was carried out for strategic factors that affect tourism activities, i.e. internal and external factors. Internal factors analysis was carried out using the Internal Factor Analysis Strategy (IFAS), while external factors used the External Factor Analysis Strategy (EFAS). Based on the IFAS and EFAS matrices then a SWOT matrix was made (Table 1). From each SWOT element, some elements which have the highest or most strategic influence were chosen. *Analytic Hierarchy Process* (AHP), is a method of decision making with multiple criteria developed by Saaty (1993). To determine the priority of ecotourism development programs, and evaluation of ecological, economic, social, cultural, and institutional aspects was based on internal and external factors that have an important value on the ecotourism development strategy around the area. The selection of respondents was based on a purposive sampling technique with the consideration that the respondent can make policy and provide input to policymakers (government, private sector, and local communities). The AHP analysis uses analysis tools in the form of Expert Choice software version 11.

RESULTS AND DISCUSSION

Potential of natural resources as marine ecotourism objects

The coastal and marine natural resources in Kotania Bay, including the potential of coral reefs, reef fish, mangrove ecosystems, and various types of wildlife, and white sand beaches as tourist destinations are potential as marine ecotourism objects and are in demand by the tourists.

Potential of mangroves and wildlife

The identification of mangrove ecosystems around Marsegu island found 12 species belonging to 7 families, dominated by the Rhizophoraceae family, some species within the family were *Rhizophora mucronata*, *Bruguiera gymnorrhiza*, and *B. cylindrical*, with the diversity index 1.76, and classified as medium category. This showed that mangroves in the Marsegu island region are in a "stable" condition. Considering the potential resources, the mangrove vegetation can be further developed as an object of tourist destination. Ecotourism activities can be carried out through the guidance of the interpretation lane. The lane interpretation is developed to provide conservation education for visitors to know mangrove species so that it becomes an educational mode. Fandeli and Muckhlison (2009) stated that the observation of flora is a great experience for the visitors to learn about the flora and climatic conditions that affect the ecosystem. The development of interpretation lane in Osi Island was a mangrove bridge with a length of 1.2 km and width of 2 meters, while Marsegu island was provided through the lane of mangrove forest in the south Kotania Bay and Pelita Jaya Bay.

Mangrove observation was carried out in conjunction with wild animal observation. The observation of animals found 33 bird species belonging to 23 families, consisted of four species of limited distribution (BST), one endemic bird species of Seram island, *P. subcorniculatus*, and one endangered bird species, *E. wallacei*. Likewise shorebirds such as *Egretta garzetta*, *Egretta sacra*, *Ducula bicolor*, and *Pteropus melanopogon*. In addition to the bird species, there are mammal species (*Pteropus melanopogon*) that make the mangrove ecosystem as their habitat. The daily activity of these species looks like a tourist attraction. Another interesting fauna is *Birgus latro* which can be found in several places on Osi and Marsegu islands, and various species of wild animals make the mangrove forest as their habitat.

Table 1. SWOT analysis matrix

Internal Factors (IFAS) / **External Factors (EFAS)**	STRENGTH (S) Determined 5-10 internal strength factors	WEAKNESS (W) Determined 5-10 internal weakness factors
OPPORTUNITIES (O) Determined 5-10 external opportunity factors	STRATEGY (S-O) Strategies that use power to take advantage of opportunities	STRATEGY (W-O) Strategies that minimize weaknesses to take advantage of opportunities
THREATS (T) Determined 5-10 external threat factors	STRATEGY (S-T) Strategies that use power to overcome threats	STRATEGY (W-O) Strategies that minimize weaknesses to take advantage of opportunities

Idajati et al. (2016) stated that the potential of mangrove forests with the wild animals can be developed into mangrove ecotourism by involving the participation of local communities. Besides, Rhormens et al. (2017) stated that mangrove ecosystems in protected areas can be one of the objects to promote the ecotourism area. This indicated that the diversity of mangroves and fauna can be an ecotourism attraction. Furthermore, Gunn (1994) reported that the diversity of flora and fauna which are endemic and frequently found in tropical regions can be the object of ecotourism activities.

Potential of coral reefs and coral fish

There were 45 species of coral reefs belonging to 23 genera and 14 families. Closure of coral reefs found in the form of hard coral included acropora and non-acropora, soft coral, dead coral (dead coral algae), algae, sponges, other biotas (OT), and abiotic. Hard coral dominates coral cover with a percentage of 69.86%. Based on the value of live coral cover, it can be seen that the health level of coral ecosystems is in a good category, while the number of lifeforms identified was 12. The diversity of coral types found around the marine area of Marsegu Island is supported by the marine physical condition including temperature, salinity, chlorophyll-a, ocean current, and brightness level with values of 28.66 °C, 34.10 ‰, 0, 31 ppb, 0.167 m/sec, and 80% respectively.

The types of corals found in the Kotania Bay have branched growth forms (ACB), such as table (ACT), submassive (ACS) with beautiful and attractive colors to tourists, especially foreign tourists for snorkeling and diving activities. This is in line with Lelloltery et al. (2018a) stated that the form of coral growth, coral species both acropora, non-acropora, soft coral with interesting shapes and colors is a tourist attraction. Burke et al. (2011) stated that diving activity is the most popular tourist activity because they can enjoy the beauty of coral reefs in their habitat. Supriharyono (2007) stated that coral reefs have a beauty value that cannot be doubted because of the mainstay of marine tourism.

Based on the observations of reef fish, 146 species were found belonging to 64 genera and 21 families. Fish species found, based on their function and role, consisted of 16 species of fish (10.95%), 81 species of major fish (55.48%), and 49 species of target fish (33.56%). Fish species found have interesting shapes and colors. The existence of fish showed that the condition of the coral reef is categorized as good (Latuconsina 2015). One species of fish whose status is threatened in nature is *Cheilinus undulatus* (IUCN, 1994). The existence of this fish is an attraction for the tourists who want to see this fish species directly in their habitat. This finding is following Wabnitz et al. (2017) who stated that the protection of areas that become habitats of endangered species is needed to minimize the loss of endemic fish species in their natural habitat.

White sand beach

The beach which is dominated by white sand can only be found on Marsegu island from east to north with a beach length of 1.68 km and is currently a location that is in demand by both domestic and foreign tourists. The beach's general characteristics are 0-3 meters of depths, 20-30 meters of widths when the low tide, bottom marine dominated by sand, and the beach slope is around 7°. Coastal land cover is dominated by forest with dominant species are *Pongamia pinnata*, *Hibiscus tiliaceus*, *Terminalia catappa*, and the northern is dominated by shrubs.

The existence of two wells for providing clean water isa little bit far, i.e. about 800 meters. The availability of clean water is one of the obstacles in the development of coastal tourism in the area, so that supporting facilities are needed to meet the needs of clean water for the visitors. The beach characteristics, white sand, on Marsegu Island are very suitable for ecotourism activities (Lelloltery et al. 2016).

Socio-economic of the local community

The local people who live around Kotania Bay belong to the coastal community of the Western Seram District. They are generally migrants and natives of Seram Island. The migrants come from Buton Island, Southeast Sulawesi Province, but have inhabited the coastal region of the Seram island since their ancestors. Their main livelihood is fishermen, so they use marine resources as a source of livelihood. To fulfill their basic needs, the community carries out seaweed farming and fish ponds. The activities of catching fish and utilizing marine resources cannot be separated from the lives of local communities. Nikijuluw (2003) stated that coastal communities have a high dependency and consumption of natural resources. The communities are having a low level of education (elementary school), and their livelihood consists of fishermen (53%), entrepreneurs 26.6%, and the rest are civil servants or police, and the army. Based on the age level, it is generally in the productive age (86.66%). This productive age is related to the potential of the workforce in the tourist area.

The participation of the community is limited to providing services such as accommodation, consumption, transportation, and tour guides (Lelloltery et al. 2018b). Their involvement is still passive, has business planning, and depends on the demand of visitors. Though it provides employment, business opportunities, additional income to the local communities. Damanik (2013) stated that ecotourism on small islands creates diverse opportunities, i.e. employment, for the communities. The creation of employment from ecotourism activities can improve economic welfare by forming a symbiotic mutualism between local communities and natural areas (Stronza and Gordillo 2008).

Community participation in providing services, i.e. shopping activities, to the visitors can provide economic benefits. For example, the total expenditure of foreign tourists is an average of IDR 979,000/day with a standard deviation of IDR 276,036/day. This value is quite efficient for the local communities. Lelloltery (2018b) stated that the service activities in supporting ecotourism have contributed to the income of the local communities.

Condition of facilities and accessibility at tourist area

Tourist facilities are available on the Osi island, i.e. accommodation facilities (homestays), transportation (motorcycle, cars, and motorboats), restaurants, shops, and stalls. The facilities are commonly owned by the local communities and managed independently to support ecotourism activities. Abdulhaji and Yusuf (2016) stated that the facilities available at the tourist area will encourage the visitors to come again and enjoy the attractions. To date, the condition of tourist facilities faces several obstacles including the limited number of sea and land transportation, unskilled human resources, and efforts to increase human resources so far not yet intensively carried out.

To date, access to tourist areas can be reached by using sea and land transportation. The road trip from Ambon city takes an hour to the seaport in the Liang village, then continued by ferry (ASDP) for 1.5 to 2 hours to the Waipirit seaport in Western Seram District. From the capital city of West Seram District take the car or motorbike for two hours to the ecotourism area. The visitors can enjoy along the mangrove forest, it can be done on foot or using a motorbike with a travel time of about 20 to 30 minutes. To explore the marine or visit the small islands and white sand beaches on Marsegu island, can be reached by traditional motorboats approximately 15 to 30 minutes.

Ecotourism development strategy

Activities to develop an area require strategic planning and study of the strategic factors of an organization which includes strengths, weaknesses, opportunities, and threats (Rangkuti 2002). Strategic factors in developing ecotourism in the Kotania Bay area were formulated based on a SWOT analysis. Based on these strategic factors, strategies and alternative policies were formulated in the development of ecotourism. Development policy priorities were carried out by AHP analysis. The analysis of strategic factors (internal and external factors) and the determination of policy priorities are presented as follows:

Internal factors

The values of strategic factors as a component of strengths and weaknesses in the development of ecotourism (Table 2).

External factors

The value of strategic factors as a component of opportunities and threats in the development of Ecotourism is provided in Table 3.

Based on Tables 2 and 3, the IFAS values for internal factors (the difference between Strength and Weakness values) was 0.271, while the EFAS values for external factors (the difference between the Opportunity and Threat values) was 0.11. The IFAS values obtained are positive which means cumulatively the strength factor is more dominant than weakness, while the EFAS values are negative which means the opportunity factor is smaller than the threat. Referring to the IFAS and EFAS values, the SPACE matrix and ecotourism mapping positions were in quadrant II, the strategy that must be applied is the diversification strategy (ST). The results of the analysis of strategic factors that influence the development of ecotourism are summarized in Table 4. Based on the matrix, there are four alternative strategies to develop ecotourism activities in Kotania Bay, but the results of the analysis of strategic factors revealed that the development should apply a diversification strategy (ST).

The results of the SWOT analysis showed that strength factors included the diversity of coastal and marine resources, with endemic species. Natural resources supported by the local wisdom of the community are a major force for the region to be developed. Support and community participation are factors that influence the success of the development of an area to the fullest. Coria and Calfucura (2012) reported that collaboration and community participation are keys to the success of ecotourism. In addition to that government support, high accessibility to ecotourism areas, and positive perceptions from the local communities are opportunities to increase efforts to the development of ecotourism areas.

Table 2. Internal factors in developing ecotourism potential

Factors		Quality	Score	Value of influence
Strength Factors				
1.	Potential aquatic resources in the form of coral reefs, reef fish, and other biota as conservation areas with small island ecosystems	0.101	4	0.404
2.	Having specific mangrove ecosystem and endemic fauna species	0.094	3	0.282
3.	Having the diversity of objects and tourist attractions	0.085	3	0.255
4.	Having the culture and local wisdom of a typical coastal community	0.084	3	0.249
5.	Support and participation of local communities	0.085	3	0.255
	Total			1.445
Weakness Factors				
1.	Limited supervision	0.08	2	0.16
2.	Limited supporting facilities and infrastructure within the region	0.101	2	0.30
3.	Promotion and publication of the area have not been done intensively	0.090	2	0.270
4.	Limited human resources both in quantity and quality	0.091	2	0.27
5.	Regional boundary arrangement has not been carried out optimally	0.09	1	0.17
	Total			1.174

Table 3. External factors in developing ecotourism potential

Factors		Quality	Score	Value of influence
Opportunity				
1.	Support of government of Western Seram District for the development of ecotourism	0.101	2	0.202
2.	Positive perceptions from the local communities and visitors related to efforts to develop ecotourism	0.085	3	0.255
3.	Easy accessibility	0.099	3	0.297
4.	The trend of tourist visits is increasing from year to year	0.101	3	0.303
5.	The tourism potential can be a tour package in Western Seram District	0.099	2	0.198
	Total			1.255
Threat				
1.	There is still environmental damage due to marine and coastal pollution activities	0.135	3	0.405
2.	Logging of mangrove forests			
3.	Local communities knowledge is still minimal about the conservation of natural resources	0.106	3	0.314
4.	The dependence of local communities is still high on natural resources	0.084	2	0.168
5.	Limited cooperation and coordination between stakeholders	0.102	2	0.204
	Total			1.365

Table 4. Matrix of SWOT analysis to develop ecotourism based on natural resource management

Internal / **External**	**Strength (S)**	**Weakness (W)**
	• High potential of coastal and aquatic resources • Having specific mangrove ecosystem and endemic fauna species • Having the diversity of objects and tourist attractions • Having the culture and local wisdom of a typical coastal community • Support and participation of local communities	• Limited supervision • Limited supporting facilities and infrastructure within the region • Promotion and publication of the area have not been done intensively • Limited human resources both in quantity and quality • Regional boundary arrangement has not been carried out optimally
Opportunity (O) • The support of the Local Government of Western Seram District for the development of ecotourism in the Regional Medium-Term Development Plan 2017-2022 • Positive perceptions from the local communities and visitors related to efforts to develop ecotourism • Easy accessibility • The trend of tourist visits is increasing from year to year • The tourism potential can be a tour package in Western Seram District	**Strategy (SO)** • Developing the potential of coastal and marine resources including coral reefs, reef fish, and marine biota as a special marine ecotourism attraction for beach tourism with snorkeling, diving as the main attractions • Improve facilities or infrastructure to the ecotourism area • Using the potential of marine resources, flora and fauna, and local wisdom of the community into a package tour • Promote the potential of ecotourism through printed and electronic media. • Improving the quality of tourism products by involving local communities	**Strategy (WO)** • Regional arrangement so that it becomes more accountable • Increasing intensive supervision of ecotourism areas with the support of local communities • Providing Facilities and Infrastructure • Intensifying promotion and publication activities • Improve the quality of human resources • Community empowerment through the provision of business capital and the establishment of tourism aware groups
Threat (T) • To date, there is still environmental damage due to marine and coastal pollution activities as well as logging of mangrove forests • The knowledge of local communities about natural resource conservation is categorized as low • Communities dependence on coastal natural resources is categorized as high • Cooperation and coordination between stakeholders is limited	**Strategy (ST)** • Developing the potential of coastal and marine natural resources sustainably • Increase participation and empowerment of coastal communities • Promoting and educating conservation to local communities • Developing partnerships among stakeholders in the effort to manage attractions, accessibility, and amenities area • Increasing the area's security by involving local communities, adding security personnel, and law enforcement	**Strategy (WT)** • Increasing stakeholder cooperation in efforts to safeguard the area • Strengthen the role and function of local government and local communities • Involve non-governmental organization (NGO) and the private sector in the development of ecotourism • Increasing the number and quality of human resources through training on tourism, environmental conservation in supporting the ability of management, the community, and the business world in the development of ecotourism

Other factors which become obstacles in the development of ecotourism are limited infrastructure and human resources, as well as low promotional activities. This condition is caused by the limited involvement of stakeholders, especially in providing infrastructure. Reihanian et al. (2012) stated that the lack of supporting infrastructure may cause ecotourism activities unsustainably. Besides, Das and Chatterjee (2015) asserted that improving infrastructure can sustainably affect ecotourism activities, and improve the living standards of local communities. Besides, there are still threats in the area in the form of fishing activities by applying destructive principles and logging activities to mangrove forests. This condition occurs due to very high community dependence on coastal and marine resources. Emmanuel and Spence (2009) reported that coastal and marine communities are highly dependent on marine and coastal resources. This causes utilization activities that are not environmentally friendly.

The results of the SWOT analysis showed that threats still outweigh opportunities in the region so that strategies are needed to overcome threats by optimizing the strength of the region. The involvement of local communities in the region and the economic benefits received by the local community from ecotourism activities are expected to be a solution to overcome the problem of community dependence on coastal and marine resources. Ghorbani et al. (2015) asserted that the collaboration and participation of local communities are the keys to guarantee sustainable ecotourism. The results of the analysis showed that ecotourism activities have not been achieved well in Kotania Bay, so that the diversification strategy obtained from the SWOT results provides policy direction for regional development.

The diversification strategy (ST) is described in several alternative activities, i.e. (i) developing the potential of coastal and marine natural resources in a sustainable manner, (ii) encouraging participation and empowering coastal communities, (iii) promoting conservation education to the local communities, (iv) developing partnerships among stakeholders in the effort to manage the attractions, accessibility, and amenity of the area, (v) increase the security of the area by involving local communities, law enforcement, and strict regulations regarding the management and development of ecotourism.

Priority ecotourism development in Kotania Bay

Ecotourism development strategies based on SWOT analysis cannot be implemented at the same time due to limited funds, time, and human resources, so that it is necessary to determine priority programs that must be implemented. The determination of priority programs for the development of ecotourism areas is carried out by taking into account the factors involved, so there must be Analytic Hierarchy Process (AHP). The results of the pairwise comparison analysis related to the priority of ecotourism development are shown in Figure 2. The priority of developing ecotourism is implemented in the form of implementing activities (Table 5).

The results of AHP analysis showed that integrated natural resource development is a priority of ecotourism development activities, which are implemented through development activities (Table 5). The priority from developing ecotourism is based on the Block system. This is intended to map the area so that it is developed following the functions and potential possessed. The establishment of these blocks facilitates supervision to minimize activities that are destructive and in turn increase protection of natural resources.

Besides, the second priority is the participation and empowerment of coastal communities. This is intended to increase the active role of the local communities in ecotourism activities because it has an impact on improving the community economy. This condition can be a useful tool to minimize community dependence on resources due to the availability of jobs. Damanik (2006) reported that community participation in ecotourism activities can provide benefits to local communities individually or collectively as well as direct conservation benefits for ecosystems.

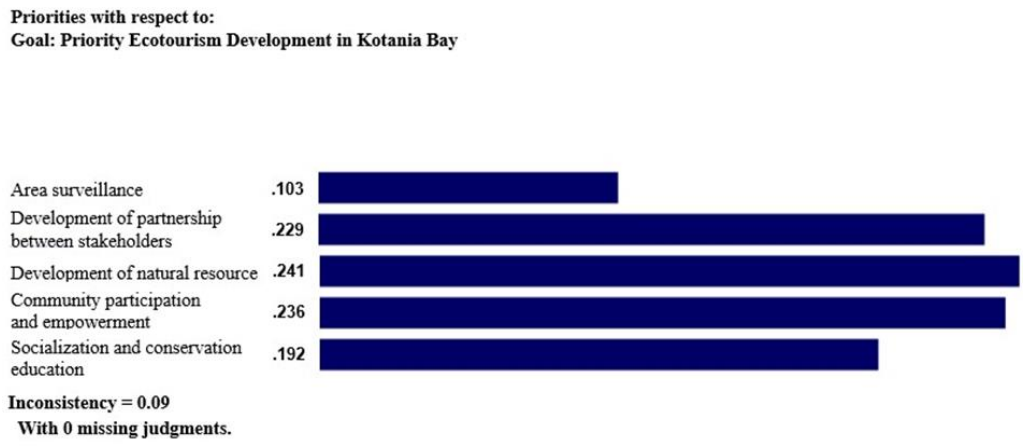


Figure 2. Priority of ecotourism development programs

Table 5. Priority of ecotourism development activities

No.	Priority development	Development activities
1	Development of potential ecotourism objects sustainably	Development with a block system Marine ecotourism block (beach, snorkeling, and diving) Mangrove ecotourism block (Path of interpretation on Osi and Marsegu Islands) Ecotourism Facility Block (Osi island and Pelita Jaya Village)
2	Participation and empowerment of coastal communities	Building economic units (Cooperative) Formation of business and aware tourists group Entrepreneurship training (culinary, souvenirs, etc) Training on seaweed cultivation and coral transplantation Assistance for business actors Determination of tourist villages
3	Partnership among stakeholders in the effort to manage attractions, amenities, and accessibility	Strengthening cooperation and coordination among stakeholders Developing promotion of natural resource potential in the Kotania Bay continuously through printed and electronic media, by all stakeholders involved Implement a "collaborative management" model
4	Conservation, socialization, and education	Socialization of the concepts of ecotourism and environmental conservation Establish the area as a research location Establish areas or spots for the protection and preservation of coral reefs Encourage the participation of local communities and stakeholders in environmentally friendly activities
5	Improve supervision and law enforcement	Socialization of the rule of law regarding tourism and the environment Conducting patrol activities continuously in the Kotania Bay Strengthening traditional institutions in the supervision of ecotourism areas in the Kotania Bay Establish regulations related to the development of ecotourism in the village (Village Regulation)

Table 5 showed that the partnership factors among stakeholders, i.e. the promotion of ecotourism programs and conservation education as well as intensive supervision from various parties are the next priorities that support the first and second priorities, so that they become a unity for the realization of the concept of sustainable ecotourism. Rhormens et al. (2017) reported that sustainable environmental monitoring and partnership factors are important principles in the management of an ecotourism area. This is following the principle of sustainable ecotourism is the sustainability of ecological, socio-cultural, economic aspects and presenting educational values to the tourism products offered (Triyuniarthi 2010).

In conclusion, the potential of coastal and marine resources in Kotania Bay has the opportunity to be developed for marine ecotourism activities by implementing a diversification strategy (ST). A diversification strategy is a strategy that utilizes strengths and minimizes threats. This strategy is implemented with priority activities as follows: (i) developing potential of coastal and marine resources sustainably, (ii) participation and empowerment of coastal communities, (iii) development of partnerships among stakeholders, (iv) conservation education for local communities, and (v) supervision of the area, with successive values are 24.1%; 23.6%; 22.9%; 19.2%; and 10.3%.

ACKNOWLEDGEMENTS

The authors declare that this study has not received any funding, and categorized it as an independent study. All authors are asserted that this study is a personal collaboration among lecturers of Universitas Pattimura and Universitas Terbuka, and was carried out independently. We also acknowledge to the staff of the Department of Forestry, and Department of Biology, Universitas Pattimura, and staff of Biology Education Department, Faculty of Education and Teacher Training, Universitas Terbuka. The authors also thank the laboratory staff of the Deep Sea Research Center, Indonesian Institute of Sciences (LIPI) who helped in analyzing of marine condition of Kotania bay.

REFERENCES

Abdulhaji Y. 2016. The influence of attractions, accessibility, and facilities on the image of Tolire lake attraction in Ternate city. Jurnal Penelitian Humano 7 (2): 134-148. DOI: 10.33387/hjp.v7i2.317. [Indonesian]

Baiquni M. 2013. Geostrategy of Indonesia and pacific islands on ecotourism collaboration. In: Baiquni M, Damanik J, Rindrasih E (eds.). Ecotourism Destinations in Archipelago Countries. Gadjah Mada University Press, Yogyakarta. [Indonesian]

Burke L, Reytar K, Spalding M, Perry A. 2011. Reefs at Risk Revisited. World Resources Institute, Washington, DC.

Coria J, Calfucura E. 2012. Ecotourism and the development of indigenous communities: The good, the bad, and the ugly. Ecol Econ 73 (15): 47-55. DOI: 10.1016/j.ecolecon.2011.10.024.

Damanik J, Weber HF. 2006. Ecotourism Planning (from Theory to Applications). Andi Publisher, Yogyakarta. [Indonesian]

Damanik J. 2013. Social and cultural dimensions of ecotourism development in small island. In: Baiquni M, Damanik J, Rindrasih E (eds.). Ecotourism Destinations in Archipelago Countries. Gadjah Mada University Press, Yogyakarta, Indonesia. [Indonesian]

Das M, Chatterjee B. 2015. Ecotourism: A panacea or a predicament? Tourism Manag Perspec 14: 3-16. DOI: 10.1016/j.tmp.2015.01.002

Emmanuel K, Spence B. 2009. Climate change implications for water resource management in Caribbean tourism. Worldwide Hospitality and Tourism Themes 1 (3): 252-268. DOI: 10.1108/17554210910980594.

English S, Wilkinson C, Baker V. 1997. Survey Manual for Tropical Marine Resources. Australian Institut of Marine Science, Townsville.

Fandeli C, Muhamad. 2009. Basic Principles for Conserving Landscapes. Gadjah Mada University Press, Yogyakarta, Indonesia. [Indonesian]

Ghorbani A, Raufirad V, Rafiaani P, Azadi H. 2015. Ecotourism sustainable development strategies using SWOT and QSPM model: A case study of Kaji Namakzar Wetland, South Khorasan Province, Iran. Tourism Manag Perspect 16: 290-297. DOI: 10.1016/j.tmp.2015.09.005

Gunn CA. 1994. Tourism Planning: Basics, Concepts, Cases. Taylor and Francis Ltd, Washington DC.

Idajati H, Pamungkas A, Kukinul VS. 2016. The level of participation in mangrove ecotourism development, Wonorejo Surabaya. International Conference, Intelligent Planning Towards Smart Cities, CITIES, Surabaya. [Indonesian]

IUCN. 1994. Guidelines for protected areas management categories. IUCN Commissions on National Parks and Protected Areas (CNPPA)-World.

Latuconsina H. 2016. Tropical Aquatic Ecology. Gadjah Mada University Press, Yogyakarta, Indonesia.

Lelloltery H, Pudyatmoko S, Fandeli C, Baiquni M. 2018a. Study of coral reef for marine ecotourism development based on region suitability and carrying capacity in Marsegu Island Nature Tourism Park, Maluku, Indonesia. Biodiversitas 19 (3): 1089-1096. DOI: 10.13057/biodiv/d190342

Lelloltery H, Pudyatmoko S, Fandeli C, Baiquni. 2016. Development of ecotourism based on conformity and carrying capacity of coastal areas (case study of Marsegu Island) Western Seram Regency. J Agric Cultivation 12 (1): 25-33.

Lelloltery H, Pudyatmoko S, Fandeli C, Baiquni. 2018b. Socio-economic study of the community and the role of stakeholders in ecotourism development in Marsegu island natural tourism park, Western Seram Regency. J Trop For 3: 302-314.

Nikijuluw VPH. 2003. Aspek Sosial Ekonomi Masyarakat Pesisir dan Strategi Pemberdayaan Mereka dalam Konteks Pengelolaan Sumberdaya Pesisir Secara Terpadu. In: Knight M, Tighe S (eds) Koleksi Dokumen Proyek Pesisir 1997-2003. Coastal Resources Center, University of Rhode Island, Narragansett, Rhode Island, USA.

Rangkuti F. 2002. Analisis SWOT Teknik Membedah Kasus Bisnis. Gramedia Pustaka Utama, Jakarta. [Indonesian]

Reihanian A, Mahmood NZB, Kahrom E, Hin TW. 2012. Sustainable tourism development strategy by SWOT analysis: Boujagh National Park, Iran. Tourism Manag Perspect 4: 223-228. DOI: 10.1016/j.tmp.2012.08.005.

Rhormens MS, Pedrini AG, Lopes NPG. 2017. Implementation feasibility of a marine ecotourism product on the reef environments of the marine protected areas of Tinhare and Boipeba Islands (Cairu, Bahia, Brazil). Ocean Coast Manag 139: 1-11. DOI: 10.1016/j.ocecoaman.2017.01.022.

Rhormens MS, Pedrini AG, Lopes NPG. 2017. Implementation feasibility of a marine ecotourism product on the reef environments of the marine protected areas of Tinhare and Boipeba islands (Cairu, Bahia, Brazil). Ocean Coastal Manag 139: 1-11. DOI: 10.1016/j.ocecoaman.2017.01.022.

Saaty TL. 1993. Decision making for leaders: analytic hierarchy process for decision making in complex situations. Binaman Presindo, Jakarta, Indonesia. [Indonesian]

Stronza A, Gordillo J. 2008. Community views of ecotourism. Ann Tourism Res 35 (2): 448-468. DOI: 10.1016/j.annals.2008.01.002

Supriharyono. 2007. Konservasi Ekosistem Sumberdaya Hayati di Wilayah Pesisir dan Laut Tropis. Pustaka Pelajar, Yogyakarta. [Indonesian]

Triyuniarthi. 2010. The Concept of Marine Ecotourism with The Approach of Developing Sustainable Tourism Products on Lelei island, Guraici Archipelago, Southern Halmahera Regency, North Maluku. [Thesis] Universitas Gadjah Mada, Yogyakarta. [Indonesian]

Wabnitz CCC, Montemayor AMC, Hanich Q, Ota Y. 2017. Ecotourism, climate change and reef fish consumption in Palau: Benefits, Trade-offs and adaptation strategies. Mar Policy 88, 323-332. DOI: 10.1016/j.marpol.2017.07.022.

Walters RDM, Samways MJ. 2001. Sustainable dive ecotourism on a South African coral reef. Biodivers Conserv 10 (12): 2167-2179. DOI: 10.1023/A:1013197926580.

Yulianda F. 2007. Ekowisata bahari sebagai alternatif pemanfaatan sumberdaya pesisir berbasis konservasi. Makalah Seminar Sains 21. [Indonesian]

NUSANTARA BIOSCIENCE
Vol. 13, No. 1, pp. 100-110
May 2021

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n130114

Evaluation of fluctuating asymmetry and sexual dimorphism of *Channa striata* using landmark-based geometric morphometric analysis from Agusan Marsh and Lake Mainit in Caraga Region, Philippines

REX BOMVET D. SAURA,[1,♥] GENEVEVE C. FALCASANTOS,[2] REX JR. M. ANDANTE[3], LUNA C. MUNDA[4], MARYCRIS M. ALIMORONG[5], BRENT JOY HERNANDO[6]

[1]Surigao State College of Technology-Mainit Campus, Surigao del Norte, Philippines. ♥email: saura.rex@yahoo.com.
[2]Asian College Foundation, Butuan City, Agusan del Norte, Philippines
[3]Senior High School, Agusan del Sur National High School, San Francisco, Agusan del Sur, Philippines
[4]Special Science, Anticala National High School, Anticala Butuan City, Agusan del Norte, Philippines
[5]Senior High School Teacher, Jabonga National High School, Jabonga, Agusan del Norte, Philippines
[6]Department of Biology, College of Arts and Science, Caraga State University, Ampayon, Butuan City, Agusan del Norte, Philippines.

Manuscript received: 26 October 2020. Revision accepted: 25 April 2021.

Abstract. *Saura EBD, Falcasantos GC, Andante RJM, Munda LC, Alimorong MM, Hernando BJ. 2021. Evaluation of fluctuating asymmetry and sexual dimorphism of* Channa striata *using landmark-based geometric morphometric analysis from Agusan Marsh and Lake Mainit in Caraga Region, Philippines. Nusantara Bioscience 13: 100-110.* Evaluation was conducted to determine the impact of ecological condition to *Channa striata* using fluctuating asymmetry (FA) employing the landmark-based geometric morphometric analysis, using TPS software, SAGE, PAST-Hotellings Test, including Physico-chemical analysis, cyanide and four base metals. Results of Physico-chemical analysis of the two habitats showed Agusan marsh with low oxygen level (3.37-4.55) and high in Iron and TDS as compared to DENR Standards. The statistical results showed high variations ($p < 0.0001$) on left and right-side having percentage of 79.22% in Agusan Marsh and 72.35% in Lake Mainit which is relatively high Fluctuating Asymmetry. In terms of sex, Agusan marsh had 84.27% and 85.41% FA in males and females while Lake Mainit having 85.12% and 80.89% in males and females. The fairly similar percentage of Fluctuating Asymmetry confirms no sexual dimorphism and no shape differences between male and female, and no peculiar characters to distinguished between sexes based on the 17 landmark points. Present study concluded that FA observed in the snout tip was due to breeding behavior, the landmark points in cephalic, pectoral, and caudal region were associated with burrowing and mobility factors within the lateral line system in these fishes which thrive in high turbid habitat and low oxygen. Furthermore, it was found that the level of FA tends to increase in disturbed environs.

Keywords: Phenotypic Plasticity, Physico-chemical parameters, Procrustes shape coordinates, Variation

INTRODUCTION

Character variation among populations describing the external morphology and accompanied differences between sexes are basic concepts in systematics. The magnitude of phenotypic differences such as body shapes, sexual dimorphism, and allometry is perhaps a reflection of the partitioning between sexes, availability of food, seasonal or climatic patterns, inter-relationship among organisms in their respective habitat which is often correlated to geographical factors (Broadley 1965; Sullivan and Best 1997).

The variability of attributes such as body shape, color, sizes and related to sex phenotypic characteristics among species are annotated as “sexual dimorphisms” (McCombe and Greer 2014). Such characteristics lead to an instinct of rivalry amongst individuals in the interior of each sex and mate selection based on morphological and behavioral traits (Ralls and Mesnick 2009; Vitt and Caldwell 2014). Geographical variation denotes dissimilarities between species in inherently founded characters through the habitat’s natural topography. Studies on geographic variation of a number of organisms highlight clarity on causes and significance of microevolution (Olvido 2012; Wu et al. 2015). At the global level, geographic variation in phenotypic plasticity is in part due to genetic differences acquired via long-term natural selection to produce adaptation to different environments (Niskanen et al. 2017). The degree of phenotypic plasticity in life-history traits differs among species and these differences are adaptive and non-adaptive in various habitats (Lymbery 1992; Seigel and Ford 2001). Habitat conditions have a tendency to reduce equilibrium stability in the developmental aspect of aquatic organisms e.g. small and random deviations from bilaterally symmetrical traits leading to Fluctuating Asymmetry (FA) (Santos et al. 2013). Stressed marginal habitats tend to influence increasing asymmetry as well as genetic perturbations (Parsons 1992). FA has been used as an ecological indicator in studies of natural and sexual selection and as a reliable bio-indicator tool relevant in environmental monitoring and conservation biology (Leary and Allendorf 1989; Tomkins and Kotiaho 2001; Beasley et al. 2013; Coda et al. 2017).

Furthermore, landmark-based Geometric morphometrics offers a powerful approach in computing morphological variables and covariation of shape (Rohlf 2000; Adams et al. 2009; Webster and Sheets 2010). Such techniques utilize statistical analysis on morphological variables based on Cartesian landmark coordinates. The traditional morphometrics separates shape from overall size, position, and orientation of the landmark configurations and the resulting Procrustes shape coordinates are used for statistical analysis (Mitteroecker and Gunz 2009).

The present study was conducted on the fish species *Channa striata* of Channidae (Bloch 1973) commonly known as Chevron Snakehead which is a non-native and invasive species to Philippines. Though in wild a burrowing dweller of bottom of mud lakes, canals, and swamps and living and breeding in turbid and low levels (Phen et al. 2005; Cagauan 2007), in other cases cultured for commercial food production (Guerrero III 2014). These fish are capable of limited movement overland by the use of a wriggling motion which they use to disperse to new water bodies on rainy nights (Sayer 2005). *C. striata* have eyes, but given the turbid habitats they live in, they may also use other sensory mechanisms to find prey and mates. They have a lateral line system that detects small changes in water pressure that help them to be aware of the objects moving in the water around them (Kasumyan 2003; Musikasinthorn 2004). The capacity of the folds of skin to distend is an important adaptation enabling the jaws to protrude and assist the fish to engulf relatively large-size prey (Mittal and Agrawal 1994).

Present study aims to compare the levels of FA, sexual dimorphism, and body variations of *C. striata* collected from two separate inland waters Agusan Marsh and Lake Mainit of Caraga Region Philippines using landmark-based geometric morphometric analysis. These include the assessment of Physico-chemical parameters, Cyanide and four base metals; Copper, Lead, Zinc, Iron of the study area. The study would provide knowledge on the character variation of *C. striata* and give relevant information useful in policy making and formulation of ecosystem-based management plan to the immediate agency for environmental protection in Agusan Marsh and Lake Mainit.

MATERIALS AND METHODS

Study Area

The study was conducted in two different types of inland waters namely Agusan Marsh, Agusan del Sur, and Lake Mainit, Surigao del Norte of Caraga Region, Philippines. The ecological aspects were recorded and coordinates of the six sampling stations on each area were obtained using geocam mobile app, as presented in Table 1 and a map was generated (Figure 1).

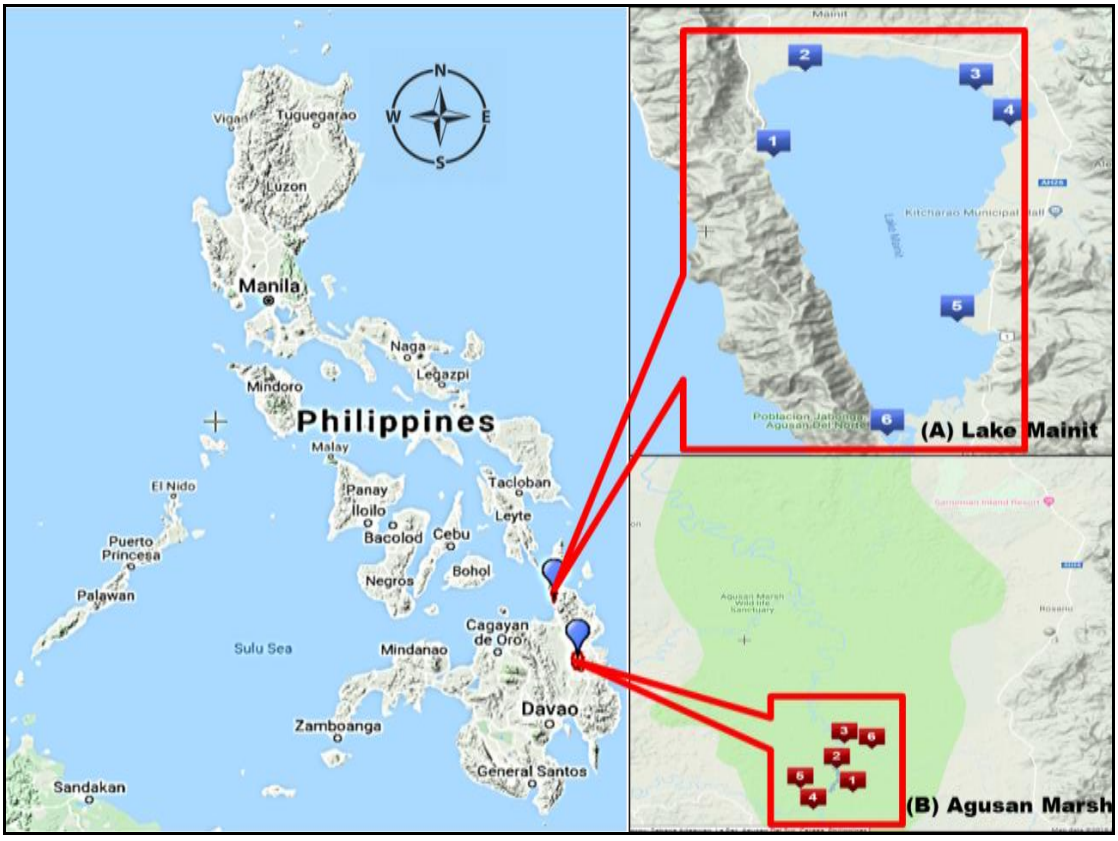


Figure 1. Map of geographically separate habitat of *Channa striata* (Chevron Snakehead) samples in Caraga Region, Philippines: A. Lake Mainit (17,060 hectares showing the six (6) water sampling stations, B. Agusan Marsh (14,835 hectares) showing the six (6) sampling stations water on the southern portion.

Table 1. Site Characteristics of the sampling stations established in Lake Mainit and Agusan Marsh, Philippines

Sampling areas	Coordinates	Site characteristics
Lake Mainit		
Station 1	9° 28' 59" N 125° 28' 49" E	River mouth of Tagbuyawan, few residents, rocky riverbanks and substrate, diverse plants in the sloping areas of the Malimono Mountain range Active fishing and navigation within the Lake. Previous site of Cyanide gold processing area in nearby areas.
Station 2	9° 31' 35" N 125° 29' 27" E	River mouth of Mayag sedges and grass dominant in the riparian area and shrub plants to trees are present in the upland areas, active fishing and navigation were observed, residential areas and agricultural land
Station 3	9° 31' 1" N. 125° 33' 2" E	River mouth of Magpayang, sedges and grass dominant in the riparian area and shrub plants to trees, coconut plants in the upper stream areas and obvious landscape of large scale mining, few live stocks, and agricultural lands like rice fields, several residential areas, and establishments. Active fishing and navigation.
Station 4	9º29'54" N 125º33'45" E	River mouth of Magtiaco with sandy river banks, sedges and grass dominant in the riparian area and shrub plants to trees, coconut plants are present in the upper stream areas and obvious landscape of small-scale gold mining, few live stocks, agricultural land like rice fields, several residential areas. Active fishing and navigation.
Station 5	9º23'51"N 125º32'40" E	River mouth of Jaliobong, cattails plants dominant in the littoral zone with floating water lilies and water hyacinth, Agricultural land and residential areas in the nearby upstream. Active fishing and navigation within the Lake.
Station 6	9º20'20"N 125º31'11" E	Kalinawan river as outlet of the lake, cattails dominated the littoral zone, near to the resort, nearby areas are present with residents, on-going operation for flood control project in the upper stream.
Agusan Marsh		
Station 1	8º11'16"N 125º54'50" E	The lake basin of Mihaba dominated by water hyacinth and few disperse trees agricultural land and mining in the nearby environs
Station 2	8º12'15"N 125º54'16" E	Creek outlet of Lake Mihaba riparian vegetation is cattails and sedges with few trees and agricultural land and mining in the upper streams. Active navigation was observed.
Station 3	9º13'16"N 125º54'29" E	Near to the intersection of by cattails and sedges with few trees and agricultural land and mining in the upper streams. Active navigation was observed
Station 4	8º10'41"N 125º53'40" E	The Loreto river end of mixing to Agusan river, river waters flowing from the northeastern of Bukidnon
Station 5	8º10'48"N 125º53'44" E	The neck of three headwaters from Bukidnon, Simulao river and Lake Mihaba drainage creek, riparian dominated by tall grass and sedges. Active navigation was observed
Station 6	9º13'22"N 125º54'14" E	Downstream of Simulao River, riparian dominated by cattails and sedges with few trees and agricultural land and mining in the upper streams. Active navigation was observed

Establishment and collection of water and Physico-chemical analysis

The water samples were collected from two different types of inland waters in CARAGA region in Lake Mainit and Agusan Marsh respectively. Six sampling stations were established in each area. The water samples were collected through depth of 1-50 cm of the water column of each sampling site. The physicochemical parameters of each sampling station in every area were done using HANNA HI98194 multi-parameter test. Water samples were collected @ 0.5 L per sample and placed directly in calibrated PET bottles. The bottles were sealed tightly and labeled with information of area, station, and date of collection and photographed for documentation. A total of six bottles with 0.5 L water samples from Lake Mainit were collected on 26.11.2018 and another six bottles from Agusan Marsh were collected on 05.12.2018. The samples were chilled in ice bucket and delivered to Philsaga Mining Assay Laboratory, Rosario, Bunawan, Agusan del Sur for the analysis of Cyanide and Four Base Metals.

Collection of fish samples and preparation for landmarking

The fresh and live samples of *C. striata* were obtained from the local fishermen folks of Agusan Marsh and Lake Mainit. A total of sixty fish samples, out of which thirty males and thirty females were collected during 13-15.11.2018 from Lake Mainit. Another sixty fish samples, with equal sex proportion, were obtained on 22.11.2018 from Agusan Marsh. The length of each fish sample was determined by measuring the specimen from its snout down to the caudal fin using measuring tape and weighed using BATVOX ACS-40 digital weighing scale with 40 kg capacity and 1/3000 F.S. precision. The individual fish was flanked on a Styrofoam and fins were pinned showing its point of origin. Photographs of the left and right side of each specimen were taken with canon eos 1200D for land-marking. Specimens were dissected individually for their sex determination.

Table 2. Description of the landmark points in the body shape of *Channa striata* adopted and modified from Saura and Andante (2018).

Coordinates	Location
1	Snout Tip
2	End of head length
3	anterior junction of the dorsal fins
4	Posterior junction of the dorsal fins
5	Dorsal insertion of caudal fin
6	Midpoint of caudal border of hypural plate
7	Ventral insertion of caudal fin
8	Posterior insertion of anal fin
9	Anterior insertion of anal fin
10	Posterior insertion in the dorsal base of pelvic fin
11.	Anterior insertion in the dorsal base of pelvic fin
12	Ventral end of lower jaw articulation
13	Posterior end of maxilla
14	Anterior margin through midline of orbit
15	Posterior margin through midline of orbit
16	Dorsal end of operculum
17	Dorsal base of pectoral fin

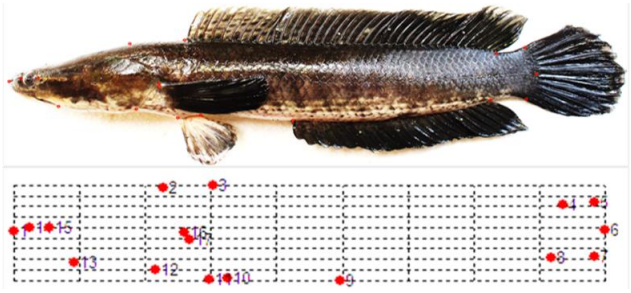


Figure 2. Actualized image of digitized selected landmark point of *Channa striata*.

Selection of Land-marking selection and digitizing

The images of fish specimens were sorted by sexes and the images processed in tpsUtil64 to create *.tps files. The resultant tps files were digitized with 17 landmarks using tpsDig2 software. The location of the landmarks and anatomical descriptions were represented in Table 2 and Figure 2. Landmarking was referred to on stickleback fish model of anyfish.com.

Shape analysis

The Procrustes superimposition was performed on the digitized image samples through tps relwarp software by subsequently pressing the clickable buttons of the consensus, partial warps, and the relative warp scores report and was saved for the statistical analysis for sexual dimorphism. Further, the digitized images were subjected to Symmetry and Asymmetry Geometric Data (SAGE) v.1.21 software following the protocols for object symmetry which is the landmark pairing protocol and matching symmetry, a side identification protocol to determine the shape conformation of individual variations (symmetric, asymmetric and error) and counting the possible covariance. In addition, the SAGE software platform provided options for the Principal Component Analysis (PCA) which was utilized to determine the affected landmark, and only the first four PCA% and deformation grid results of *C. striata* from Agusan Marsh and Lake Mainit and respective sexes were obtained.

Statistical analysis

The cumulative report saved from relative warp analysis was subjected to PAST 3 software for discriminant analysis and to derived plot for sexual dimorphisms and Hotellings T^2 test was used to determine significant differences of the sex shape differences. Further, the Procrustes method was employed and the significant differences of fluctuating asymmetry values respective to habitat and sexes were assessed using SAGE v.1.21 software. Permutation default statistical analysis i.e. Procrustes ANOVA procedure for understanding interaction and side effects were carried out in SAGE software.

Data analysis

The physicochemical parameters, content of cyanide, and four base metals, Lead, Copper, Zinc, Iron in water samples were compared to DENR standards (national water quality standards) above or below the maximum contamination level. Furthermore, the data of relative warp analysis, discriminant analysis, sexual dimorphism, and FA obtained from both the sexes of *C. striata* both Lake Mainit and Agusan Marsh were describe and compared.

RESULTS AND DISCUSSIONS

Fluctuating asymmetry

A total of one hundred twenty *C. striata* fish samples were obtained for this study, sixty from Lake Mainit and another sixty (60) from Agusan marsh. The length and weight of the fish samples from pond and Lake Mainit was presented in Table 3.

Table 3. Total Length and total weight of *Channa striata* from Lake Mainit and Agusan Marsh collected in the month of November 2018.

Fish samples	N	Total length (cm)			Total weight (g)		
		Range	Mean	SD ± SE	Range	Mean	SD ± SE
Lake Mainit	60	30.0-41.9	34.96	2.3 ± 0.3	205-480	318.83	64.3 ± 8.3
Agusan Marsh	60	29.5-41.1	36.2	2.8± 0.3	180-565	391.1	89.7± 11.6

Note: N-total number of samples

Furthermore, Procrustes method was used to assess the FA value of the right and left side of *C. striata* pooled samples both from Agusan Marsh and Lake Mainit are presented in Table 4. The individual symmetry and sides of Left and Right body shape in Agusan Marsh and Lake Mainit were not statistically significant. Out of the three effects considered, two effects (Individual and Sides) showed high significance ($p < 0.0001$) in both Agusan Marsh and Lake Mainit pooled samples. It indicated the incidence of FA in *C. striata* which was attributed to the effects of environmental stressors which can cause developmental instability to the species. Similar reports of developmental instability caused by the effects of stressed environment were observed in goby fish *Glossogobius giuris* from Agusan river samples (Jumawan et al 2016) and in the wings of *Aedes albopictus* from selected barangays in Iligan City, Philippines (Quirog and Tabugo 2015).

The principal component analysis (PCA) was employed on both pooled samples Agusan Marsh and Lake Mainit to determined landmark points affected (Table 5). Samples of *C. striata* from Agusan Marsh showed a total of 79.22% of FA from PC1-PC4. PC1 showed that all areas covered by these landmarks were found to have greater asymmetry. On the other hand, Lake Mainit *C. striata* showed a total FA interaction of 72.35% from PC1 to PC4. Affected landmarks were greater in PCA 3. This indicated the bilateral asymmetry of both male and female species of each habitat. The Agusan marsh *C. striata* population experienced a higher level of environmental stress. The asymmetrical shape of *C. striata* from Agusan Marsh and Lake Mainit fishes were compared and shown in Figure 3.

The individual symmetry and sides of left and right body shape of male and female in Agusan Marsh and Lake Mainit were not statistically significant shown in Table 6 and Table 7. Both male and female from two populations, out of the three effects considered, two shows high significance ($p < 0.0001$) both individuals and sides which is depicted.

Table 4. Procrustes ANOVA results of pooled fish sample of *Channa striata* from two habitats.

Effect	Sum of Squares	Degrees of freedom	Mean square	F-value	p-value
Agusan Marsh					
Individuals	0.1216	1770	0.0001	3.2236	0*
Sides	0.0134	30	0.0004	20.8811	0*
Individual x Sides	0.0377	1770	0	0.06928	1^{ns}
Measurement Error	0.11075	3600	3.0764	---	---
Lake Mainit					
Individuals	0.1922	1770	0.0001	5.8043	0*
Sides	0.0044	30	0.0001	7.8103	0*
Individual x Sides	0.0331	1770	0	0.4114	1^{ns}
Measurement Error	0.16366	3600	4.5462	---	---

Note: *highly significant ($p < 0.0001$), ns not significant.

Table 5. The results of first four (4) Principal Components of *Channa striata* from Agusan Marsh and Lake Mainit.

PCA	Individual (symmetry)	Sides (directional asymmetry)	Interaction (fluctuating asymmetry)	Landmarks affected
Agusan Marsh				
PC1 (%)	44.31	100%	43.15	1, 2, 5, 6, 7, 9, 11, 12, 13, 14,15, 16, 17
PC2 (%)	17.61		18.93	1, 2, 3, 5, 7, 9, 10, 11, 12, 14, 15, 16
PC3 (%)	13.93		9.69	1, 6, 7, 10, 11, 14, 15, 16, 17
PC4 (%)	5.83		7.43	2, 5, 7, 8, 9
Overall%	81.68		79.22	
Lake Mainit				
PC1 (%)	61.01	100%	29.88	1, 2, 3, 6, 10, 13, 14, 15,16, 17
PC2 (%)	15.69		20.91	1, 2, 7, 9, 10, 11, 12, 14, 16, 17
PC3 (%)	6.56		12.33	1, 2, 3, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 17
PC4 (%)	5.17		9.23	1, 2, 3, 5, 9, 11, 12, 16, 17
Overall%	88.52		72.35	

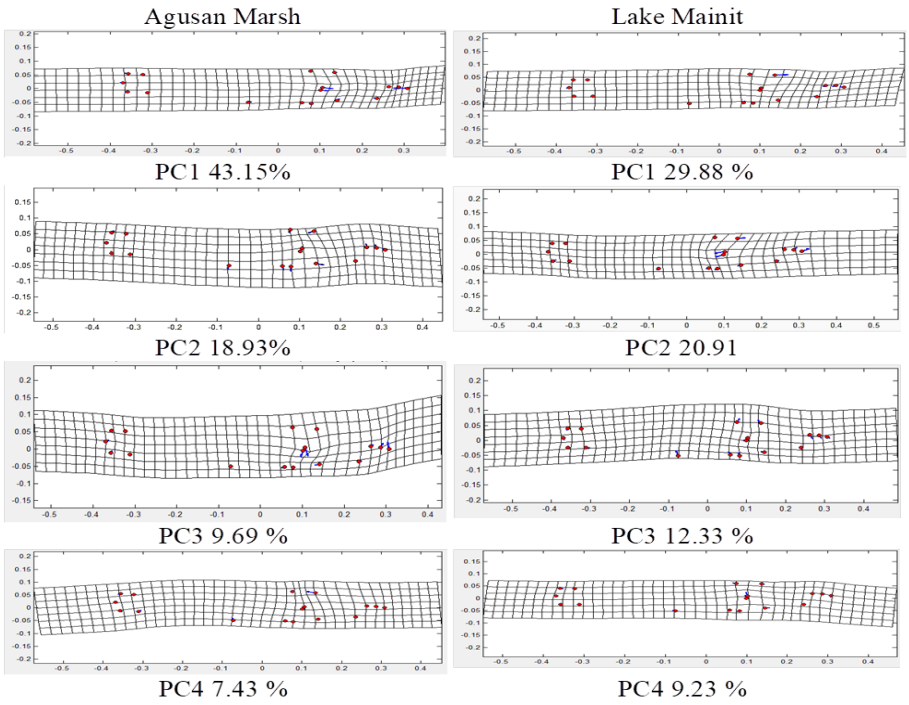


Figure 3. PCA implied deformation for individual × side interaction of fluctuating asymmetry of *Channa striata* from Agusan Marsh (first column) and Lake Mainit (second column) (zoom in to see blue arrows implies degree)

Table 6. Procrustes ANOVA results of *Channa striata* from Agusan Marsh in terms of sexes.

Effect	Sum of squares	Degrees of freedom	Mean square	F-value	P-value
Male					
Individuals	0.0652	870	0.0001	3.7276	0*
Sides	0.0075	30	0.0002	12.418	0*
Individual × Sides	0.0175	870	0	0.6188	1^{ns}
Measurement Error	0.0585	1800	3.2497	---	---
Female					
Individuals	0.0524	870	0.0001	2.69	0*
Sides	0.0064	30	0.0002	9.8481	0*
Individual × Sides	0.0195	870	0	0.7712	1^{ns}
Measurement Error	0.0523	1800	2.9031	---	---

Note: *highly significant ($p < 0.0001$), ns not significant

Table 7. Procrustes ANOVA results of *Channa striata* from Lake Mainit in terms of sexes

Effect	Sum of Squares	Degrees of Freedom	Mean Square	F-value	p-value
Male					
Individuals	0.0761	870	0.0001	4.6312	0*
Sides	0.0036	30	0.0001	6.2693	0*
Individual × Sides	0.0164	870	0	0.388	1^{ns}
Measurement Error	0.0876	1800	4.8652	---	---
Female					
Individuals	0.1071	870	0.0001	7.4611	0*
Sides	0.0032	30	0.0001	6.3652	0*
Individual × Sides	0.0144	870	0	0.3905	1^{ns}
Measurement Error	0.0761	1800	4.227	---	---

Note: *highly significant ($p < 0.0001$), ns not significant

Furthermore, Table 8 and 9 show the results of Principal Component Analysis of *C. striata* samples in terms of sexes from Agusan Marsh and Lake Mainit respectively. Both sexes of *C. striata* sample from Agusan Marsh showed close percentage of FA (Male, PC1, 84.41%) and (Female, PC4,85.41%). PC1 showed that all areas covered by these landmarks were found to have greater asymmetry in males and PC3 in females. On the other hand, Lake Mainit *C. striata* male showed a total FA of 85.12% from PC1 to PC4 and its affected landmarks were greater in PCA 1. While in female *C. striata* had a total FA of 80.89% from PC1 to PC4 in which PC3 had greater affected landmarks. The asymmetrical shape of *C. striata* from Agusan Marsh and Lake Mainit were compared and depicted in Figures 4 and 5.

The degree of closeness of FA is merely on behavior and characteristics and their preferred habitat. As observed in the deformation grid and PCA most of the affected portion of the body with many and variation was the anterior part of the body extended up to the middle areas which may be due to burrowing behavior and association of the anterior part with the lateral line system of the fish which enable them with their predatory skills and ability to live in stressed swampy and muddy habitat. The increase of Fluctuating Asymmetry variation of *C. striata* populations from two sites was followed by influence of their habitat quality. Noteworthy, that the samples from Agusan Marsh had higher (7%) difference from Lake Mainit due to the fact that observed habitat had high turbidity, and local residence also attested that selected sampling rivers and creeks within the Agusan Marsh remained turbid throughout the year (Primavera and Tumanda Jr. 2008), unlike Lake Mainit that had clear waters (LMDA 2018).

Sexual dimorphism

Based on the cumulative report retrieved from relative warp analysis and discriminant analysis using PAST software, the resultant graphs showed overlap in the discriminant plot (Fig. 6) meaning shape differences between two sexes of *C. striata* specimens from Agusan Marsh and Lake Mainit are minimal. Hotellings T^2 test results also revealed no significant sexual dimorphism for the body shape of the *C. striata* in all specimens from Agusan Marsh and Lake Mainit (Table 10). This is in line with the study of Saura and Andante (2018) who showed similar less significant sexual dimorphism in *C. striata in* Bunawan, Agusan River. Parker (1992) has pointed towards sperm competition, size buffering among other features in answer to the sexes being often so alike in size. However, some studies reported *C. striata* females are larger than males (Chaudhry 2010; Dumalagan 2017).

Table 8. The results of Principal Components of *Channa striata* from Agusan Marsh in terms of sexes

PCA	Individual (symmetry)	Sides (Directional Asymmetry)	Interaction (Fluctuating Asymmetry)	Landmarks affected
Male				
PC1 (%)	44.68	100%	43.81	1, 2, 5, 6, 7, 9, 11, 12, 13, 14,15, 16, 17
PC2 (%)	21.90		20.45	1, 2, 3, 5, 7, 9, 10, 11, 12, 14, 15, 16
PC3 (%)	14.62		12.94	1, 6, 7, 10, 11, 14, 15, 16, 17
PC4 (%)	5.31		7.07	2, 5, 7, 8, 9
Overall%	86.51		84.27	
Female				
PC1 (%)	52.62	100%	47.80	1, 2, 3, 6, 10, 13, 14, 15,16, 17
PC2 (%)	16.57		20.31	1, 2, 7, 9, 10, 11, 12, 14, 16, 17
PC3 (%)	10.09		9.07	1, 2, 3, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 17
PC4 (%)	5.31		8.23	1, 2, 3, 5, 9, 11, 12, 16, 17
Overall%	84.59		85.41	

Table 9. The results of Principal Components of *Channa striata* from Lake Mainit in terms of sexes.

PCA	Individual (symmetry)	Sides (Directional Asymmetry)	Interaction (Fluctuating Asymmetry)	Landmarks affected
Male				
PC1 (%)	54.20	100%	32.81	1, 2, 5, 6, 7, 9, 11, 12, 13, 14,15, 16, 17
PC2 (%)	19.25		26.02	1, 2, 3, 5, 7, 9, 10, 11, 12, 14, 15, 16
PC3 (%)	8.05		13.09	1, 6, 7, 10, 11, 14, 15, 16, 17
PC4 (%)	5.62		7.43	2, 5, 7, 8, 9
PC5 (%)	---		5.77	
Overall%	87.12		85.12	
Female				
PC1 (%)	67.25	100%	30.39	1, 2, 3, 6, 10, 13, 14, 15,16, 17
PC2 (%)	14.68		22.07	1, 2, 7, 9, 10, 11, 12, 14, 16, 17
PC3 (%)	6.59		11.82	1, 2, 3, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 17
PC4 (%)	---		10.12	1, 2, 3, 5, 9, 11, 12, 16, 17
PC5 (%)	---		6.49	
Overall%	88.52		80.89	

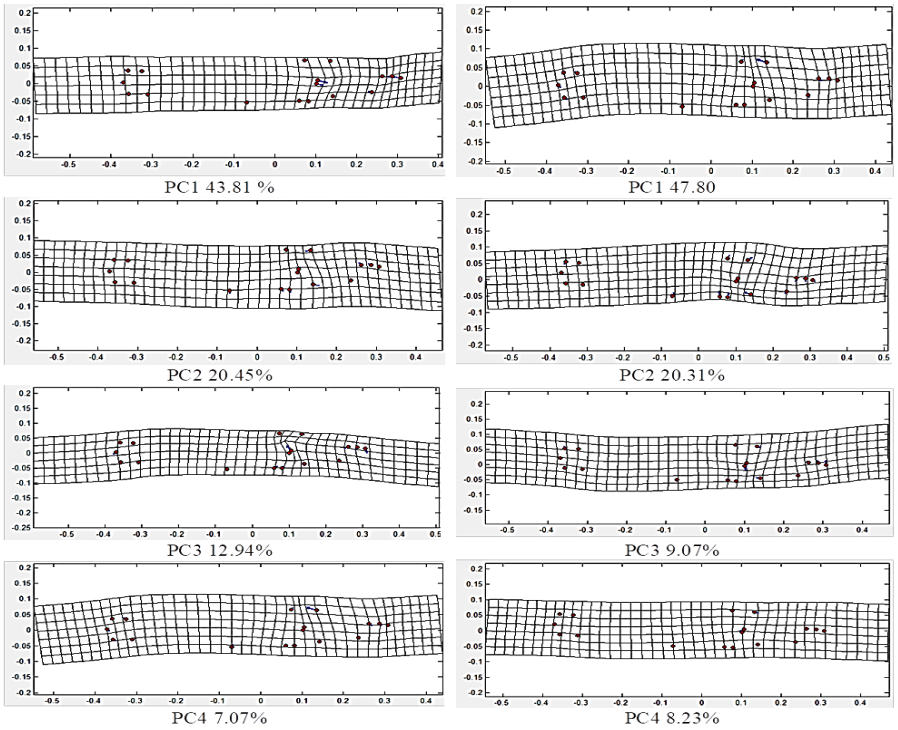


Figure 4. The male (first column) and female (second column) fishes from Agusan Marsh with the affected landmarks shown in PCA-deformation grid.

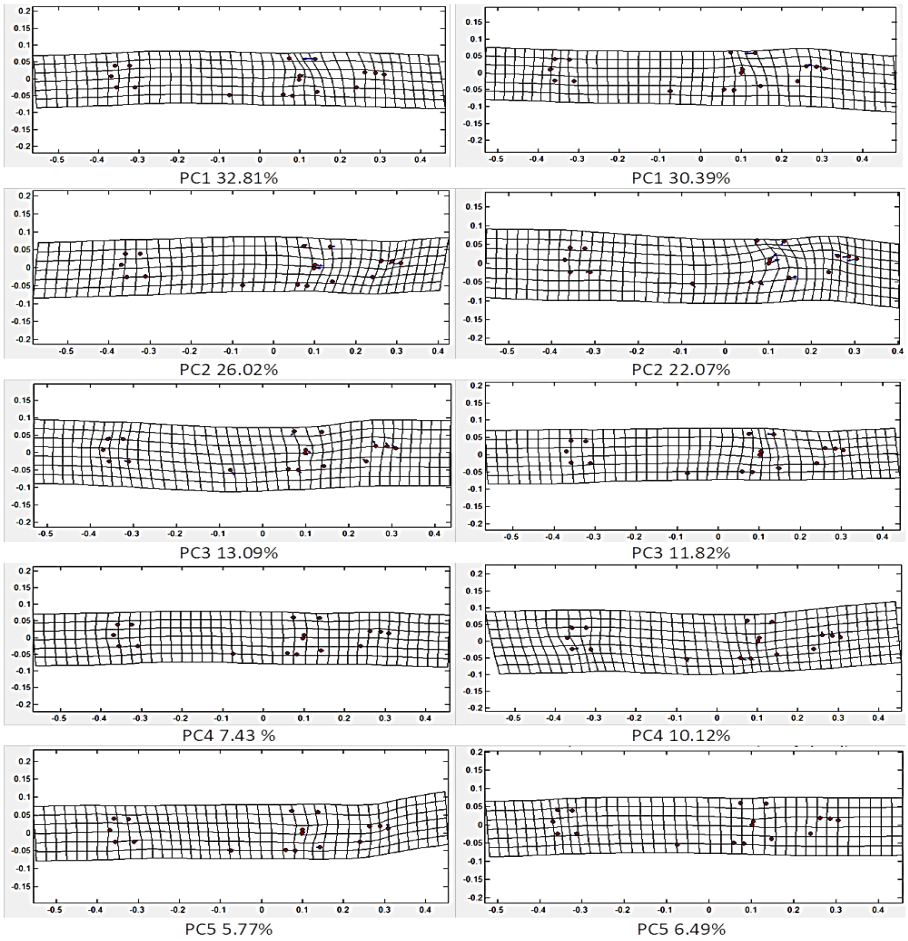


Figure 5. The male (first column) and female (second column) fishes from Lake Mainit with the affected landmarks shown in PCA-deformation grid.

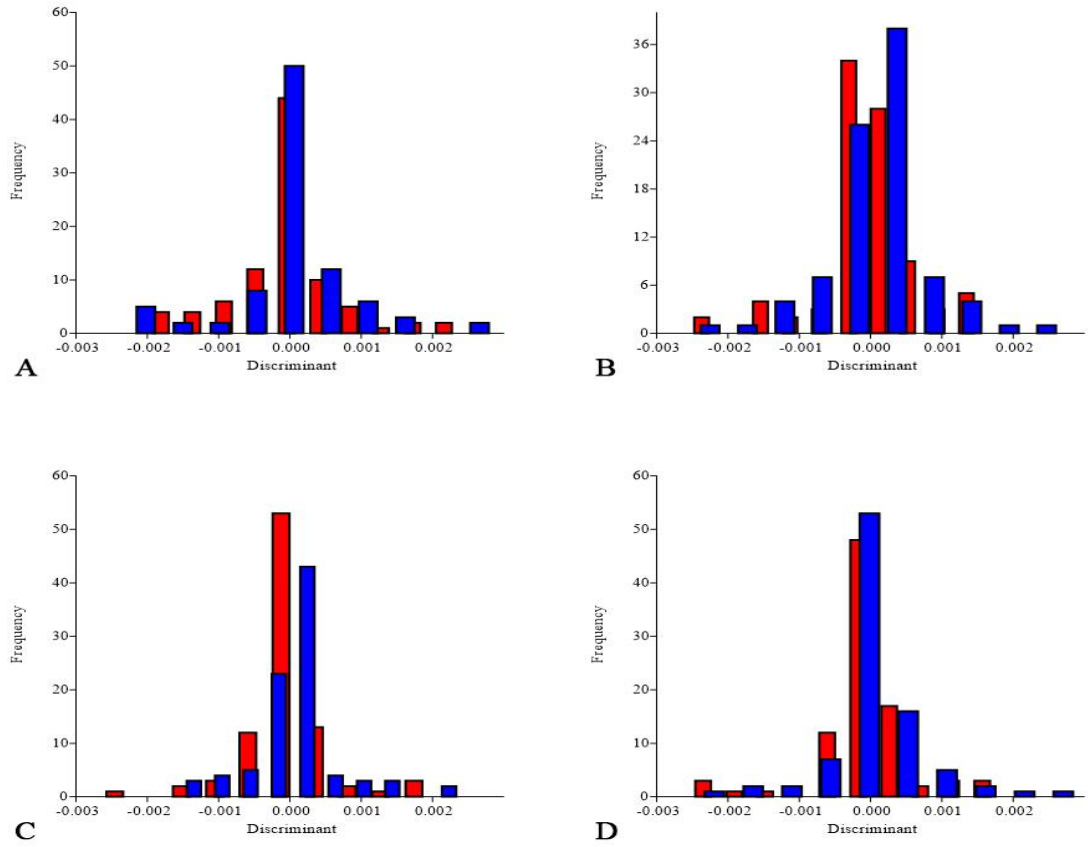


Figure 6. Discriminant plot of sexual dimorphism: A (*left* flank) and B (*right* flank) from Agusan Marsh; C (*left* flank) and D (*right* flank) from Lake Mainit.

Table 10. Result of Discriminant analysis of Sexual Dimorphism of *Channa striata*.

Discriminant analysis	*Channa striata*			
	Agusan Marsh		Lake Mainit	
	Left flank	Right flank	Left flank	Right flank
Hotelling's T^2	2.9337	2.1325	1.7599	2.1011
F-value	3.1131	2.263	1.8676	2.2296
p-value	1	1	1	1

Table 11. Physico-Chemical Parameters of both sampling areas gathered in the month of December 2018.

Sampling area	Parameters					
	TSS (ppm)	TDS (ppm)	DO (ppm)	pH	Salinity (ppm)	Temperature (^{O}C)
*Tagbuyawan	10.5.	101	7.86	7.73	0.03	29.35
*Mayag	3.4	100	7.52	8.07	0.03	29.50
*Magpayang	11.8	113	8.93	7.07	0.03	29.30
*Magtiaco	72.4	125	12.1	7.35	0.01	28.95
*Jaliobong	15.0	101	7.73	7.67	0.01	29.66
*Kalinawan	31.0	105	7.97	7.48	0.01	29.16
**Lake Mihaba	47.8	92	3.37	6.9	0.05	28.52
**Lake Mihaba Creek (outlet)	37.4	73	3.42	6.93	0.04	28.81
**Agusan river (downstream)	61.0	153	3.44	8.14	0.11	28.94
**Loreto river (downstream)	30.0	145	3.46	7.87	0.11	28.65
**Agusan river (upstream)	63.2	226	3.42	8.02	0.17	29.06
** Sumilao (downstream)	57.60	104	4.55	7.8	0.07	28.72

Note: Legend: *Lake Mainit sampling stations, ** Agusan Marsh sampling stations

Table 12. Comparisons of Physico-chemical parameters and concentrations of CN, Fe, Zn, Cu, Pb to DENR standards (as sampled in Lake Mainit sampling stations on November 11, 2018, and Agusan Marsh sampling stations on December 05, 2018.

Parameters	Available range		DENR
	Lake Mainit	Agusan Marsh	
TSS (ppm)	3.4-10.5	30-63.2	80
TDS (ppm)	100-125	73-226	96-100
DO (ppm)	7.52-12.1	3.37-4.55	5
pH	7.07-8.07	6.9-8.14	6.5-9.0
Salinity (ppm)	0.01-0.03	0.04-0.17	Nd
Fe (ppm)	<0.01-0.09	0.46-3.39	2
Zn(ppm)	<0.01	<0.01	1.5
Cu (ppm)	<0.01	<0.01	0.02
Pb (ppm)	<0.01	<0.01-0.01	0.02
CN^-(ppm)	<0.01	<0.01	0.1

Note: nd: no data

Physico-chemical, cyanide and four base metals

Table 11 shows the result of the physicochemical parameter in the sampling area of Lake Mainit and Agusan Marsh using multiparameter test. In Lake Mainit sampling stations, Magtiaco river mouth recorded the highest total suspended solids, total dissolved solids and dissolved oxygen level. It may be due to the heavy coarse load and runoff from the landslide in small-scale mining areas of upstream mountainous areas. during Typhoon (Sanba) Basyang of February 2018. Salinity, pH and temperature were mostly the same among other sampling sites. In Agusan Marsh sampling area, upstream and downstream of Agusan river were observed with high total suspended solids, total dissolved solids, pH and temperature due to the fact that Agusan River is the receiver of almost eleven tributaries including the other sampling sites. The dissolved oxygen and salinity were mostly the same among other sampling sites in Agusan Marsh sampling area.

Furthermore, the analysis of four base metals and Cyanide indicated that Zinc, Copper, Lead, and Cyanide levels were in less than detection limit of 0.01 ppm in Lake Mainit and Agusan Marsh sampling stations, except the value of Lead was 0.01 in Lake Mihaba Creek (outlet) of Agusan Marsh. In terms of Iron content, Magtiaco (0.09 ppm) in Lake Mainit had the highest value while other locations of the said sampling site had values ranging from <0.01-0.01 ppm. Agusan Marsh sampling stations had varied Iron content all above the detectable limit of 0.01 ppm ranging from 0.46 in Agusan river (upstream) to 3.39 in Lake Mihaba.

Table 12, shows the range and comparison of the water parameters that were tested in the selected sampling sites to the DENR recommended water quality standards and contamination limit on toxic and deleterious substances. The range of the parameter values was observed high mostly in Agusan Marsh TSS, TDS, Salinity and Iron (Fe) while Dissolve oxygen had low values compared to Lake Mainit. Out of the four base metals, only Iron (Fe) was recorded above detectable limit and observed high in Agusan Marsh. Cyanide was below detectable limit and acceptable to the DENR standards. Total dissolved solids in both Lake Mainit and Agusan Marsh were not acceptable to the standard set by DENR that should not surpass 96-100 ppm except in Lake Mihaba and its outlet creek that was in the acceptable limit (see table 3). Total suspended solids, TSS, pH, Zn, Cu, Pb, CN^-values of Lake Mainit and Agusan Marsh were acceptable to the recommended standards. On the other hand, in terms of Dissolved Oxygen level, Agusan Marsh is not acceptable to the recommended standards. At the same time, Iron (Fe) level specifically in Lake Mihaba and its outlet in Agusan Marsh were not acceptable to the DENR standards. Salinity however owns at the level characterized by freshwater.

The values of selected physicochemical parameters differed from each habitat such as dissolved oxygen level and Iron which recorded higher in Agusan Marsh compared to Lake Mainit. These differences due to the surroundings and the ecosystem type of each habitat. The Agusan Marsh is an extensive floodplain in the middle of the Agusan River Basin in eastern Mindanao which served as stopped drainage of waters from rivers, creeks and tributaries mainly in the provinces of Agusan del Norte, Agusan del Sur and Compostela Valley and development for agriculture, construction of dams and reservoirs for irrigation, deliberate or accidental introductions of exotic species, e.g., tilapia, carps, janitor fish, and golden apple snail, and logging in the watershed areas. (Primavera and Tumanda Jr. 2008). While Lake Mainit is an oligotrophic lake based on its physicochemical, natural productivity and optical properties and morphometric characteristics (Tumanda Jr. et al. 2003). The study provided that *C. striata* which is a food source and bioresources for livelihood generation can be propagated in freshwater aquaculture even in a stressful environment.

In conclusion, the landmark-based geometric morphometric was a useful tool in describing the fluctuating asymmetry and sexual dimorphism of *Channa striata*. The statistical results showed high variations ($p < 0.0001$) on the left and right side of the bilateral represented by 79.22% in Agusan Marsh specimens and 72.35% in Lake Mainit specimens which may be considered as relatively high Fluctuating Asymmetry. The affected landmark points and variation are observed in the cephalic, pectoral, and caudal regions which may be due to burrowing behavior and association of the lateral line system among fishes that thrive in high turbid waters and

low oxygen. Further, the physicochemical analysis reveals that Agusan Marsh had low dissolved oxygen, TDS, and Iron deviates to the national water quality standards while Lake Mainit is within permissible limits. On the other hand, the total FA in terms of sexes, Agusan marsh had 84.27% and 85.41%, while Lake Mainit had 85.12% and 80.89% respectively in males and females. The fairly similar FA% based on the 17 landmark points of all *C. striata* population confirms to less or no sexual dimorphism of fish in the study area. The present study contributes essential information about the body variations of *C. striata* and the condition of Agusan marsh basin and Lake Mainit and importance of FA in determining the habitat condition the fact that organisms, *C. striata* preferred high turbidity and thrived in stress condition. Further study is needed regarding Fluctuating Asymmetry of *C. striata* in relation to seasonal variations.

REFERENCES

Adams DC, Rohlf FJ, Slice DE. 2009. Geometric Morphometrics: Ten Years of Progress Following the 'Revolution'. Ital J Zool 71: 5-16. DOI: 10.1080/11250000409356545.

Beasley DAE, Bonisoli-Alquati A, Mousseau TA. 2013. The use of fluctuating asymmetry as a measure of environmentally induced developmental instability: A meta-analysis. Ecol Indic 30: 218-226. DOI: 10.1016/j.ecolind.2013.02.024.

Broadley DG. 1965 Serpentine Systematics-A Review of Taxonomic Characters. J Herpetol Assoc Afr 1: 7-10. DOI: 10.1080/04416651.1965.9650678.

Cagauan AG. 2007. Exotic aquatic species introduction in the Philippines for aquaculture-A threat to biodiversity or a boon to the economy?. J Environ Sci Manag 101: 48-62.

Chaudhry S. 2010. *Channa striata*. The IUCN Red List of Threatened Species 2010: e.T166563A6237224. https://www.iucnredlist.org/species/166563/6237224

Coda JA, Martínez JJ, Steinmann AR, Priotto J, Gomez MD. 2017. Fluctuating Asymmetry as an Indicator of Environmental Stress in Small Mammals. Mastozool Neotrop 24: 313-321. DOI: 10.1016/B978-0-08-045337-8.00117-0.

Dumalagan FA, Garcines JV, Boyles LZ. 2017. Reproductive Biology, Length-Weight Relationship and Condition Factor of *Channa striata* (Bloch, 1793) from tributaries of Lake Kilobidan, Agusan Marsh, Philippines. Intl J Comput Commun Instrum Eng 4: 78-81. DOI: 10.15242/ijccie.ae0117114.

Guerrero III RD. 2014. Impacts of Introduced Freshwater Fishes in the Philippines (1905-2013): A Review and Recommendations Philipp J Sci 143: 49-59.

Jumawan JH, Abastillas SOMAL, Dicdican MJA, Cabag MB, Gamolo EM, Velasco JPB, et al. 2016. Fluctuating asymmetry in the body shapes of goby fish *Glossogobius giuris* from Agusan River, Butuan City, Agusan del Norte, Philippines. AACL Bioflux 9: 133-142.

Kasumyan AO. 2003. The Lateral Line in Fish: Structure, Function, and Role in Behavior. J Ichthyol 43: S175-S213.

Leary RF, Allendorf FW. 1989. Fluctuating asymmetry as an indicator of stress: Implications for conservation biology. Trends Ecol Evol 4: 214-217. DOI: 10.1016/0169-5347(89)90077-3

Lymbery AJ. 1992. The environmental control of colouration in a bushcricket, *Mygalopsis marki* Bailey (Orthoptera: Tettigoniidae). Biol J Linn Soc 45: 71-89. DOI: 10.1111/j.1095-8312.1992.tb00632.x.

McCombe PA, Greer JM. 2014. Sexual dimorphism in the Immune System. In: Mackay IR, Rose NR (eds.). The Autoimmune Diseases 5th. Academic Press, Elsevier, Amsterdam. DOI: 10.1016/B978-0-12-384929-8.00024-1

Mittal AK, Agrawal N. 1994. Modifications in the epithelia of lips and associated structures of the predatory murrel (*Channa striata*). J Appl Ichthyol 10: 114-122. DOI: 10.1111/j.1439-0426.1994.tb00150.x.

Mitteroecker P, Gunz P. 2009. Advances in Geometric Morphometrics. Evol Biol 36: 235-247. DOI: 10.1007/s11692-009-9055-x.

Musikasinthorn P. 2004. Channoidei (snakeheads). In: Hutchins M et al. (eds). Grizmek's Animal Life Encyclopedia Vol. 5, 2nd ed. Gale Virtual Reference Library, Detroit, USA.

Niskanen M, Ruff CB, Holt B, Sládek V, Berner M. 2017. Temporal and Geographic Variation in Body Size and Shape of Europeans from the Late Pleistocene to Recent Times. In: Ruff CB (eds). Skeletal Variation and Adaptation in Europeans: Upper Paleolithic to the Twentieth Century. John Wiley & Sons, Inc., New York DOI: 10.1002/9781118628430.ch4

Olvido AE, Mousseau TA. 2012. Geographical Variation. In: Encyclopedia of Life Sciences. John Wiley & Sons Ltd, Chichester. DOI: 10.1002/9780470015902.a0001736.pub3.

Parker G. 1992. The Evolution of Sexual Size Dimorphism In Fish. J Fish Biol 41: 1-20. DOI: 10.1111/j.1095-8649.1992.tb03864.x.

Parsons PA. 1992. Fluctuating Asymmetry: A Biological Monitor of Environmental And Genomic Stress. Heredity 68: 361-364. DOI: 10.1038/hdy.1992.51.

Phen C, Thang TB, Baran E, Vann LS. 2005. Biological Reviews of Important Cambodian Fish Species, Based on Fishbase 2004. WorldFish Center and Inland Fisheries Research and Development Institute, Phnom Penh, Cambodia.

Ralls K, Mesnick S. 2009. Sexual Dimorphism. In: Perrin WF, Würsig B, Thewissen JGM (eds). Encyclopedia of Marine Mammals (2nd ed). Academic Press, Elsevier, Amsterdam. DOI: 10.1016/B978-0-12-373553-9.00233-9.

Quirog DG, Tabugo SR. 2015. Population Analysis Via Fluctuating Asymmetry in The Wings of Aedes albopictus from selected barangays in Iligan City, Philippines. J Entomol Zool Stud 3: 34-39.

Santos JC, Alves-Silva E, Cornelissen TG, Fernandez GW. 2013. The Effect of Fluctuating Asymmetry and Leaf Nutrients On Gall Abundance and Survivorship. Basic Appl Ecol 14: 489-495. DOI: 10.1016/j.baae.2013.06.005.

Sayer MDJ. Adaptations of Amphibious Fish for Surviving Out of Water. Fish Fish 6: 186-211.

Seigel RA, Ford NB. 2001. Phenotypic plasticity in reproductive traits: geographical variation in plasticity in a viviparous snake. Funct Ecol 15: 36-42. DOI: 10.1046/j.1365-2435.2001.00492.x.

Sullivan RM, Best TL. 1997. Effects of environment on phenotypic variation and sexual dimorphism in *Dipodomys simulans* (Rodentia: Heteromyidae). J Mammal 78: 798-810. DOI: 10.2307/1382937.

Tomkins JL, Kotiaho JS. 2001. Fluctuating Asymmetry. In: Encyclopedia of Life Sciences. DOI: 10.1038/npg.els.0003741.

Vitt LJ, Caldwell JP. 2014. Communication and Social Behavior. In: Vitt LJ, Caldwell JP (eds). Herpetology an Introductory Biology of Amphibians and Reptiles (4th ed). Academic Press-Elsevier, Amsterdam. DOI: 10.1016/B978-0-12-386919-7.00009-5.

Webster M, Sheets DH. 2010. A Practical Introduction to Landmark-Based Geometric Morphometrics. In: Alroy J, Hunt G (eds). Quantitative Methods in Paleobotany. The Paleontological Society Papers. Cambridge University Press, UK.

Wu Z, Yu D, Wang Z, Li X, Xu X. 2015. Great influence of geographic isolation on the genetic differentiation of *Myriophyllum spicatum* under a steep environmental gradient. Sci Rep 5: 15618. DOI: 10.1038/srep15618.

NUSANTARA BIOSCIENCE
Vol. 13, No. 1, pp. 111-120
May 2021

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n130115

Assessing the susceptibility of the selected gourami (*Osphronemus goramy*) to *Aeromonas hydrophila*

RITA FEBRIANTI[1,2,♥], IKHSAN KHASANI[2], KEUKEU KANIAWATI ROSADA[1,♥♥]

[1]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: rita19002@mail.unpad.ac.id, ♥♥email: keukeu@unpad.ac.id
[2]Research Institute for Fish Breeding, Ministry of Marine and Fisheries. Jl. Raya 2 Sukamandi, Subang 41263, West Java, Indonesia

Manuscript received: 12 March 2021. Revision accepted: 26 May 2021.

Abstract. *Febrianti R, Khasani I, Rosada KK. 2021. Assessing the susceptibility of the selected gourami (*Osphronemus goramy*) to* Aeromonas hydrophila. *Nusantara Bioscience 13: 111-120.* A breeding program to improve the growth performance of the gourami fish was carried out through selection methods that produced faster growth gourami (selected population). The purpose of this study was to determine the susceptibility of the selected gourami to *Aeromonas hydrophila* infection based on tolerance limits (LD_{50}) and investigated clinical signs post-injection of the pathogenic bacteria. The challenge test by intramuscular injection of *A. hydrophila* was done to the fingerling fish (15-20 g) for 14 days post-infection. The population of the tested fish was obtained from six families, selected gourami (SP), and non-selected control (CP) groups. Phosphate buffered saline (PBS) as control and several doses of the pathogen, 10^2, 10^4, 10^5, 10^6, 10^7, and 10^8 CFU/mL of *A. hydrophila*, were injected into the fish. Fish mortality and clinical signs were observed daily. The fish mortality was confirmed by isolating bacteria in the fish which showed clinical signs, followed by biochemical characterization of the isolated bacteria using API 20E and PCR. The LD_{50} of *A. hydrophila* to the selected population (9.70×10^5 CFU/mL) was higher than that of the control (6.50×10^4 CFU/mL). On the final day of the test, the accumulation mortality of CP (63.33±5.77%) higher than that in SP (33.33±5.77%). Based on the output test statistics, it was known that there was a significant difference between the mortality and clinical signs of SP and CP. The data suggested that the selected gourami were more resistant to *A. hydrophila* infection than that of the control. The *A. hydrophila* infection caused most of the major clinical signs, including mass mortality of the fish. The biochemical and PCR test ensure that fish mortality was caused by *A. hydrophila* infection.

Keywords: *Aeromonas hydrophila*, challenge test, clinical signs, fish breeding, selected gourami

INTRODUCTION

Giant gourami (*Osphronemus goramy* Lacepede 1801) is an Indonesian native fish with high economic value. The national productivity of the gourami consistently increased, from 35.09% in 2015 to 198.97% in 2017, and 133.48% in 2018 (Ministry of Marine Affairs and Fisheries 2018). Therefore, the intensive farming of the fish has a high prospect to be developed. However, the slow growth character of the gourami is a serious problem to develop industrial farming of the fish. Disease attack is one of the obstacles in fish farming. Diseases can be caused by bacteria, viruses, parasites, and fungi. Bacterial disease in fish had been known since 1980. *Aeromonas hydrophila* bacteria causes Motile Aeromonad Septicemia (MAS) disease in gourami (Öztürk and Altinok 2014; Kusdarwati et al. 2018). *Aeromonas hydrophila* bacterial infection causes death and reduces the quality of the harvested fish (Purwaningsih et al. 2014). MAS disease is reported to cause mass mortality of cultured gourami in Indonesia. In 2016, the disease caused the loss of 47 tons of the gourami in West Sumatra (Department of Marine Affairs and Fisheries, Padang Pariaman Regency 2015), and mortality of 87-100% of the gourami population in the Banyumas area, the district of Central Java (Khumaidi and Hidayat 2018).

Intensive fish farming has a high prospect to be developed. Even though, the prevalence of disease outbreaks is relatively higher in this system. One of the major diseases in freshwater fish farming is Motile Aeromonad Septicemia (MAS) causing by *A. hydrophila*. The pathogenic bacteria cause tissue damage (invasiveness) and produce toxins (toxigenic) (Fernandez and Figueras 2020). The attack of *A. hydrophila* bacteria causes several clinical signs. Clinical signs are indications of the presence of a disease in the form of disease characteristics. According to Rozi et al (2018), the clinical signs of *A. hydrophila* bacteria attack is the presence of septicemic hemorrhage which is characterized by wounds on the body's surface, gills, ulcers, abscesses, exophthalmia, and flatulence. *A. hydrophila* bacteria cause disease outbreaks in catfish (*Clarias* sp.*)* (Kusdarwanti et al. 2017), tilapia (*Oreochromis niloticus*) (Hardi et al. 2018), gourami (*Osphronemus goramy*) (Rozi et al. 2018), and snakehead fish (*Ophicephalus striatus*) (Rao and Benarjee 2016). The determination of the observation period is based on the statement of Kusumawaty et al. (2016), that *A.hydrophila* bacterial infection in gourami is acute.

In the context of prevention and control of MAS disease, several strategies alternative has been done, including the management of integrated fish health, the use of MAS-free fish, and the vaccination (Ma et al. 2019). On the other hand, the specific pathogen resistance (SPR) of several cultured fishes has been developed in the world. Nevertheless, the SPR of MAS disease is not yet be

reported. Based on our observation in the gourami population, there were wide variations in the resistance level of the fish to bacterial disease. Refer to Hendry et al. (2011), the high variation of a specific character (disease resistance) in the population suggested that a selection method may be conducted to improve the character. In the natural disease outbreak, there were 10-20% of fish survive (survivor) (Subhan et al. 2020). Furthermore, the survivor can establish a based population to develop the SPR population (Moss et al. 2012). Based on the previous studies in the cultured species, selective breeding to produce MAS-resistant gourami may be conducted. Selective breeding for increased disease resistance is a promising strategy that has not been widely used in aquaculture. At the same time, improving growth performance is critical for efficient production (Hua et al. 2019). A study about the susceptibility of selected gourami, the specific strain of Indonesia, to *A. hydrophila* is not yet be conducted.

The breeding program to improve the growth character of the gourami has been conducted in Indonesia for several years. The breeding program was done through selection, hybridization, and genetic engineering (Nugroho et al. 2012; Sularto et al. 2016; Arifin et al. 2017). The main purpose of selection is to change the quantitative phenotype of the population mean by exploiting genetic additives that have a beneficial trait from elders to tillers. For this purpose, the base population with high genetic variation is important to produce superior varieties. Several years of breeding programs to improve the growth character of gourami had been conducted and resulted in a selected population. Besides growth, resistance to disease and tolerance to pond conditions are important factors to aquaculture productivity (Reid et al. 2019). Therefore, a study to evaluate the susceptibility level to the disease on the selected gourami was conducted. LD_{50} is the amount of a toxic agent (such as a poison, virus, or radiation) that is sufficient to kill 50 percent of a population of animals. The purpose of the recent study was to evaluate the resistance level of the selected gourami, based on tolerance limits (LD_{50}) and investigated clinical signs post-injection of the pathogenic bacteria.

MATERIALS AND METHODS

Experimental location

This research was conducted in February 2020 at the research facilities of the Research Institute for Fish Breeding, West Java, Indonesia, including the gourami hatchery, The Microbiology Laboratory, The Genetics Laboratory, and The Water Quality Laboratory. The Research Institute is located at latitude 6°22'20.6"S 107°37'18.5"E, and an altitude of 25 meters above sea level (GPS Coordinates 2017).

Procedures

Research design

A total of 420 fingerlings were used for both the selected gourami (SP) and non-selected control (CP) groups. The SP and CP groups were chosen because the SP group was the result of the selection that had faster growth than the CP group. In addition, disease resistance is a promising strategy that has not been widely used in cultivation. The healthy gourami fingerlings weighed 16.2±2.12 g of body weight and measured standard length 9.80±0.48 cm. A completely randomized design was used in this study with seven treatments and three replicates. The treatments were infection of *A. hydrophila* to the gourami in several doses:

(i) SPP: Selected gourami injected with Phosphate Buffered Saline (PBS)
(ii) SP-10^2: Selected gourami infected with 10^2 CFU/mL of *A. hydrophila*
(iii) SP-10^4: Selected gourami infected with 10^4 CFU/mL of *A. hydrophila*
(iv) SP-10^5: Selected gourami infected with 10^5 CFU/mL of *A. hydrophila*
(v) SP-10^6: Selected gourami infected with 10^6 CFU/mL of *A. hydrophila*
(vi) SP-10^7: Selected gourami infected with 10^7 CFU/mL of *A. hydrophila*
(vii) SP-10^8: Selected gourami infected with 10^8 CFU/mL of *A. hydrophila*
(viii) CPP: Control gourami injected with Phosphate Buffered Saline (PBS)
(ix) CP-10^2: Control gourami infected with 10^2 CFU/mL of *A. hydrophila*
(x) CP-10^4: Control gourami infected with 10^4 CFU/mL of *A. hydrophila*
(xi) CP-10^5: Control gourami infected with 10^5 CFU/mL of *A. hydrophila*
(xii) CP-10^6: Control gourami infected with 10^6 CFU/mL of *A. hydrophila*
(xiii) CP-10^7: Control gourami infected with 10^7 CFU/mL of *A. hydrophila*
(xiv) CP-10^8: Control gourami infected with 10^8 CFU/mL of *A. hydrophila*

Fish and acclimatization

The population of the tested fish was obtained from six families, each for SP and CP. All the families resulted from one spawning batch in the controlled spawning pond of the gourami in the Research Institute for Fish Breeding. The fish were healthy and were free from *A. hydrophila*. Before the experimental tests, the fish were acclimatized for 14 d in fiber tanks (200 L capacity) under laboratory conditions. According to the research design, the normal fish, based on behavior, feeding response, and health status, were distributed in the 40 L of glass aquariums according to the research design.

Aeromonas hydrophila *and preparation of the culture medium*

Aeromonas hydrophila bacteria (American Type Culture Collection 35654 (ATCC 35654) was obtained from National Reference Laboratory the Biosafety in Microbiological and Biomedical Laboratories from the U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institute for Health, belonging to the Collection of Bacterial

Enteropathogens and maintained in cryopreservation. The liquid culture medium *Tripton Soya Broth* (TSB-Oxoid, England) was prepared according to the instructions of the manufacturer by dissolving 30 g in 1 L of distilled water. The sterilization was processed by autoclaving at 121°C for 15 min (Daihan Scientific Co., Ltd, Korea). The RS medium base (RS agar-Himedia, Mumbai) was prepared according to the manufacturer's instructions by dissolving 45.43 g in 990 mL of distilled water and it should not be sterilized using an autoclave. After removal and cooling between 45-50 °C, one vial of Novobiocin Supplement (FD096) was added followed by homogenization and distribution to the sterile Petri plate.

Preparation of A. hydrophila *suspensions*

Bacterial suspensions were prepared by transferring a heave containing three to five colonies of *A. hydrophila* isolated in Petri dishes containing the RS agar medium after 24 h of cultivation at 31°C to tubes containing 10 mL of BHI broth and reincubated for 18 h in a bacteriological incubator (Memmert, Germany). After incubation of the bacterial suspensions with the culture, the logarithmic growth phase was measured by turbidity caused by bacterial growth; Spectrophotometer (Hitachi U1500, Japan) was designed to measure the optical density of a suspension of microorganisms. A bacterial growth test was carried out by growing a bacterial isolate from an agar slant, then inoculating one loop of 30 mL TSB medium and incubating it for 24 h at room temperature. Inoculation of the results of liquid culture on TSB media into new TSB media in a test tube of 2 mL of isolate on 8 mL of liquid TSB. Incubation at room temperature and measuring OD (optical density) with a spectrophotometer at a wavelength of 620 nm every two hours for 30 h (Zubaidah et al. 2019). Before being used as a pathogen test, *A. hydrophila* bacteria was restored through the test of Postulate Koch on SPF gourami varieties. The test of Postulate Kohn was carried out twice. Furthermore, this procedure was carried out by injecting 0.1 mL of inoculum into the test gourami intramuscularly (IM) (Taukhid et al. 2016).

Susceptibility study of the gourami to A. hydrophila

For the pathogenicity test, 420 gourami fishes were introduced into 42 research containers with 60 L water, and *A. hydrophila* was added intramuscularly injected of fish with PBS, 10^2, 10^4, 10^5, 10^6, 10^7, and 10^8 CFU/mL. The mortality rate of the fish was recorded more than 24 h post-infection, and LD_{50} was determined. LD_{50} was estimated using the Dregsted Behrens method (Maryadi 2011). This study was carried out according to the principles adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Committee on the Ethics of Animal Use (CEUA/UFAC: 08/2014).

Clinical signs of the gourami after A. hydrophila *infection*

The symptoms of infection were observed, and after 24-96 h. The observations and measurements were carried out every 24 h. The mortality rate of the fish was determined at the end of the study. Water quality parameters including temperature, pH, dissolved oxygen, ammonia, nitrite, and nitrate were monitored at the beginning and end of the study. Observation of the clinical signs of gourami was carried out every 14 days (Taukhid et al. 2016).

Confirmation test of the fish death

The confirmation test aims to confirm the death of the gourami was caused by *A. hydrophila* bacteria. This test is performed by isolating bacteria from the liver, spleen, and kidneys, then purifying and characterizing it biochemically (API 20 E kit) and molecularly (PCR). The PCR method used is a method developed by (Alpha DNA Montreal, Quebec) using primary pairs of 16S F 5 'GGG AGT GCC TTC GGG AAT CAG A 3' and 16S R: 5'-TCA CCG CAA CAT TCT GAT TTG -3 ' with amplification of preheating temperature of 94°C for 2 min, denaturation temperature of 94°C for 1 min, the annealing temperature of 56°C for 30 sec, extension temperature of 72°C for 45 sec, final extension temperature of 72°C for 5 min with 30 cycles (Hussain et al. 2013). Furthermore, the PCR results were run by electrophoresis for 35 min with a voltage of 60 V and 400 A with a 1.5% agarose gel medium. The PCR product is seen in the High-Performance Ultraviolet Transilluminator with an amplicon target of approximately 356 bp. The data was processed with three replications of the measured data and analyzed descriptively.

Data analysis

Water quality data were reported as mean ± SD. Observation of the clinical signs of the gourami was carried out every day by descriptive analysis to compare between groups. Water quality cultivation was used as a supporting parameter. Clinical signs were analyzed with a Mann-Whitney u test using SPSS. The data LD_{50} obtained from the research observation was analyzed using two-way ANOVA followed by Tukey's post hoc test to analyze data sets. The confirmation test was done to confirm the death of gourami is caused by *A. hydrophila*.

RESULTS AND DISCUSSION

Susceptibility study of the gourami to *A. hydrophila*

In aquaculture, disease outbreaks in aquaculture are affected by three factors, such as host, pathogen, and environment. This study evaluated several doses of the pathogenic bacteria (*A. hydrophila*) and two populations of the gourami in the optimal condition.

The susceptibility of SP to *A. hydrophila* was indicated by 100 % mortality at a dose of 1×10^8 CFU/mL after 44 h. Different *A. hydrophila* susceptibility seen in CP was indicated by 100 % mortality at a dose of 1×10^8 CFU/mL and 1×10^7 CFU/mL after 24 h. The *A. hydrophila* bacterial at a dose of 1×10^2 CFU/mL did not cause the death of the SP and CP. The LD_{50} value of *A, hydrophila* to the CP, and SP were 6.50×10^4, and 9.70×10^5 CFU/mL respectively. The result of the analysis shows that F arithmetic is bigger than the F table (5 % and 1 %). Pairwise testing between SP and CP was performed by treatment when a significant interaction was found and multiple comparison adjustment was made using Tukey's

method. There was a significant ($p < 0.05$), with a calculated F value of 96.100 > 4.35 F table, it can be concluded that there were differences in mortality results based on the type of population and the concentration of infected bacteria. There was a significant ($p < 0.05$), with an F count of 146.650 > 2.87 F table, it can be concluded that there were differences in mortality results based on the type of gourami population. There was a significant ($p < 0.05$), with an F value of 14.350 > 2.87 F table, it can be concluded that there was an interaction based on the type of gourami population and the concentration of bacteria injected in determining mortality in gourami. This indicates that the *A. hydrophila* batch-test conducted for 14 days in gourami had a significant effect on the susceptibility of gourami. The mortality of the SP and CP during the LD_{50}-96h of *A. hydrophila* is presented in Table 1.

Clinical signs of the gourami after *A. hydrophila* infection

In addition to causing death in the test fish, bacterial infection with lower doses can cause physiological disturbances which are indicated by several clinical symptoms, including loss of balance (LB), loss of appetite (LA), weakness (W), ulcers (U), necrosis (N), and hemorrhagic (H). Based on the output test statistics, it was known that the Asymp. Sig. (2-tailed) <0.05 for parameters LB, LA, W, U, N, and H. Thus there was a significant difference between the clinical signs of SP and CP. CP clinical symptoms were severe than SP. The clinical signs of the SP and CP post *A. hydrophila* infection presented in Table 2.

The early clinical signs that arise in this study were loss of balance or buoyancy control, floating upside down, or 'sitting' on the tank floor (most fish are normally only slightly negatively buoyant and take little effort to maintain the position the water column). As the disease progresses, the following may be observed: change in coloration, cloudy skin (indicates excess mucous production due to an irritant bacterial. Several hours after *A. hydrophila* injection, several clinical signs were observed in the infected gourami presented in Figure 1.

The bioassay pathogenic test must be conducted at the optimum level of water quality parameters to certain that the pathogen causes fish mortality. Refer to (Republic of Indonesia 2017), the main water parameters of the treatment tanks during this study were maintained at the optimum level for the giant gourami. They were 28.1-29.0°C of temperature, 8.00-8.42 of pH, 5.9-8.5 mg/L of DO, 0.0020-0.0163 mg/L of ammonia, 0.0097-0.0540 mg/L of nitrite, and 0.0430-0.9634 mg/L of nitrate. Based on the optimum level of water quality, the A. hydrophila infection causes the mortality of the fish during the recent study. The water quality parameters during this study were presented in Table 3.

Confirmation test of the fish death

Confirmation of fish mortality is carried out to ensure death due to the bacterial injection. All SP and CP fish were isolated from the dead fish from the liver, kidney, and spleen organs. As many as 100% of all SP and CP fish were isolated from the dead fish from the organs of the test results from the fermentative test, catalase test, gram stain test, PCR test, and API 20 E test showed that A.hydrophila caused the death of gourami. The fermentative test showed the color of the media turned yellow (Figure 2.A-B). The catalase test on bacteria that caused clinical signs showed positive results due to the formation of bubbles (Figure 2.C-D). Pure isolated bacteria were then stained and the result was that the bacteria were rod-shaped, short-chain, and red when tested with gram stain (Figure 2.E). The results of the PCR and API 20 E tests showed that fish mortality was caused by infection with *A. hydrophila* bacteria (Hussain et al. 2013). The results of confirmation bacterial isolation *A. hydrophila* of liver, kidney, and spleen organs of fish showed a band around 356 bp presented in Figure 3. Tests using API 20 E showed very good identification results with a percent ID value of 99% presented in Table 4.

Discussion

The LD_{50} use of host death provides a nonequivocal endpoint and measurement has the advantage that it allows comparisons across microbes (Casadeval 2017). LD_{50} figures are frequently used as a general indicator of a substance's acute toxicity. A lower LD_{50} is indicative of increased toxicity (Aisiah et al. 2020). Growth and survival are the major character in the aquaculture system and most selection programs focused on these traits (Krishna et al. 2011; Gjedrem et al. 2012). Based on these data, the LD_{50} value of *A. hydrophila* to the control, and selected gourami were 6.50×10^4 and 9.70×10^5 CFU/mL, respectively. At this time, the average mortality in the control gourami had reached 63.33±5.77% and was significantly higher ($p < 0.05$) compared to that of selected gourami (33.33±5.77%). After 96 h post-infection, the gourami in both the control and selected groups showed normal behavior.

Table 1. Mortality of the selected gourami (SP) and control (CP) during the LD_{50}-96h of *Aeromonas hydrophila*

Pathogen concentration (CFU/mL)	Number of fishes (0 h)	Number of dead fishes (96h)	Mortality (%)	LD_{50} (CFU/mL)
SPP	30	0	0.00±0.00	9.70×10^5
SP-10^2	30	0	0.00±0.00	
SP-10^4	30	25	16.67±0.58	
SP-10^5	30	23	23.33±5.77	
SP-10^6	30	20	33.33±5.77	
SP-10^7	30	14	53.33±5.77	
SP-10^8	30	30	100.00±0.00	
CPP	30	30	0.00±0.00	6.50×10^4
CP-10^2	30	1	3.33±5.77	
CP-10^4	30	7	23.33±5.77	
CP-10^5	30	13	43.33±5.77	
CP-10^6	30	19	63.33±5.77	
CP-10^7	30	30	100.00±0.00	
CP-10^8	30	30	100.00±0.00	

Pairwise testing between SP and CP was performed by treatment when a significant interaction was found and multiple comparison adjustment was made using Tukey's method. Based on the output test statistics, it was known that the significant <0.05 for mortality. Thus there was a significant difference between the mortality of SP and CP. The susceptibility of CP was higher than SP. On the final day of the test, the accumulation mortality of CP had reached 63.33±5.77%, which was considerably higher than that in SP (33.33±5.77%) (Tabel 1). Fish susceptibility is manifested in fish mortality. Based on Table 1, the highest mortality occurred at a bacterial dose of 10^8 CFU/mL of 100%. Determining of clinical signs was done by observing the wounds on the outside of the body and the behavior of the gourami seeds infected with *A. hydrophila* bacteria.

Figure 1. Clinical signs in the *O. goramy* were intramuscularly injected with *Aeromonas hydrophila*: A. Ulcers, B. Injury on caudal fin with hemorrhagic foci, C. Swimming at the bottom, D. Lesion at the site of *A hydrophila* inoculation, E. Weakness, and F. Integumentary injuries with depigmentation and hemorrhagic foci

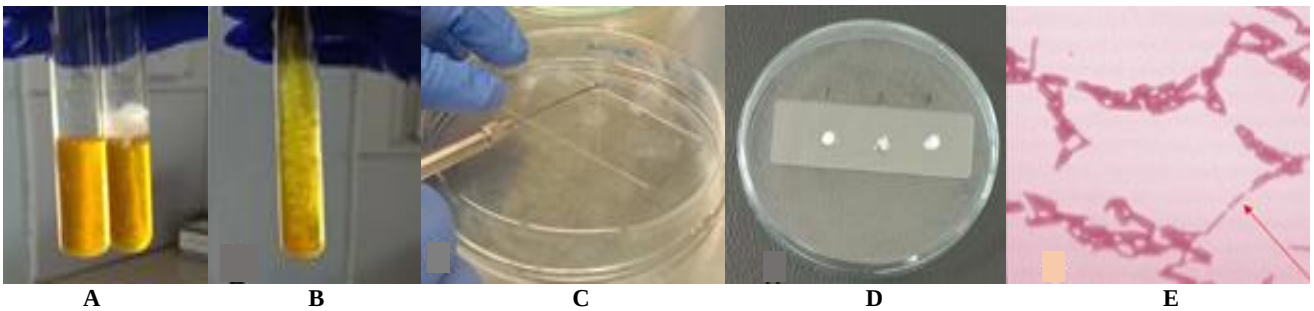


Figure 2. Confirmation of clinical signs and fish mortality by detection of bacteria: A. OF test, B. Test fermentative (TSIA), C. KOH test, D. Catalase test, and E. Gram stain test

Table 2. The clinical signs of the selected gourami (SP) and control (CP) post *Aeromonas hydrophila* infection

Observation time (hours)	Clinical signs		Footnote
	SP	CP	
0	LA-,W-,H-,N-, LB-, U-	LA-,W-,H-,N-, LB-, U-	LA: Loss appetite
12	LA-,W+, H-, N-, LB-,U-	LA+,W++, H+, N+, LB+,U+	W: Weakness
24	LA+, W+, H-, N-, LB+, U+	LA++, W++, H++,N++, LB++, U++	H: hemorrhagic
48	LA+, W+, H+, N+, LB+, U+	LA++, W++, H++,N+++, LB++, U++	NF: Necrosis
72	LA++, W+, H++, N++, LB+, U++	LA++, W++, H+++, N+++, LB++, U+++	LB: Loss of balance
96	LA+, W+, H++, N++, LB+, U++	LA+++, W++, H+++, N+++, LB+, U+++	U: Ulcers
120	LA+, W++, H+, N+, LB-, U+	LA++, W+++, H+++, N++, LB+, U++	-: no symptoms
144	LA+, W++, H+, N-, LB-, U+	LA++, W+++, H++, N+, LB+, U++	+: low
168	LA-, W-, H-, N-, LB-, U+	LA++, W+, H+, N+, LB+, U++	++: moderate
192	LA-, W-, H-, N-, LB-, U-	LA-, W-, H-, N-, LB-, U-	+++: severe
216	LA-, W-, H-, N-, LB-, U-	LA-, W-, H-, N-, LB-, U-	
240	LA-, W-, H-, N-, LB-, U-	LA-, W-, H-, N-, LB-, U-	
264	LA-, W-, H-, N-, LB-, U-	LA-, W-, H-, N-, LB-, U-	
288	LA-, W-, H-, N-, LB-, U-	LA-, W-, H-, N-, LB-, U-	
312	LA-, W-, H-, N-, LB-, U-	LA-, W-, H-, N-, LB-, U-	
336	LA-, W-, H-, N-, LB-, U-	LA-, W-, H-, N-, LB-, U-	

Table 3. Water quality conditions of the selected gourami (SP) and control (CP) during the LD_{50}-96h of *Aeromonas hydrophila*

Patogen concentration (CFU/mL)	PARAMETER					
	Temperature (°C)	DO (mg/L)	pH	Ammonia (mg/L)	Nitrite (mg/L)	Nitrate (mg/L)
SPP	28.7-29.0	8.2-8.5	8.02-8.20	0.0023-0.0132	0.0385-0.0540	0.0532-0.7850
SP-10^2	28.4-28.9	7.4-7.7	8.05-8.10	0.0130-0.0123	0.0110-0.0234	0.0430-0.9634
SP-10^4	28.5-28.7	6.9-7.0	8.40-8.42	0.0090-0.0130	0.0097-0.0172	0.0843-0.5870
SP-10^5	28.2-28.6	6.0-7.1	8.02-8.30	0.0024-0.0130	0.0122-0.0242	0.0432-0.9630
SP-10^6	28.1-28.8	6.1-6.4	8.22-8.37	0.0073-0.0090	0.0154-0.0340	0.3760-0.6260
SP-10^7	28.2-28.6	6.0-6.3	8.10-8.21	0.0035-0.0086	0.0145-0.0321	0.1876-0.6589
SP-10^8	28.2-29.0	5.9-6.3	8.00-8.10	0.0022-0.0163	0.0148-0.0367	0.1743-0.3398
CPP	28.3-28.9	8.1-8.4	8.01-8.20	0.0026-0.0142	0.0388-0.0540	0.0532-0.7856
CP-10^2	28.2-28.8	7.3-7.6	8.06-8.10	0.0128-0.0143	0.0114-0.0238	0.0432-0.9484
CP-10^4	28.4-28.8	6.8-7.1	8.39-8.42	0.0092-0.0132	0.0104-0.0176	0.0840-0.5670
CP-10^5	28.3-28.5	6.1-7.2	8.12-8.30	0.0028-0.0130	0.0126-0.0244	0.0532-0.9632
CP-10^6	28.2-28.9	6.2-6.4	8.22-8.37	0.0073-0.0090	0.0154-0.0348	0.3840-0.6266
CP-10^7	28.3-28.7	6.1-6.3	8.14-8.21	0.0038-0.0088	0.0148-0.0324	0.1882-0.7658
CP-10^8	28.2-28.9	5.9-6.2	8.00-8.20	0.0020-0.0163	0.0152-0.0382	0.1842-0.3698
Threshold value (Republic of Indonesia 2017)	25.0-30.0	> 3.0	6.50-8.50	≤ 0.02	≤ 0.06	≤ 20

Table 4. Identification of bacteria using the API 20 E kit

Reference: Very good identification	% ID	T	Test against			
Significant Taxa						
Aeromonas hydrophila/caviae/sobria 2	99.0	1.0				
Next taxon	% ID	T	Test against			
Aeromonas hydrophila/caviae/sobria 1	99.0	0.69	LDC 25%	CIT 25%	VP 25%	ARA 75%
Complementary test(s)	Glucose g		ESC (HYD)		0/129 R	Methyl Red
Aeromonas caviae	-		+		+	+
Aeromonas hydrophila	+		+		+	86 %
Vibrio fluvialis	0%		NT		-	NT
Aeromonas sobria	+		-		+	-

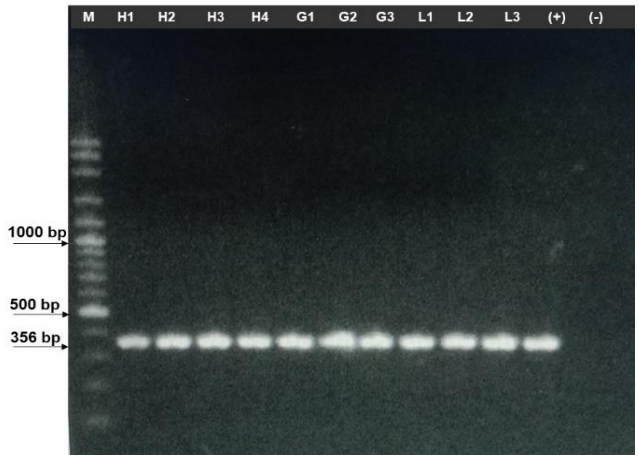


Figure 3. Gel electrophoresis of product using 16S F & 16S R primers, M denotes 100 bp DNA ladder (Marker), H1-H4: liver, G1-G3: kidney, L1-L3: spleen, (+): positive control, and (-): negative control

This study showed that the selected gourami was higher resistant than the control. Because, selected gourami had high genetic variation, it will provide a great opportunity to get the right combination of crosses with good disease resistance (Suprapto and Kairudin 2017). Selected gourami can be used as a candidate for superior gourami. According to Sularto et al. (2020), the growth of selected gourami in nursery and resistance phases was higher than that of the control. This corresponds to the results of the study of the LD_{50} value of selection gourami were higher than pure gourami (Sularto et al. 2020). High genetic variation will provide a great opportunity to get the right cross combination with a good combination of traits (Suprapto and Kairudin 2017). Based on the result, the selection program to increase resistance to gourami. On the other hand, Sularto et al. (2020) reported the SP groups had better growth than CP. Based on the result, the selection program to increase resistance to gourami did not negatively affect the growth of gourami. Therefore, a breeding program to improve the resistance level of the gourami to specific pathogen could be applied in the giant gourami. Besides that, other selection lines based on the growth character of the gourami have improved 11,18% of their productivity (Sularto et al. 2020).

The correlation pattern between disease resistance and growth is host-specific and pathogen-specific (Gjedrem 2015). On the other hand, Suebsong et al. (2019) reported the same thing selective breeding programs have significant potential to make tilapia more resistant to *Streptococcus agalactiae*. Heritability estimates for G0 were 0.22 using the Cox model. At the same time, the researchers noted that selection response indicated that the risk of death decreased to 54 percent, survival time increased to 3.4 days. Survival rate increased to 21%, suggesting that breeding tilapia that are more resistant to *S. agalactiae* is possible. On the other hand, faster growth also occurring in disease-resistant populations has been reported in several aquaculture species. Parker et al. (2011) reported that Line 1 grows at twice the rate of non-selected oysters, has a higher standard metabolic rate, and has a significantly higher survival when exposed to elevated levels of PCO_2 and temperature predicted to occur as a result of climate change. Differences in reproductive status between fifth-generation fast growth oysters and non-selected oysters were found by Dove and O'Connor (2012). The authors are monitoring changes in reproductive condition in Lines 1-3 to determine if this also occurs in SROs bred for disease resistance. Huang et al. (2012) reported that that Pacific whiteleg shrimp having 21% higher resistance to WSSV grew 34.51% faster than that of the control. Another study showed that the growth of oyster resistant (*Crassostrea gigas*) to herpesvirus 1 (OsHV-1) was 15% faster than that of the control (Lionel et al. 2015). Furthermore, Khasani et al. (2017) reported that the growth of giant freshwater prawns (*Macrobrachium rosenbergii*) resistant to vibriosis was 46% higher than that of the control. The neutral correlation between growth and resistance to bacterial cold-water disease was also observed in rainbow trout. Besides that, the growth of Atlantic salmon (*Salmo salar*) resistance to *Piscirickettsia salmonis* and *Caligus rogercresseyi* was similar to that of the normal fish (Yanez et al. 2014). Another case reported that genetic correlations between body weights and WSSV resistance in Pacific Whiteleg Shrimp and *Liptopenaeus vannamei* were negative (Trang et al. 2019).

The LD_{50} of *A. hydrophila* in this study is lower than in several previous studies. The LD_{50} of Bogor gourami was 10^8 CFU/mL (Taukhid et al. 2016), tilapia was 4.9×10^6 CFU/mL (Mangunwardoyo et al. 2016), Arapaima gigas was 1.8×10^8 CFU/mL (Dias et al. 2016), strain K14 in African catfish was 4.977×10^5 CFU/mL (Wulandari et al. 2014), and strain ASB01 in snakehead was 2.69×10^5 CFU/mL (Olga 2014). Citterio and Biavasco (2015) classify *A. hydrophila* into virulent and non-virulent bacteria. *A. hydrophila* isolates with an LD_{50} of $10^{4.5}$-$10^{5.5}$ CFU/mL were declared virulent, while *A. hydrophila* isolates with an LD_{50} of 10^7 CFU/mL or more were declared non-virulent. The mortality in gourami infected with *A. hydrophila* proves that the bacteria are pathogenic and very virulent in fish. Characteristics of bacteria that are pathogens include transmissibility, adherence to host cells, persistence, invasion of host cells and tissues, toxigenicity, and the ability to evade or survive the host's immune system (Jawetz et al. 2014). This difference is probably due to differences in serotype and biotype of bacteria, fish species, and temperature (Olga 2014). Besides that, the difference in test results and LD_{50} calculations is thought to be due to the very different sources of origin of bacteria and host fish used (Makrinos and Bowden 2016; Li et al 2017; Yengkhom et al. 2019). Changes in the physical and chemical characteristics of the environment can increase the abundance and virulence of pathogenic organisms as can genetic mutation, factors which must have an important influence on the outcome of a situation in which pathogens challenge fish in the water (Tripathi et al. 2018). However, another influence, namely the degree of susceptibility of the host, may also be instrumental in determining whether or not pathogenic challenge results in disease (Tipathi et al.

2018). Therefore, disease predisposition in fish can be said to be the end result of an interaction between host susceptibility, pathogen virulence, and environmental factors (stressors) (Gjedrem 2015). Stress weakens fish immune systems, and increases susceptibility to disease (Abram et al. 2017).

Further clinical signs are the appearance of white spots on the body of the fish, mucus accumulation on the body, skin lesions, white pectoral fins, and red spots. Other clinical signs include a dorsal fin, injury to the injection site, and ulcers (Figure 1.A). Internal symptoms that arise due to *A. hydrophila* infection include the presence of yellow fluid in the abdominal cavity, pale red and tender kidneys, brownish-red liver, heart, gills, pale intestines, swollen stomach filled with water or necrosis of the fins and tail, ulcer (Figure 1.B). Changes in swimming patterns that occur after the fish are infected with *A. hydrophila* in the form of fish tend to be aggressive with a dorsal fin that is the weakness in the bottom of the aquarium (Figure 1.C). Cloudy skin (indicates excess mucous production due to an irritant bacterial (Figure 1.D) due to bacterial infection of the kidneys. Weakness on the surface of the aquarium (Figure 1.E), as well as a reddish color all over the fish's body (Figure 1.F). Aeromonas bacteria can attack fish fins, tegument, and intestines. According to Rozi et al. (2018), these bacteria are capable of rupturing little blood vessels, resulting in ulcerative lesions in the tegument with a hemorrhagic aspect, causing a reddish color on the body. In this study, besides the ulcerative lesions observed on the body, exophthalmia, and mucus excess were also observed. Rozi et al. (2018) have described these manifestations as clinical signs of *A. hydrophila* infection and, according to Oliveira et al. (2011), the high proliferation of these bacteria on fish intestine can cause excessive mucus liberation. Based on the output test statistics, it was known that the Asymp. Sig. (2-tailed) <0.05 for parameters LB, LA, W, U, N, and H. Thus there was a significant difference between the clinical signs of SP and CP. CP clinical symptoms were severe than SP.

Aeromonas hydrophila belongs to the group of pathogenic bacteria with high virulence. The virulence level of the bacteria is determined by the ability of bacteria to produce enzymes and certain toxins it plays a role in the invasion and infection process (Leitão 2020). In the first step, the pathogen attaches to the fish scales and produces the chitinase enzyme. The enzyme destroys the chitin of the scales layer. A further step, the bacteria enter the fish's body through the bloodstream. This process is assisted by the lecithinase enzyme which has a specific function to penetrate the bloodstream. Furthermore, the toxin will spread through the bloodstream (Andriani et al. 2020). The disease caused by *A. hydrophila* bacteria is called *Motile Aeromonad septicemia* (MAS) because the infection spreads throughout the body through the bloodstream, infects fish through the surface of the body or wounded gills, and then enters the blood vessels and other internal organs (Ulfiana et al. 2012). According to Yengkhom et al. (2019), disease malignancy is influenced by interrelated factors, such as bacterial virulence, types of bacteria, and the degree of stress affected by fish populations, physiological conditions of the host, and the degree of genetic resistance that cannot be separated in specific populations of fish.

Haryani et al. (2012) also mentioned that the first reaction of animals in cellular and vascular to bacteria that enter the body that causes damage to tissue is inflammation (Table 2). This tissue damage is thought to occur due to toxins released by these bacteria and carried throughout the body through the bloodstream. This was also stated by Andriani et al. (2020), that the toxin was spread throughout the body through the bloodstream causes hemolysis and rupture of blood vessels resulting in redness or red spots on the body of the fish. The appearance of clinical signs can affect the appetite of fish. Fish experience changes in appetite after injection. According to Hardi et al. (2017), fish infected with bacteria take a longer time digesting food than uninfected fish. In infected fish, the feed condition is still intact at 5 min after giving. This is due to disruption of the digestive enzymes of the fish due to a bacterial infection of the brain that regulates intestinal peristalsis. Bacterial infection of the brain can inhibit the work of the lateral hypothalamus which regulates diet. If the hypothalamus which is in the telencephalon (forebrain) is infected, the fish appetite will decrease (Hardi et al. 2018). Based on Figure 3, gram staining results indicate that *A. hydrophila* bacteria were gram-negative, short rod-shaped, non-spore, motile, and have one flagellum, living in the temperature range 25-30ºC (Yamazaki et al. 2021). *A. hydrophila* bacteria are aerobic and facultatively anaerobic. The 16S rRNA primer was used to amplify 356 bp. Fragment of the 16S rRNA gene is conserved for the genus *Aeromonas* to confirm the presence of *Aeromonas* spp. (Hussain et al. 2013).

Selected gourami that is resistant to disease and has a higher tolerance to environmental disturbances is a key factor to increase the survival in gourami farming. The average mortality in the non-selected control had reached (63.33±5.77%) and was significantly higher ($p < 0.05$) compared to that of selected gourami (33.33±5.77%). In conclusion, the result of this study demonstrated that non selected group was more susceptible than the selected groups.

ACKNOWLEDGEMENTS

The DIPA budget funded this study with the Number: SP DIPA-032.12.2.403832 / 2020. We thank Sularto, and laboratory technicians and the gourami team have helped a lot in completing data.

REFERENCES

Abram QH, Dixon B, Katzenback BA. 2017. Impacts of low temperature on the teleost immune system. Biology 6 (4): 1-15. DOI: 10.3390/biology6040039.

Andriani Y, Handaka AA, Rosidah, Himyati R. 2020. Potential of Telang plant (*Clitoria ternatea*) for treatment of *Aeromonas hydrophila* infection on Koi fish (*Cyprinus carpio*). Revista Agrotec 11 (1): 23-29. DOI: 10.20884/1.oa.2020.16.1.751.

Aisiah S, Prajitno A, Maftuch M, Yuniarti A. 2020. Effect of Nauclea subdita (Korth.) Steud. leaf extract on hematological and histopathological changes in liver and kidney of striped catfish infected by *Aeromonas hydrophila*. Vet World 13 (1): 47-53. DOI: 10.14202/vetworld.2020.47-53.

Arifin OZ, Imron I, Muslim N, Hendri A, Aseppendi A, Yani A. 2017. Phenotype and genotype characteristics of gourami, *Osphronemus goramy*, black Galunggung strain, white Galunggung, and its hybrid. J Aquac Res 12 (2): 99-110. DOI: 10.15578/jra.12.2.2017.99-110. [Indonesian]

Casadevall A. 2017. The pathogenic potential of a microbe. MSphere 2 (1): 1-7. DOI: 10.1128/msphere.00015-17.

Citterio B, Biavasco F. 2015. *Aeromonas hydrophila* virulence. Virulence 6 (5): 417-418. DOI: 10.1080/21505594.2015.1058479.

Department of Marine Affairs and Fisheries, Padang Pariaman Regency. 2015. Padang Pariaman Minapolitan Master Plan 2015 - 2020. Department of Marine Affairs and Fisheries, Padang Pariaman District, Padang Pariaman. [Indonesian]

Dias MKR, Sampaio LS, Proietti AA, Yoshioka ETO, Rodrigues DP, Rodriguez AFR, Ribeiroe, Faria FSEDV, Ozório ROA, Tavares-Dias M. 2016. Lethal dose and clinical signs of *Aeromonas hydrophila* in *Arapaima gigas* (Arapaimidae), the giant fish from Amazon. Vet Microbiol 188: 12-15. DOI: 10.1016/j.vetmic.2016.04.001.

Dove MC, O'Connor WA. 2012. Reproductive cycle of Sydney rock oysters, *Saccostrea glomerata* (Gould 1850) selectively bred for faster growth. Aquaculture 324-325: 218-225. DOI: 10.1016/j.aquaculture.2011.10.022.

Fernández-Bravo A, Figueras MJ. 2020. An update on the genus Aeromonas: Taxonomy, epidemiology, and pathogenicity. Microorganisms 8 (1): 129. DOI: 10.3390/microorganisms8010129.

Gjedrem T, Robinson N, Rye M. 2012. The importance of selective breeding in aquaculture to meet future demands for animal protein: A review. Aquaculture 350-353: 117-129. DOI: 10.1016/j.aquaculture.2012.04.008.

Gjedrem T. 2015. Disease resistant fish and shellfish are within reach: A review. J Mar Sci Eng 3: 146-153. DOI: 10.3390/jmse3010146.

GPS (Global Positioning Systems) Coordinate. 2017. Google Play Store.www.google.com/gps. Accessed January 2020.

Hardi EH, Kusuma IW, Suwinarti W, Saptiani G, Sumoharjo S, Lusiastuti AM. 2017. Utilization of several herbal plant extracts on Nile tilapia in preventing *Aeromonas hydrophila* and *Pseudomonas* sp. bacterial infection. Nusantara Biosci 9 (2): 220-228. DOI: 10.13057/nusbiosci/n090219.

Hardi EH, Nugroho RA, Saptiani G, Sarinah RIA, Agriandini M, Mawardi, M. 2018. Identification of potentially pathogenic bacteria from tilapia (*Oreochromis niloticus*) and channel catfish (*Clarias batrachus*) culture in Samarinda, East Kalimantan Indonesia. Biodiversitas 19 (2): 480-488. DOI: 10.13057/biodiv/d190215.

Haryani A, Roffi G, Ibnu DB, Ayi S. 2012. Effectiveness test of Papaya leaves (*Carica papaya*) for treatment of *Aeromonas hydrophila* bacterial infection in Chef carp (*Carassius auratus*). J Fish Mar Affairs 3 (3): 213-220.

Hendry AP, Kinnison MT, Heino M, Day T, Smith TB, Fitt G, et al. 2011. Evolutionary principles and their practical application. Evol Appl 4 (2): 159-183. DOI: 10.1111/j.1752-4571.201000165x.

Hua K, Cobcroft JM, Cole A, Condon K, Jerry DR, Mangott A, Strugnell J. M. 2019. Review the future of aquatic protein: Implications for protein sources in aquaculture diets. One Earth 1 (3): 316-329. DOI: 10.1016/j.oneear.2019.10.018.

Huang YC, Yin Z, Weng S, He J, Li S. 2012. Selective breeding and preliminary commercial performance of *Penaeus vannamei* for resistance to white spot syndrome virus (WSSV). Aquaculture 364-365: 111-117. DOI: 10.1016/j.aquaculture.2012.08.002.

Hussain IA, Jeyasekaran G, Shakila RJ, Raj KT, Jeevithan E. 2013. Detection of hemolytic strains of *Aeromonas hydrophila* and *A. sobria* along with other *Aeromonas* spp. from fish and fishery products by multiplex PCR. J Food Sci Technol 51 (2): 401-407. DOI: 10.1007/s13197-013-1190-9.

Khasani I, Lusiastuti AM, Zairin M, Alimuddin A. 2017. Resistance to vibriosis, production performance and tolerance to stress of the selected giant freshwater prawn (*Macrobrachium rosenbergii*). Aquac Res 49 (1): 273-281. DOI: 10.1111/are.13458.

Krishna G, Gopikrishna G, Gopal C, Jahageerdar S, Ravichandran P, Kannappan S, Hayes B. 2011. Genetic parameters for growth and survival in *Penaeus monodon* cultured in India. Aquaculture 318: 74-78. DOI: 10.1016/j.aquaculture.2011.04.028.

Khumaidi A, Hidayat A. 2018. Identification of causes of mass death of Gurami fish (*Osphronemus gouramy*) in Gurami Fish Cultivation Sentra, Desa Beji, Kedung Banteng District, Banyumas District, Central Java. J Aqua Sci 3 (2): 145-153. DOI: 10.31093/joas.v3i2.53.

Kusdarwanti R, Kismiyati, Sudarno, Kurniawan H, Prayogi YT. 2017. Isolation and identification of *Aeromonas hydrophila* and *Saprolegnia* sp. on Catfish (*Clarias gariepinus*) in Floating cages in Bozem Moro Krembangan Surabaya. IOP Conf Ser Earth Environ Sci 55 (1): 012038. DOI: 10.1088/1755-1315/55/1/012038.

Kusdarwati R, Rozi D, Dinda N, Nurjanah I. 2018. Antimicrobial resistance prevalence of *Aeromonas hydrophila* isolates from motile *Aeromonas septicemia* disease. IOP Conf Ser Earth Environ Sci 137 (1): 012076. DOI: 10.1088/1755-1315/137/1/012076.

Kusumawaty D, Pancoro A, Suhandono S. 2016. Evaluation of identification techniques for the fish pathogen, *Aeromonas hydrophila*, from Indonesia. Malays J Microbiol 12 (3): 191-198. DOI: 10.21161/mjm.77915.

Leitão JH. 2020. Microbial virulence factors. Intl J Mol Sci 21 (15): 1-6. DOI: 10.3390/ijms21155320.

Li T, Li H, Gatesoupe FJ, She R, Lin Q, Yan X, Li J, Li X. 2017. Bacterial signatures of "Red-Operculum" disease in the gut of Crucian carp (*Carassius auratus*). Microbiol Aquat Sys 74: 510-521. DOI: 10.1007/s00248-017-0967-1.

Lionel D, Max N, Elise M. 2015. Mass selection for survival and resistance to OsHV-1 infection in *Crassostrea gigas* spat in field conditions: Response to selection after four generations. Aquaculture 446: 111-121. DOI: 10.1016/j.aquaculture.2015.04rr.029.

Ma J, Bruce TJ, Jones EM, Cain KD. 2019. A review of fish vaccine development strategies: Conventional methods and modern biotechnological approaches. Microorganisms 7 (11): 569. DOI: 10.3390/microorganisms7110569.

Mangunwardoyo W, Ismayasari R, Riani E. 2016. Pathogenicity and virulence test of *Aeromonas hydrophila* Stanier on Tilapia (*Oreochromis niloticus* Lin.) through postulate koch. J Aquac Res 5 (2): 145-255. DOI: 10.15578/jra.5.2.2010.145-255.

Marine and Fisheries Ministry. 2018. Gourami cultivation, activator regional economy and improve welfare. [Online] Available at http:// www.djpb. kkp.go.id [accessed October 25, 2019].

Makrinos DL, Bowden TJ. 2016. Natural environmental impacts on teleost immune function. Fish Shellfish Immunol 53: 50-57. DOI: 10.1016/j.fsi.2016.03.008.

Maryadi H. 2011. Study of the Development of Clinical Symptoms and Pathology in Tiger Grouper (*Epinephelus fuscoguttatus*) Infected with *Streptococcus iniae*. [Thesis]. Bogor Agricultural University, Bogor. [Indonesian]

Moss SM, Moss DR, Arce SM, Lightner DV, Lotz JM. 2012. The role of selective breeding and biosecurity in the prevention of disease in penaeid shrimp aquaculture. J Invertebr Pathol 110: 247-250. DOI: 10.1016/j.jip.2012.01.013.

Nugroho E, Nafiqoh N, Gustiano R. 2012. Productivity of several varieties of gourami (*Osphronemus gourami*). Proceedings of the Aquaculture Technology Innovation Forum 2012: 1095-1101.

Olga. 2014. Pathogenicity of *Aeromonas hydrophila* ASB01 bacteria in snakehead fish (*Ophicephalus striatus*) (The Pathogenicity of *Aeromonas hydrophila* ASB01 on snakehead (*Ophicephalus striatus*) Aquat Sci 14 (1): 33-39.

Oliveira SRD, Souza RTYBD, Brasil EM, Andrade JIAD, Nunes EDSS, OnoEA, Affonso EG. 2011. LD5o of the bacteria *Aeromonas hydrophila* to matrinxã, *Brycon amazonicus*. Acta Amazon 41 (2): 321-326. DOI: 10.1590/s0044-59672011000200019.

Öztürk RÇ, Altinok I. 2014. Bacterial and viral fish diseases in Turkey. Turkish J Fish Aquat Sci 14 (1): 275-297. DOI: 10.4194/1303-2712-v14_1_30.

Parker LM, Ross PM, O'Connor WA. 2011. Populations of the Sydney rock oyster, *Saccostrea glomerata*, vary in response to ocean acidification. Mar Biol 158 (3): 689-697. DOI: 10.1007/s00227-010-1592-4.

Purwaningsih U, Indrawati A, Lusiastuti AM. 2014. Protection of monovalent vaccines and whole cocktails against co-infection of *Mycobacterium fortuitum* and *Aeromonas hydrophila* in gourami, *Osphronemus gourami*. J Aquac Res 9 (2): 283-294. DOI: 10.15578/jra.9.2.2014.283-294.

Rao K, Benarjee PG. 2016. Haematological changes in South Indian freshwater murrel, Channa punctatus have both EUS and *A. hydrophila* infection. J Parasit Dis 41 (2): 329-335 DOI: 10.1007/s12639-016-0798-x.

Republic of Indonesia. 2017. Government Regulation No. 32 concerning Fish Farming, President of the Republic of Indonesia. Minister of the Environment, Jakarta. [Indonesian]

Reid GK, Gurney-Smith HJ, Marcogliese DJ, Chopin T, Brewer-Dalton K, Moccia RD, Flaherty M. 2019. Climate change and aquaculture : Considering biological response and resources. Aquac Environ Interact 11: 569-602. DOI: 10.3354/aei00332.

Rozi R, Rahayu K, Daruti DN, Stella MSP. 2018. Study on characterization, pathogenicity and histopathology of disease caused by *Aeromonas hydrophila* in gourami (*Osphronemus gourami*). IOP Conf Ser Earth Environ Sci 137: 012003. DOI: 10.1088/1755-1315/137/1/012003.

Subhan U, Mauladani S, Rosidah, Suryadi IBB, Joni IM. 2020. Promising application of fine bubbles (FBs) to control *Aeromonas hydrophila* on striped catfish seed (*Pangasianodon hypophthalmus*). AIP Conf Proceed 2219: 090002. DOI: 10.1063/5.0003059.

Suebsong W, Poompuang S, Srisapoome P, Koonawootrittriron S, Luengnaruemitchai A, Johansen H, Rye M. 2019. Selection response for *Streptococcus agalactiae* resistance in Nile tilapia *Oreochromis niloticus*. J Fish Dis 42 (11): 1553-1562. DOI: 10.1111/jfd.13074.

Sularto, Febrianti R, Suharyanto, 2016. Heritability estimation and selection response of Goramy crosses (*Osphronemus goramy* Lac.). J. Aquac Res 11 (1): 23-28. DOI: 10.15578/jra.11.1.2016.23-28.

Sularto, Febrianti R, Listiyowati R. 2020. Research and Development of Superior Strain Gourami in Society. Fish Breeding Research Institute. Research Activity Technical Report 2020, Sukamandi, Subang.

Suprapto S, Kairudin NM. 2017. Genetic variation, heritability, genetic action and genetic progress of soybean (*Glycine max* Merrill) on ultisol. Indon J Agric Sci 9 (2): 183-190. DOI: 10.31186/jipi.9.2.183-190.

Taukhid, Gardenia L, Andriyanto S. 2016. The efficacy of the "trivalent" combination vaccine (*Aeromonas hydrophila*, *Streptococcus agalactiae*, and *Mycobacterium fortuitum*) for the prevention of potential bacterial diseases in freshwater fish culture. J Aquac Res 11 (4): 373-385. DOI: 10.15578/jra.11.4.2016.373-385.

Trang TT, Hung NH, Ninh NH, Knibb W, Nguyen NH. 2019. Genetic variation in disease resistance against white spot syndrome virus (WSSV) in *Liptopenaeus vannamei*. Front Genet 10: 1-10. DOI: 10.3389/fgene.2019.00264.

Tripathi GS, Tripathi G, Singh P. 2018. Assessment of different water quality parameters under bottle aquaponic system and normal controlled conditions with *Trichopodus trichopterus* (Blue gourami). J Entomol Zool Stud 6 (6): 898-902.

Ulfiana R, Mahasri G, Suprapto S. 2012. Occurrence rate of *Aeromonasis* in Koi fish (*Cyprinus carpio*) infected by *Myxobolus* koi at different degrees of infection. J Fish Mar Sci 4 (2): 169-174. DOI: 10.20473/jipkv4i2.11568.

Wulandari A, Prayitno SB, Sarjito. 2014. Pathogenicity of K14 isolate isolated from African catfish (*Clarias gariepinus*) originating from Demak. J Aquac Manag Technol 3 (2): 143-149.

Yamazaki K, Kashimoto T, Niwano A, Yamasaki M, Nomura M, Akeda Y, Ueno S. 2021. Expansion of necrosis depending on hybrid motor-driven motility of *Aeromonas hydrophila* in a murine wound infection model. Microorganisms 9 (1): 1-12. DOI: 10.3390/microorganisms9010010.

Yanez JM, Lhorente JP, Bassini LN, Oyarzu NM, Neira R, Newman S. 2014. Genetic covariation between resistance against both *Caligus rogercresseyi* and *Piscirickettsia salmonis*, and body weight in Atlantic salmon, Salmo salar. Aquaculture 433: 295-298. DOI: 10.1016/j.aquaculture.2014.06.026.

Yengkhom O, Shalini KS, Subramani PA, Michael RD. 2019 Stimulation of nonspecific immunity, gene expression, and disease resistance in Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758), by the methanolic extract of the marine macroalga, *Caulerpa scalpelliformis*. Vet World 12: 271-276. DOI: 10.14202/vetworld.2019.271-276.

Zubaidah A, Samsundari S, Hidayaturrahmi H. 2019. Effectiveness of strychnine bush extract (*Strychnos ligustrina* Blume) against to prevalence and survival rate of Dumbo catfish (*Clarias gariepinus*) infected with *Aeromonas hydrophila*. Indones J Trop Aquat 2 (1): 1. DOI: 10.22219/ijotav2i1.5601.

NUSANTARA BIOSCIENCE
Vol. 13, No. 1, pp. 121-128
May 2021

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n130116

Genetic diversity and morphological variation of *Pinus gerardiana* along the environmental gradient from Zhob, Balochistan, Pakistan

JANAT GUL, SHAZIA SAEED, ALIA AHMED♥, SAADULLAH KHAN LEGHARI, ABDUL BASIT, ABDUL REHMAN, MUHAMMAD ZAHID KHAN

Department of Botany, University of Balochistan. Quetta 87300, Pakistan. Tel./fax.: +92-81-921-1264. ♥email: aliaahmed_botany@yahoo.com

Manuscript received: 29 March 2021. Revision accepted: 28 May 2021.

Abstract. *Gul J, Saeed S, Ahmed A, Leghari SK, Basit A, Rehman A, Khan MZ. 2021. Genetic diversity and morphological variation of* Pinus gerardiana *along the environmental gradient from Zhob, Balochistan, Pakistan. Nusantara Bioscience 13: 121-128. Pinus gerardiana,* an evergreen gymnosperm, is an economically- and ecologically valuable tree found in the Takhte Suleman Mountain Range, Zhob northeastern edge of the Balochistan, Pakistan. The present study is based on the assessment of the impact of environmental gradients with special reference to altitudinal gradient and soil variables on morphological, phytochemicals and genetic variation of *P. gerardiana*. A total of 27 genotypes of *P. gerardiana* were collected from the three elevation zones ranging from 2000 - 3500 m above sea level. The genetic diversity was assessed by combined markers, the random amplified polymorphic DNA (RAPD) and the Inter Simple Sequence Repeat (ISSR). Polymorphic bands analyzed the data to generate a dendrogram, based on the unweighted pair group method with arithmetic mean (UPGMA). Morphological characters assessed the morphological characters. Phytochemicals were investigated; the total phenolic content and total flavonoid contents were estimated and compared amongst all accessions. Our results revealed variations along altitudinal gradients and related to soil characteristics. The populations at the Middle altitude zone have greater diversity than populations at lower and higher altitudes. The resulting altitudinal variation can be attributed to various geographical and environmental conditions. These results can help in conservation and cultivation of this economically important tree.

Keywords: Altitudinal gradient, soil variables, phytochemicals, *Pinus gerardiana*, Zhob Forest, Pakistan

INTRODUCTION

The *Pinus gerardiana* Wall. ex D.Don, locally known as "Chilgoza" or "neoza pine", is considered one of the most promising trees with ecological and economical values around the world. This tree plays a very important role in the economic progress and livelihood of communities living close to the Forest (Shalizi and Khurram 2016). The tree is utilized for food, medicine and timber by the native communities of the area and wildlife. The tree contributes to the local income and revenue, sustains the soil surface, sustains a suitable microclimate, provides shelter, and is a refuge for animals. In Pakistan, 20% of the forest consists of Pinus trees, which can survive in harsh, cold environmental conditions, including excessive drought, high wind, and severe cold. The mountains of Sulaiman hold the world's largest expanse of Chilgoza (over 260 km^2) (Ahmed et al. 2011). The Chilgoza forests are under constant threats and pressure from the timber Mafia. This species is listed as highly threatened due to excessive cutting. During their explorations, many different analysts have identified the adverse anthropogenic activities in the area with regards to this tree, such as collecting for fuelwood, overgrazing and burning, which are responsible for the significant destabilizing influence in the forests (Ahmed et al. 2009; Beg and Mirza 1984; Hussain 2013).

The environmental factors, soil variables and amount of precipitation have influenced the growth of the Chilgoza pine tree. Natural regeneration of Chilgoza pine is very poor or entirely lacking in this zone (Kumar et al. 2013; Kumar et al. 2016). Genetic diversity provides the template for adaptation and evolution of populations and species.

Genetic variation is the key factor for the conservation of biodiversity (Thomas et al. 1999). In recent years, PCR-based molecular markers have allowed the use of DNA sequences in genetic analyses to provide a better understanding of the genetic diversity and differentiation of natural populations (Malik et al. 2008). Genetic make-up plays an important role in diverse ecosystems (Meloni et al. 2006). Recent research identifies better the role of molecular markers to assess the genetic diversity amongst and within the population. Nowadays, different molecular primers are in use for analysis of genetic diversity. RAPD markers are quite suitable for DNA fingerprinting as they are rapid and easy to assess (Kernodle et al. 1993). The Inter Simple Sequence Repeat (ISSR) is also used efficiently as no prior sequence is required (Adams et al. 2003). Loss of genetic variability is a major problem in biological diversity conservation because it inhibits a species from responding to natural selection and limits its evolutionary potential. If the harmful source of genetic diversity is known, different resources can be used efficiently in conservation.

In view of the aforementioned, the main objective of this study was, therefore, to assess the genetic diversity and population structure of economically important tree *P. gerardiana*. This would aid in defining conservation

studies for the declining *P. gerardiana* population in Balochistan, Pakistan. Intraspecific variation at morphological and molecular level through morphological and molecular markers, assessment of ecological diversity of the area and its impact on the genetic diversity help for a better understanding of the genetic structure of the species.

MATERIALS AND METHODS

Study site and ecological diversity of site

Zhob Forest of Balochistan, Pakistan is part of the Sulaiman Mountain Range that located in the eastern edge of the Iranian Plateau, where the Indus River separates it from the subcontinent (Figure 1). Its elevation is approximately 3,380 m above sea level (Table 1). The study area exhibits a dry temperate climate characterized by long winters from October to April and short summers from June to August. Though rain is scarce, precipitation is received mostly in the form of snow during winter. The data pertaining to meteorological conditions of the area during the study period are presented (Figures 2.A-B).

Table 1. Geographical attributes of the study sites

Sampling site	Site no.	Code	Latitude (N)	Longitude (E)	Elevation (m asl.)
Lower elevation zone	1	L1	31.55°	69.91°	2000
	2	L2	31.56°	69.92°	2050
	3	L3	31.55°	69.92°	2090
Mid semi-arid zone	4	M1	31.58°	69.93°	2800
	5	M2	31.57°	69.92°	2950
	6	M3	31.57°	69.93°	2960
Upper arid zone	7	U1	31.53°	69.91°	3440
	8	U2	31.57°	69.82°	3365
	9	U3	31.54°	69.91°	3380

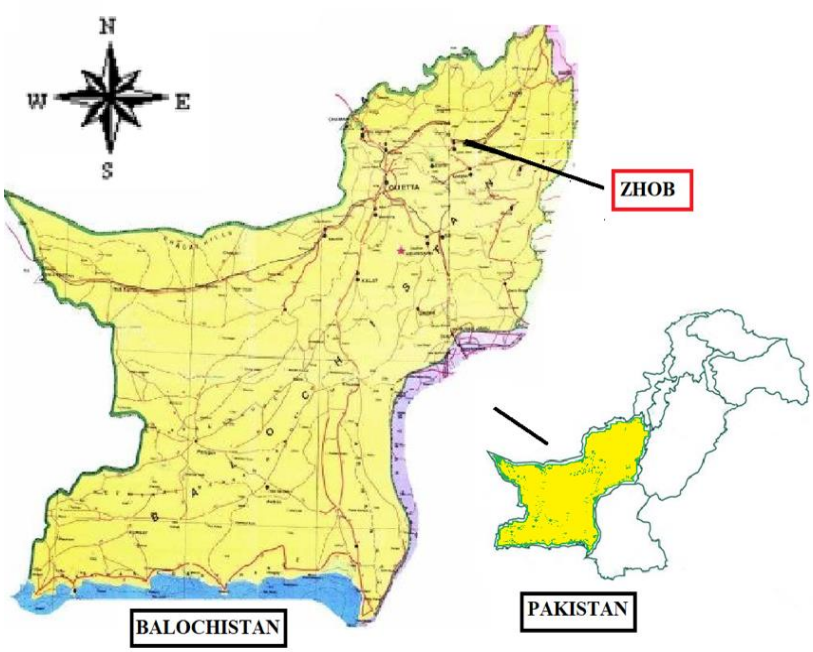


Figure 1. Map of study site in Zhob Forest, Balochistan, Pakistan

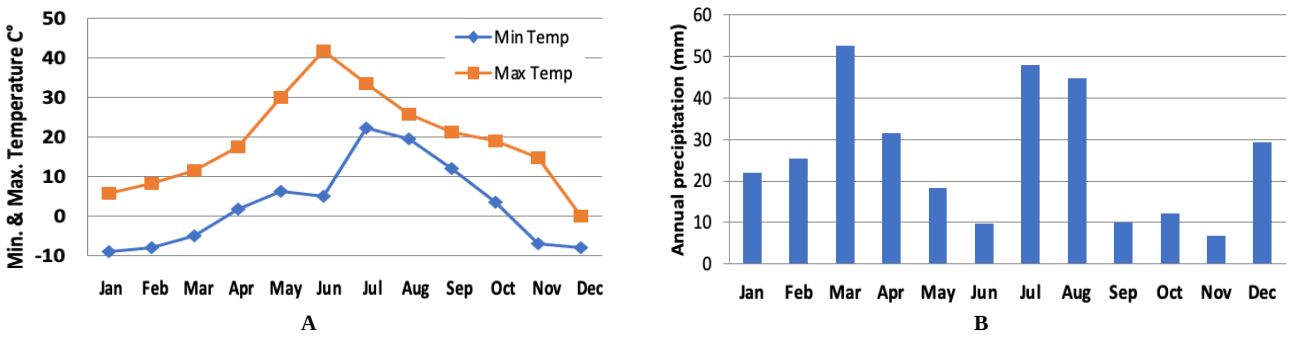


Figure 2.A. Temperature and B. Annual precipitation of the study site in 2018-2019

Sample collection

A survey was conducted during 2018-2019 to locate the populations of *P. gerardiana.* The whole distribution area of the species was divided into three zones, *viz*. lower elevation zone, mid semi-arid zone and upper arid zone. Data from three to five sites were recorded from each elevation zone. Soil samples were collected from each site for soil characterization. Fresh leaf samples for DNA extraction were taken from three randomly chosen trees in each site.

Ecological characteristics

Different ecological characteristics of the study sites were measured including microclimatic conditions and edaphic factors such as soil.

Elevation and aspects

Elevations of study sites were measured during sampling with the help of Global Positioning System (GPS). Aspects of the area were noted using Compass.

Temperature and annual precipitation

Temperature and annual precipitation data were collected for the years 2018-2019 from the metrological survey center, arid zone Quetta, Balochistan Pakistan.

Soil analysis

Composite soil samples were collected from the surface layer 0-30 cm depth. Samplings were made with systematically-randomized method (Zare et al. 2011). Samples were dried at room temperature, sieved with 2 mm sieve and then stored in zip bag for further analysis. Physical and other Characteristics were examined at "Peer Mehar Ali Shah Arid Agriculture University", Rawalpindi, Pakistan.

Survey of price list

To check the economic importance and economic value of plant, market survey was carried out. Price list was compared with open market (Table 2).

Morphological diversity analysis

To analyze the morphological diversity, tree samples were collected from nine sites at three different elevation zones. Samples were pressed and mounted on herbarium sheets. Data were analyzed by using morphological characters (S1).

Table 2. Market survey of *Pinus gerardiana* during 2018-2019

Year	Price in Zhob market	Price in open market
2018	1500-3000 PKR /kg (10-20 USD/kg)	6000-6500 PKR/kg (37-41 USD/kg)
2019	2500-3600 PKR/kg (16-24 USD/kg)	6200-7600 PKR/kg (39-48 USD/kg)

Phytochemical analysis

Total flavonoid content (TFC)

The TFC was estimated by Ordonez et al. (2006) technique using equation ($Y=0.0255x$, $R^2=0.9812$) absorbance at 420 nm.

Total phenolic content (TPC)

The TPC was calculated by the method of Slinkard and Singleton (1977) at 765 nm absorbance and expressed as mg/g tannic acid equivalent using the equation ($Y=0.1216x$, $R^2=0.9365$).

Genetic diversity analysis

Fresh pine needles were collected for DNA extraction and assessment of genetic diversity was undertaken by using molecular markers. The method of Saeed et al. (2017) was used for extraction and purification.

PCR reaction

A total of thirteen primers were used, including twenty RAPD and ten ISSR, amongst them thirteen were polymorphic (Table 3). The reaction mixture was used by also following the method of Saeed et al. (2017).

Statistical analysis

For molecular markers, the amplified bands were scored as 1 (present) and 0 (absent), and data were clustered (dendrograms) based on similarity matrices using the paired group method with the help of software NTSYS 2.10 (Rohlf 1998). For soil data and phytochemical parameters, Agglomerative hierarchical clustering was performed using Minitab software.

Table 3. Details of polymorphic primers used in the study of *Pinus gerardiana*

Primer name	Sequence 3'—5'	TA (°C)	TB	P	PB %
OPA-2	TGCCGAGCTG	36	11	7	0.64
OPA-3	AGTCAGCCAC	34	7	3	0.43
OPA-4	AATCGGGCTG	34	12	5	0.42
OPA-7	GAAACGGGTG	34	6	3	0.50
OPA-8	GTGACGTAGG	36	4	2	0.50
OPA-13	CAGCACCCAC	36	11	8	0.73
OPA-17	GACCGCTTGT	36	10	7	0.70
OPA-20	GTTGCGATCC	36	7	6	0.86
UBC-810	(GA)8T	52	6	2	0.33
UBC-832	(AT)7 TYC	46	12	8	0.67
UBC-844	(CT)8RC	47	10	7	0.70
UBC-850	(GT)8YC	47	7	4	0.57
UBC-857	(ACA)5CYG	52	6	4	0.67
Total			109	66	0.61

Note: TA (°C): annealing temperature, T: Total bands, P: Number of polymorphic bands, PB: percentage of polymorphism

RESULTS AND DISCUSSION

This is the first comprehensive report on morphological, chemical and genetic diversity of an economically important tree, *Pinus gerardiana*, from the Zhob Forest, Balochistan, Pakistan. *P. gerardiana* is an ecologically- and economically valuable species, distributed in different parts of the world. In Pakistan, it is distributed in high mountainous zones of the Pakistani-Afghan border, Sulaiman Mountain Range, and Kashmir.

Economic importance

Pinus gerardiana is used as edible nuts and as medicinal plants in folk medicines by the local community of the study area. Economically, it is a very important nut. The local communities of the study area are categorized in the low-earning income bracket. No proper source of income has been identified, except for the collection of pine nuts, cutting of pine trees, and grazing in order to sustain their livelihood. The study area is in a remote, difficult geographical zone of the mountain, with a low literacy rate and under the threat of floods and cross-border disturbance. Moreover, no proper health facilities are available. A price survey was made in the year 2018-19. In 2018, it ranged from 1500-3000 PKR/kg (10-20 USD/kg) and increases to 2500-3600 PKR/kg ((16-24 USD/kg) in 2019. The price of nuts in the open market of Quetta and the rest of Pakistan is almost double that of the local market.

Environmental characteristics

The environmental parameters of the study sites varied along the altitudinal gradient (Table 4). The pH ranges from 6.64 to 7.45 from high elevation to low elevation. The total organic matter (2.63 ± 0.11%) was highest at high elevation. Soil at mid-elevation has high sand content (52.12 ± 4.18%), high elevation has high percentage of silt and clay (36.15 ± 2.65 and 18.12 ± 2.66, respectively). Sodium, potassium, and nitrogen were also reported highest at the high elevation zone. The concentrations of heavy metals, nickel and zinc (0.10 ± 0.04 and 0.04 ± 0.00 mg kg^{-1}, respectively) were high at the low elevation zone.

Morphological diversity

Morphology of the studied samples revealed that no variation was found in the studied population. In qualitative characters, no differences were found. The cone shape was nearly spherical, and cone color at maturation was dark brown, Seed shape is like a banana and the seed color was light to dark brown. Leaves were modified into needles that are dark green in color. Seed color varied along the altitudinal gradient. Seeds of high elevation were dark brown to black, while at low elevation, their color was light brown to reddish. Leaves were in fascicles of three needles. Quantitative characters showed a variation that may be due to the age of the tree or may be due to the impact of other environmental factors (biotic and abiotic factors). The height of tree was up to 40 ft (13 m) approximately. DBH of tree was up to 4 m. Leaf length 13 cm. Fruit size was 5 cm in length and 10 mm in width. The cone size ranged from 12 cm in length to 7 cm in width.

On the basis of quantitative characters (S1), cluster analysis was performed for all collected samples from three elevations. The cluster analysis revealed that there was a variation among the samples (Figure 2). This variation may due to anthropogenic activities or may be due to the impact of other environmental factors (biotic and abiotic factors). The age of the tree is the major factor in the variation of collected samples. Environmental factors and the soil type may also influence the size or growth of tree. As the moisture increases, the growth ring size also increases.

Genetic diversity

In the present results, 18 out of 30 primers produced clear bands in all collected samples. Two different marker systems, i.e., RAPD and ISSR were used for the first time on *P. gerardiana* from three elevation zones of Sulman Mountain Range. The amplified band size ranged from 200 to 1500 bp for RAPD and 150 to 1600 bp for ISSR (Figures 3 and 4). We used combined RAPD and ISSR markers to generate a dendrogram by cluster analysis. A combined marker system approach to detect polymorphism could be useful in removing errors and targeting various sites of genome as was previously used by (Saeed et al. 2020; Saeed et al. 2017).

Table 4. Mean values of environmental parameters along altitudinal gradient

Variables	Low elevation (Mean ± S.E)	Mid elevation (Mean ± S.E)	High elevation (Mean ± S.E)
Topographic variables			
Elevation (m)	2000 ± 50	2650 ± 45	3200 ± 45
Slope	32.50 ± 2.32	44.50 ± 5.75	48.45 ± 4.35
Edaphic variables			
pH	7.45 ± 0.12	7.23 ± 0.14	6.64 ± 0.17
Total organic matter (%)	1.62 ± 0.54	2.37 ± 0.14	2.63 ± 0.11
Sand (%)	50.3 ± 2.47	52.12 ± 4.18	45.32 ± 3.36
Silt (%)	33.21 ± 2.61	32.01 ± 2.57	36.15 ± 2.65
Clay (%)	16.07 ± 1.35	15.36 ± 1.45	18.12 ± 2.66
Sodium (mg kg-1)	11.45 ± 1.37	15.36 ± 2.34	17.33 ± 1.45
Potassium (mg kg-1)	6.74 ± 1.77	8.73 ± 1.75	11.18 ± 1.35
Nitrogen (%)	3.17 ± 0.44	3.28 ± 0.08	3.81 ± 0.74
Nickel (mg kg-1)	0.10 ± 0.04	0.08 ± 0.00	0.09 ± 0.03
Zinc (mg kg-1)	0.04 ± 0.00	0.02 ± 0.04	0.03 ± 0.01

Note: S.E.: standard error

Out of the twenty-five markers from the tested primers, thirteen primers had amplified polymorphic bands. Eight were from RAPD and five from ISSR and exhibited polymorphism, showing reproducible bands amongst nine *P. gerardiana* accessions. Table 4 identifies the characteristics of banding patterns obtained from the primers. Thirteen markers amplified 109 total bands and 66 were polymorphic (61% polymorphism). The total number of RAPD and ISSR bands scored per primer also varied. The overall data revealed an average of 8.38 bands obtained per primer.

Based on the UPGMA tree (Figure 5), the cluster is delimited into three main clusters showing the variation within the species along the altitudinal gradient. The Middle elevation zone retains the highest genetic diversity. It may be due to better environmental conditions and fewer anthropogenic activities.

The UPGMA clustering has been based on RAPD and ISSR populations. The cluster consists of two groups cluster A and cluster B. Cluster A consists of two subgroups of populations from the High elevation and Middle elevation zones, while cluster B comprised of populations from the low elevation zone.

Chemical diversity

All accessions of *P. gerardiana* were characterized as having significantly varied levels of total phenolic content (TPC), expressed as tannin equivalent and flavonoids contents as quercetin equivalent within and amongst populations (Figure 6). The TPC ranged from 40 to 58 mg^{-1} within the population. Flavonoids also showed a diverse pattern amongst all sites, ranging from 104 to 123 mg g^{-1}. Based on combined data of TPC and flavonoid contents, the dendrogram generated two main clusters: A and B. Cluster A comprised the population from high. Cluster B comprised of accessions from the middle elevation zone and low elevation zones (Figure 7).

Discussion

Pinus gerardiana grows in the tropical to subtropical regions of Pakistan. The seeds are used as edible nuts and medicine by the local people in the vicinity of the study sites. The *P. gerardiana* tree is facing the threat from anthropogenic activities like other conifers and conservation action needs to be undertaken on an urgent basis. The tree grows in high elevation cold climatic zones (Kumar et al. 2016). The present study aimed to investigate the genetic variability and the impact of environmental gradient with special reference to altitude and soil for this economically important tree. Molecular, phytochemical, and morphological variations were assessed on different samples collected along three elevation zones. From earlier reports, no evidence had been found on the genetic variation of *P. gerardiana* by using markers from a comparison of three different elevations. The regeneration pattern was studied for conservation as well as the population structures by (Akbar et al. 2014; Aziz et al. 2017).

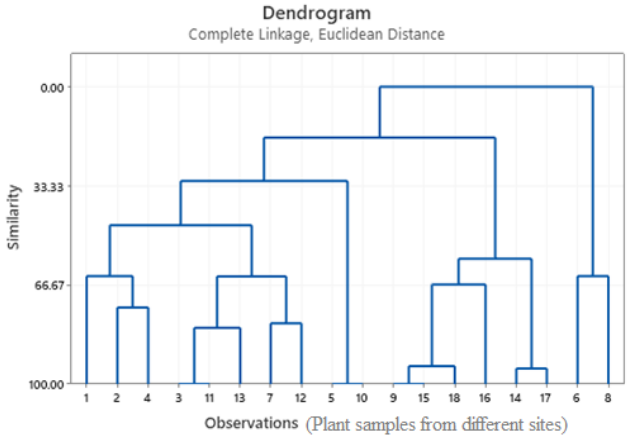
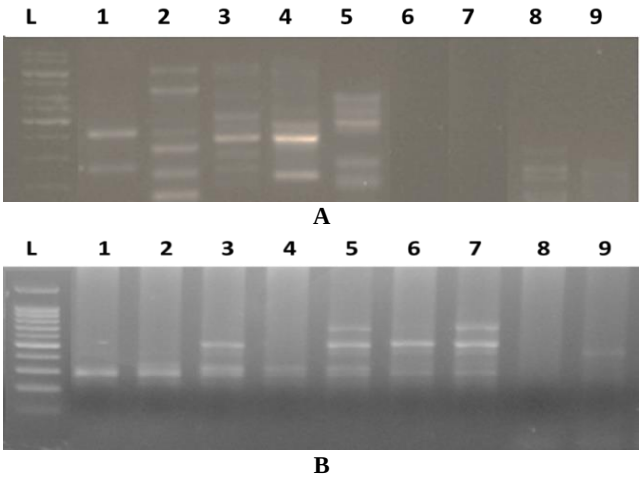


Figure 3. Tree diagram, based on quantitative morphological characters. Site number as coded in Table 1 (larger site numbers correspond to higher altitudes).



Figures 4. PCR amplification of *Pinus gerardiana*. A. RAPD OPA-1, B. ISSR- UBC-857 population from sites 1-9. With 1500 bp Leader.

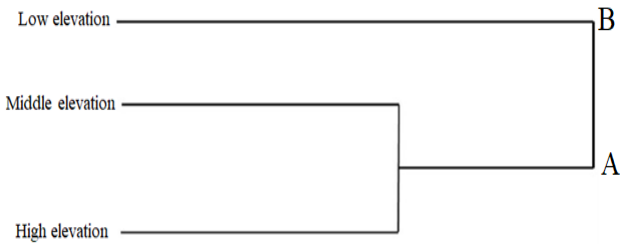


Figure 5. NTSYS- dendrogram of cluster analysis, based on data generated from RAPD and ISSR amongst three elevations

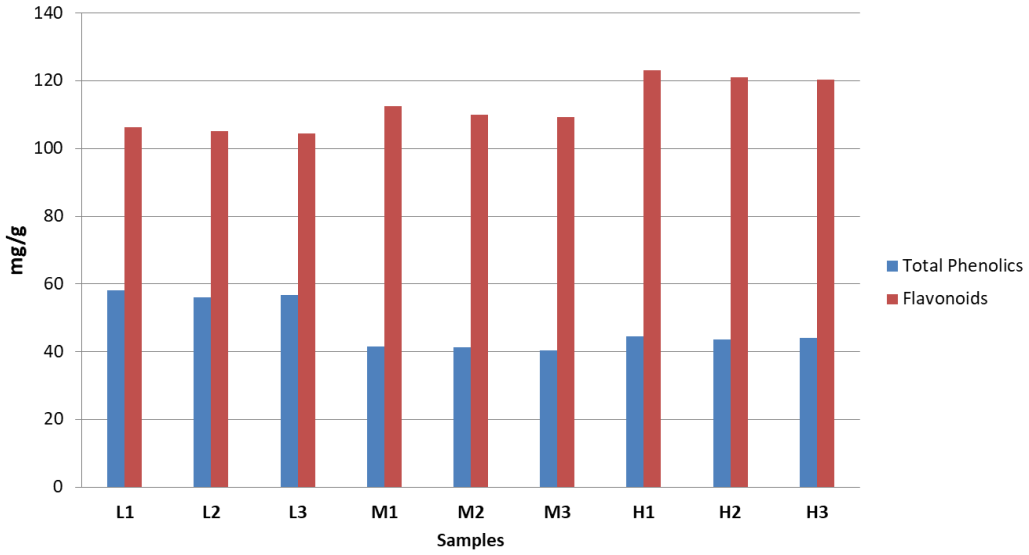


Figure 6. Patterns of phytochemical variations amongst different populations along elevation gradients. Site codes as: L: Low elevation, M: Middle, H: High.

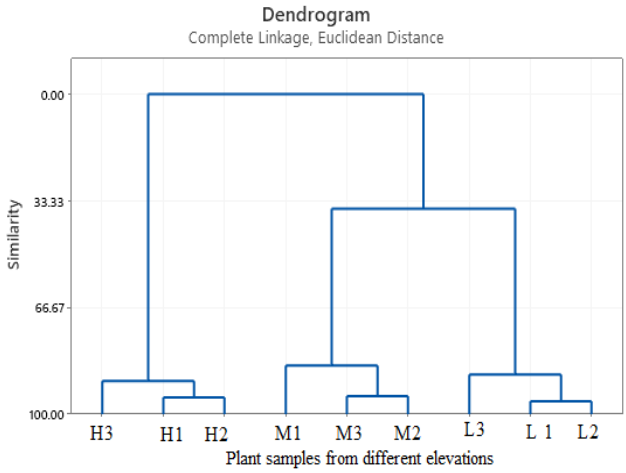


Figure 7. Dendrogram constructed using phytochemical diversity of samples from different populations along elevation gradients. Site codes as: L: Low elevation, M: Middle, H: High

Due to the importance of this valuable seed, it is greatly in demand, not only in the local market, but also from national and international markets (Peltier and Dauffy 2009). Earlier reports on an open market survey showed its prices to be approximately US$ 20-30 per kg. Open market prices are very high compared with the local market and best quality seed from the area is exported to other parts of the country and around the world (Akbar et al. 2013; Khan et al. 2015). In the global market, Pakistan, India and Afghanistan contribute the largest quantities of nut (Akbar et al. 2014; Kumar et al. 2014). Sample from different ecological sites shows the variation that may be the result of different climatic conditions and can be used to improve cultivation of the species. Conifers generally express a high degree of genetic diversity and are considered to be most variable group amongst the gymnosperms (Hamrick and Godt 1990). They express a high degree of variation amongst populations and a lesser extent of diversity (Bakshi and Konnert 2011). The genetic variation of *Pinus* may also be due to altitudinal gradients orchestrated with the change in climatic conditions associated with the variation in altitude (Loya-Rebollar et al. 2013).

Genetic variations were observed amongst the samples studied from different ecological regions that varied in altitude in our study. These variations of the population, along with altitudinal and environmental gradients, would be of great help in conserving the species for future climatic change (Sáenz-Romero et al. 2011). Species genetic resource conservation is required as geographically separated populations are expected to have different genetic compositions. Hamrick et al. (1992) stated that high genetic variation amongst the population may be due to woody plants with large geographical ranges with wind-assisted seed dispersal. Geographical distances and ecological consequences plays important role in the variability of component.

The present research shows intra-species genetic variation of *P. gerardiana* using RAPD and ISSR marker techniques. Eighteen genotypes from three elevation zones were assessed. Thirteen markers amplified 109 total bands and 66 were polymorphic (61%). The results suggest that RAPD markers show high genetic polymorphism in their capacity for producing polymorphic amplicons. Similar results were obtained by (Sinha et al. 2013) on the genetic polymorphism of *Pinus roxburghii*. Their study also justified the importance of genetic variations in both ex-situ *and* in situ conservations. In the present study, we found that chemical variation along altitudinal gradients may be a response to UV radiation. This may suggest the species adaptation to strong UV radiation and low-temperature environments at higher elevations. Flavonoid content is the main source for plant protection from UV

radiation, and protects plant tissues. Moreover, many studies have provided new evidence that UV light induces the synthesis of flavonoids (Berli et al. 2010; Saeed et al. 2018).

Population structure of *P. gerardiana* is affected by different ecological factors like elevation, climate and soil variability. Similar findings were reported earlier (Kumar et al. 2013; Kumar et al. 2016; Sáenz-Romero et al. 2011; Sharma 2005) for *P. gerardiana.* In the present study, high genetic diversity within populations may be attributed to the effect of the environmental factors (Hahn et al. 2012; Sani et al. 2018) and anthropogenic activities in agreement with (Saeed et al. 2017). Climatic factors are associated with micro-geographical genetic differences, which may cause the phenotypic plasticity buffers against environmental changes over a plant's life cycle. Further, it weakens over time as climatic event changes.

Measurement of genetic diversity through molecular markers is difficult as it shows the adaptations of environment and other conditions. (Jump and Penuelas 2014). Earlier (Aziz et al. 2017) suggested the conservation of *P. gerardiana* for the future of this valuable Pine forest. This condition would have further broader implications, both for the ecosystem and livelihood of the local people.

In conclusion, based on our findings, it is concluded that there is an important genetic and phytochemical variation along the altitudinal gradient among the *P. geradiana* populations. Such patterning of genetic and phytochemical differentiation could result from the environmental (temperature, precipitation and soil characteristics) and the human disturbance variation along the altitudinal gradient. Our findings could help to design a conservation program that should include implementation of sustainable management plans, considering the large ecological and economic local importance of this pine species.

ACKNOWLEDGEMENTS

This research project was partially supported by University of Balochistan, UBRF Research Grant Program UBRF-17/018.

REFERENCES

Adams RP, Schwarzbach AE, Pandey RN. 2003. The concordance of terpenoid, ISSR and RAPD markers, and ITS sequence data sets among genotypes: An example from *Juniperus*. Biochem Syst Ecol 31: 375-387. DOI: 10.1016/S0305-1978(02)00157-6

Ahmed M, Khan N, Wahab M, Hamza S, Siddiqui MF, Nazim K, Khan MU. 2009. Vegetation structure of *Olea ferruginea* Royle forests of lower Dir district of Pakistan. Pak J Bot 41: 2683-2695.

Ahmed M, Palmer J, Khan N, Wahab M, Fenwick P, Esper J, Cook E. 2011. The dendroclimatic potential of conifers from northern Pakistan. Dendrochronologia 29: 77-88. DOI: 10.1016/j.dendro.2010.08.007

Akbar M, Ahmed M, Shaukat SS, Hussain A, Zafar MU, Sarangzai AM, Hussain F. 2013. Size class structure of some forests from Himalayan range of Gilgit-Baltistan. Sci Tech Dev 32: 56-73.

Akbar M, Khan H, Hussain A, Hyder S, Begum F, Khan M, Ali A, Hussian SA, Khan G, Abbas Q. 2014. Present status and future trend of chilghoza forest in Goharabad, District Diamer, Gilgit-Baltistan, Pakistan. J Biodiver Environ Sci 5: 253-261.

Aziz MA, Adnan M, Hussain SK, Hashem A, Alqarawi AA, Abd-Allah EF. 2017. Comparative regeneration status of *Pinus gerardiana* in two forest-use types of Sulaiman mountain range near Pak-Afghan border region: Historical, current and future perspectives. Pak J Bot 49: 227-236.

Bakshi M, Konnert M. 2011. Genetic diversity and differentiation through isozymes in natural populations of *Pinus wallichiana* A.B Jacks (Blue Pine) in India. Annals For Res 54: 23-37. DOI: 10.15287/afr.2011.95

Beg A, Mirza HK. 1984. Some more plant communities and the future of dry oak forest zone in Swat valley. Pak J For 34: 25-35.

Berli FJ, Moreno D, Piccoli P, Hespanhol-Viana L, Silva MF, bressan-Smith R, Cavagnaro JB, Bottini R. 2010. Abscisic acid is involved in the response of grape (*Vitis vinifera* L.) cv. Malbec leaf tissues to ultraviolet-B radiation by enhancing ultraviolet-absorbing compounds, antioxidant enzymes and membrane sterols. Plant Cell Environ 3 (1): 1-10. DOI: 10.1111/j.1365-3040.2009.02044.x

Hahn T, Kettle CJ, Ghazoul J, Frei ER, Matter P, Pluess AR. 2012. Patterns of genetic variation across altitude in three plant species of semi-dry grasslands. PloS One 7: e41608. DOI: 10.1371/journal.pone.0041608

Hamrick J, Godt M. 1990. Allozyme diversity in the grasses. Population biology of grasses, Cambridge University Press, Cambridge.

Hamrick JL, Godt MJW, Sherman-Broyles SL. 1992. Factors influencing levels of genetic diversity in woody plant species. Population genetics of forest trees. New Forest 6: 95-124. DOI: 10.1007/BF00120641

Hussain A. 2013. Phytosociology and dendrochronological study of Central Karakoram National Park, Northern Areas (Gilgit-Baltistan), Pakistan. Federal Urdu University Of Arts, Science & Technology, Islamabad.

Kernodle S, Cannon R, Scandalios J. 1993. Concentration of primer and template qualitatively affects product in RAPD-PCR. Biotechniques 14 (3): 362-364.

Khan H, Akbar M, Zaman M, Hyder S, Khan M, Nafees M, Raza G, Begum F, Hussain S, Khan S. 2015. Diameter size class distributions of *Pinus gerardiana* Wall. Ex D. Don from Gohar Abad Valley district Diamer, Gilgit-Baltistan. Pak J Biodivers Environ Sci 6: 50-56.

Kumar R, Shamet G, Avasthe R, Singh C. 2013. Ecology of chilgoza pine (*Pinus gerardiana* Wall) in dry temperate forests of northwest Himalaya. Ecol Environ Conserv 19: 1063-1066.

Kumar R, Shamet G, Mehta H, Alam N, Kaushal R, Chaturvedi O, Sharma N, Khaki B, Gupta D. 2016. Regeneration complexities of *Pinus gerardiana* in dry temperate forests of Indian Himalaya. Environ Sci Pollut Res 23: 7732-7743. DOI: 10.1007/s11356-015-6010-5

Kumar R, Shamet G, Mehta H, Alam N, Tomar J, Chaturvedi O, Khajuria N. 2014. Influence of gibberellic acid and temperature on seed germination in Chilgoza pine (*Pinus gerardiana* Wall.). Indian J Plant Physiol 19: 363-367. DOI: 10.1007/s40502-014-0119-2

Loya-Rebollar E, Sáenz-Romero C, Lindig-Cisneros R, Lobit P, Villegas-Moreno J, Sánchez-Vargas N. 2013. Clinal variation in *Pinus hartwegii* populations and its application for adaptation to climate change. Silvae genetica 62: 86-95. DOI: 10.1515/sg-2013-0011

Malik A, Shamet G, Ali M. 2008. Seed Stratification of *Pinus gerardiana* Wall.: Effect of stratification duration and temperature. Indian Forester 134: 1072-1078.

Meloni M, Perini D, Filigheddu R, Binelli G. 2006. Genetic variation in five Mediterranean populations of *Juniperus phoenicea* as revealed by inter-simple sequence repeat (ISSR) markers. Annals Bot 97: 299-304. DOI: 10.1093/aob/mcj024

Ordonez A, Gomez J, Vattuone M. 2006. Antioxidant activities of *Sechium edule* (Jacq.) Swartz extracts. Food Chem 97: 452-458. DOI: 10.1016/j.foodchem.2005.05.024

Peltier R, Dauffy V. 2009. The Chilgoza of Kinnaur. Influence of the *Pinus gerardiana* edible seed market chain organization on forest regeneration in the Indian Himalayas. Fruits 64: 99-110. DOI: 10.1051/fruits/2009005

Saeed S, Ahmed A, Laghari S, Ali G, Begum S. 2020. Genetic and chemical variation of *Seriphidium quettense* endemic taxa of two mountainous regions of Quetta, Pakistan. Appl Ecol Environ Res 18: 2413-2423. DOI: 10.15666/aeer/1802_24132423

Saeed S, Ahmed A, Shah SH, Begum S, Zeeshan M, Khan W. 2018. Phytotoxic potential of wild medicinal plants from different

altitudinal gradients of Quetta Balochistan Pakistan on *Convolvulus arvensis* L. Pak J Weed Sci Res 24 (4): 323-334.

Saeed S, Barozai MYK, Ahmed A, Tareen RB, Ali S. 2017. Impact of ecological diversity on genetic and phytochemical variation injuniperus excelsa from high elevation zones of Quetta Valley, Pakistan. Pak J Bot 49: 201-206.

Sáenz-Romero C, Beaulieu J, Rehfeldt GE. 2011. Altitudinal genetic variation among *Pinus patula* populations from Oaxaca, Mexico, in growth chambers simulating global warming temperatures. Agrociencia 45: 399-411.

Sani SGAS, Chang PL, Zubair A, Carrasquilla-Garcia N, Cordeiro M, Penmetsa RV, Munis MFH, Nuzhdin SV, Cook DR, von Wettberg EJ. 2018. Genetic diversity, population structure, and genetic correlation with climatic variation in chickpea (*Cicer arietinum*) landraces from Pakistan. Plant Genome 11: 1. DOI: 10.3835/plantgenome2017.08.0067

Shalizi MN, Khurram S. 2016. Socio-economic importance of chilgoza pine forest of Afghanistan: A survey-based assessment. Asian J Sci Technol 7: 3556-3559.

Sharma V. 2005. Effect of artificial stratification of Chilgoza pine (*Pinus gerardiana*) seeds on its germination. Short Rotation Forestry for Industrial and Rural Development, ISTA, Nauni, Solan, India 266-270.

Sinha D, Singh J, Tandon P, Kakkar P. 2013. Genetic diversity of *Pinus roxburghii* sarg. collected from different Himalayan regions of India assessed by random amplified polymorphic DNA analysis. Toxicol Int 20 (3): 208-213. DOI: 10.4103/0971-6580.121667

Slinkard K, Singleton VL. 1977. Total phenol analysis: Automation and comparison with manual methods. Am J Enol Viticult 28: 49-55.

Thomas B, Macdonald S, Hicks M, Adams D, Hodgetts R. 1999. Effects of reforestation methods on genetic diversity of lodgepole pine: An assessment using microsatellite and randomly amplified polymorphic DNA markers. Theoretic Appl Genet 98: 793-801.

NUSANTARA BIOSCIENCE
Vol. 13, No. 1, pp. 129-137
May 2021

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n130117

Thymol quantitative analysis in medicinal formulation types through employing of nano-technology and antimicrobial activity in some pathogenic bacterial isolates

MOHAMMED HASAN BARRAK[1], MOHAUMAN MOHAMMAD AL-RUFAIE[1,♥], ZAHRAA YOSIF MOTAWEQ[2]

[1]Department of Chemistry, Faculty of Science, Kufa University. Najaf, Iraq. ♥email: mohaumanmajeed@yahoo.com, muhaimin.alrufaie@uokufa.edu.iq
[2]Department of Biology, Faculty of Science, Kufa University. Najaf, Iraq

Manuscript received: 25 April 2021. Revision accepted: 30 May 2021.

Abstract. *Barrak MH, AL-Rufaie MM, Motaweq ZY. 2021. Thymol quantitative analysis in medicinal formulation types through employing nano-technology and antimicrobial activity in some pathogenic bacterial isolates. Nusantara Bioscience 13: 129-137.* This study included a method for estimating thymol (THY) in its pure state and in some of its pharmaceutical preparations that were quick, easy, and sensitive, This method is based on nanoparticles that have been modified by oxidation and reduction reactions, In a sodium hydroxide base medium, with polyvinylpyrrolidone as a stabilizer. The thymol drug works as a reducing agent to dilute the ore mineral salt from silver nitrate (Ag^+) to silver nanoparticles; the oxidation-reduction reaction product for thymol has the highest absorption at 410 nm**.** The calibration curve was calculated, and the following information was determined, indicating that the Beer-Lambert Law was followed within the focus range of 0.25 to 50 parts per million. Sandal's sensitivity was 0.052 µg/cm^2, his molar absorptivity was 2.2883 x 10^3 L / mol.cm, his standard deviation rate was 0.402%, and his correlation coefficient was 0.9989. The biological effect on a number of Gram-negative and Gram-positive bacteria was studied, and the findings showed that the samples prepared were effective against these bacteria.

Keywords: Bacterial, formulation, medicinal, nano-technology, pathogenic, quantitative, thymol

INTRODUCTION

Pharmacologically classified as 2-isopropyl-5-methyl phenol is crystalline in colorless form monoterpene phenol, it has been used in traditional medicine and has been shown to have different pharmaceutical characteristics including antibacterial, antioxidant, antispasmodic, free radical scavenging, analgesic, antifungal, anti-inflammatory, antitumor activity and antiseptic, thymol (THY) possesses pharmacological properties and its numerous therapeutic activities biochemical and molecular diseases contra specific diseases: neurological, cardiovascular, metabolic, malignant, rheumatologic and gastrointestinal, the notable thymol influences are primarily due to it is anti-inflammatory activity (by stitching cytokine and chemokine recruitment).

Antioxidant (by free radical scavenging, enhancing enzymatic additionally non-enzymatic antioxidants, as well as metal chelation ions), antihyperlipidemic effects (through elevated rates of high-density lipoprotein cholesterol as well as lower amounts of low-density lipoprotein cholesterol in circulation as well as membrane stabling) (through Ionic Homeostasis maintenance) (Meeran et al. 2017) Figure 1.

Thymol (THY) was previously estimated by (Chromatographic (LC) with Electrochemical Detection) (Gao et al. 2010), (Chromatographic HPLC) (Hajimehdipoor et al. 2010), (Ultrasound) (Roosta et al. 2015), (Chromatographic GC-MS) (Jiménez-Salcedo and Tena 2017), (Chromatographic HPLC-UV) (Angelo et al. 2016), (Chromatographic GC-MS with HS-SPME) (Fiori et al. 2013), (Voltammetric) (Ziyatdinova et al. 2017), (Electrochemical) (Aghamohseni et al. 2019), (Spectrophotometric) (Dhahir and Hussein 2012), (Spectrophotometric Batch and Flow Injection) (Al-Ward and Al-Abachi 2012).

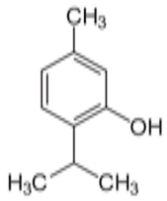


Figure 1. Chemical structure of thymol

Table 1. The pharmaceutical preparations that were studied

Drug Formulations samples	Declared composition	Company
Listerine antiseptic Fresh Burst wash mouth	Per 0.064% thymol	ADA, American dental association
Listerine antiseptic cool mint wash mouth	Per 0.064% thymol	ADA, American dental association
Zak Mouth and Dental wash 240 mL	Per 0.12% thymol	Zak Egypt

MATERIALS AND METHODS

Materials and reagents

All of the substances used in this study were inexpensive and of the highest purity, and they were used without further disinfection throughout, the 0.01 M $AgNO_3$ solution was generated by dissolving 0.4246 g in deionized water in a 250 mL volumetric vial with a standardized solution, Sodium hydroxide 0.001 M was made by dissolving 0.0199 g in 500 mL deionized water, and a Polyvinylpyrrolidone (0.2%) solution was made by breaking down 0.2 g in deionized water in a 100 mL volumetric vial, and completed to the mark in all volumetric vials, They were from the same company, reagent grade BDH, and they were delivered together, thymol was prepared as a 250 ppm standard and used solution by breaking down 25 mg of bulk drug in 100 mL water collected from SDI (State Drug Industries and Medical Appliances Company) (Iraq), the studied technique was applied to thymol using three different types of drug formulations, These types are illustrated in Table 1.

Collection and diagnosis of bacterial isolates

Multidrug-resistant (MDR) pathogenic bacterial isolates include: From stool, burns, wounds, synovial fluids, blood, and urine, two gram-negative bacteria (*Klebsiella pneumoniae* and *Escherichia coli*) were isolated, while two gram-positive bacteria (*Enterococcus faecalis* and *Staphylococcus aureus*) were isolated (Olurinola 1996; MacFaddin 2009; Deepthi and Narasimha 2013; Vu et al. 2018; Kavitha et al. 2019).

All bacterial isolates were stored on BHI broth supplemented with (15%) glycerol at (-20 °C) and later confirmed using an automated bacterial recognition instrument Vitek-2 compact system GP additionally GN card. Before use, the isolates were sub-cultured on BHIA and incubated at 37 °C for 24 hours, in the laboratories of the Biology Department of the College of Science at the University of Kufa.

Chemical samples for application were prepared as follows:

Test tube 1: polyvinylpirrolidone 1 mL, sodium hydroxide 0.5 mL, thymol 1 mL, then dilution by distilled water to 9.3 mL, then Silver nitrate 0.7 mL.

Test tube 2: polyvinylpirrolidone 1 mL, sodium hydroxide 0.5 mL, then dilution by distilled water to 9.3 mL, then Silver nitrate 0.7 mL.

Test tube 3: Thymol 1 mL, then dilution by distilled water to the signal 10 mL.

Test tube 4: Silver nitrate 0.7 mL then dilution through deionized water to the signal 10 mL.

Antibacterial activity experimental

The bacterial suspensions were prepared according to Ramalivhana et al. (2014) explained. The antibacterial activity of test tubes was compared to bacterial isolates using the agar well diffusion method (Murray et al. 1995; Kavitha et al. 2019). The test tubes were compared to bacterial isolates in MHA medium to see how biologically active they were.

Agar well diffusion assay

The micropipette was used to distribute 100 µL of bacterial suspensions BHIB on the surfaces of the MHA plate, and wells were punctured in all of the culture plates using a sterile cork borer. One well was a perforation in the middle of the plate, with 100 µL Gentamicin added as a positive control; another well had 100 µL (DMSO) added as a negative control, and the residual wells had 100 µL test tubes alone. The cultivation plates were then incubated for 24 hours at 37°C. In millimeters, the clear inhibition zone around wells has been measured. The experiments were carried out in three different ways (Olurinola 1996).

Apparatus

The main equipment used in this research includes: (i) T80 UV-Visible Spectrophotometer. PG Instruments Ltd. (Double beam). (ii) 303 PD UV-Visible Spectrophotometer. Apel. Japan (Single beam). (iii) UV-1650PC UV-Visible Spectrophotometer, SHIMADZU. Japan (Double beam). (iii) Electric Balance. Matter Toledo. Switzerland. (iv) Shaking water bath, Model: vs-1205 wl. scientific Co. Ltd. (v) pH meter, Spinbot thephaw.

Procedure for calibration curve

In volumetric flasks with a capacity of 10 mL, 0.2% PVP was added The sodium hydroxide solution was then added to these volumetric flasks in a volume of 0.5 mL, then, in these volumetric flasks, different volumes of thymol were added, ranging from (0.01 mL to 3 mL), and then dilution to 9.3 mL with distilled water then 0.7 mL of silver nitrate (0.01 M) in each volumetric flask, after 40 minutes at 35°C, the absorbance of each solution was measured at 410 nm against a reagent blank.

Zak mouth and dental wash

A sample of 240 mL mouth and teeth wash and the proportion of thymol in it (0.12% C), 21 mL of it was taken and placed in a volume volumetric flask 100 mL, then diluted with distilled water to a mark, Then, in thymol measurements, take different volumes and treat them in the same way as before.

Listerine antiseptic cool mint wash mouth

A sample of 250 mL Cool mint wash mouth and the proportion of thymol in it (0.064% C), 39 mL of it was taken and placed in a volume volumetric flask 100 mL, then diluted with distilled water to a mark, Then after, different volumes are taken and treated in a previous manner for thymol measurements.

Listerine antiseptic fresh burst wash mouth

A sample of 250 mL Fresh Burst wash mouth and the proportion of thymol in it (0.064% C), 39 mL of it was taken and placed in a volume volumetric flask 100 mL, then diluted with distilled water to a mark, then, in thymol measurements, different volumes are taken and treated in the same way.

RESULTS AND DISCUSSION

Absorption spectra

When the Colorless thymol solution (C), Blank (B) (PVP, NaOH, Distilled water for dilution and $AgNO_3$) colorless solution and (A) sample of (PVP, NaOH, thymol, Distilled water for dilution and $AgNO_3$), the red-colored product, as well as the reactants, are scanned in a UV-VIS spectrophotometer to emphasize the reaction

Figure 2 A, B, and C show the spectra of the aqueous solution of pure thymol in the spectral region of 190-800 nm, blank solution and colored product (the prepared on addition PVP then NaOH then thymol then Distilled water for dilution and $AgNO_3$), the red product with maximum absorption of 410 nm differs significantly from the maximum absorption of both reactants, as shown in this figure. The usefulness of this redshift for a product can be used as a thymol assay procedure.

Optimization of reaction conditions

Effect of different silver nitrate volumes

The effect of different silver nitrate volumes required to achieve optimum absorbance is investigated; the experiment is carried out with $AgNO_3$ (0.01 M) volumes ranging from 0.1 mL to 2 mL, Figure 3, when 1.5 mL of silver nitrate (0.01 M) is added, the maximum absorbance is reached, so this method uses 0.7 mL of silver nitrate (0.01 M).

Effect of polyvinylpirrolidone in different volumes

The effects of different polymers (Polyvinylpirrolidone PVP, Polyurethane PU, and Polyvinyl chloride PVC) on color product formation were investigated, 1 mL of (0.2%) concentration was added to see if the polymers used had any effect on the formation of thymol, PVP proved to be the most absorbent polymer for the color solution. The best volume of the base form was then determined, the effects of various volumes of PVP (0.1, 0.3, 0.5, 0.7, 1,..., and 2 mL) on the formation of thymol.

Figure 4, when 0.1 mL is added, the maximum absorbance is reached. As a result, 1 mL of Polyvinylpyrrolidone (0.2%) is used in this method, we selected PVP as a stabilizer for preventing of silver nanoparticles agglomeration, the H+ ions are produced in the silver nitrate reaction by analyses. As a result, removing H+ will promote Ag-NP formation. When adding PVP to the solution, It helps in the stabilization of silver ions by forming Ag (PVP)+ complexes and removing H+ produced by H(PVP)+ during the oxidation process (Nezhad et al. 2010).

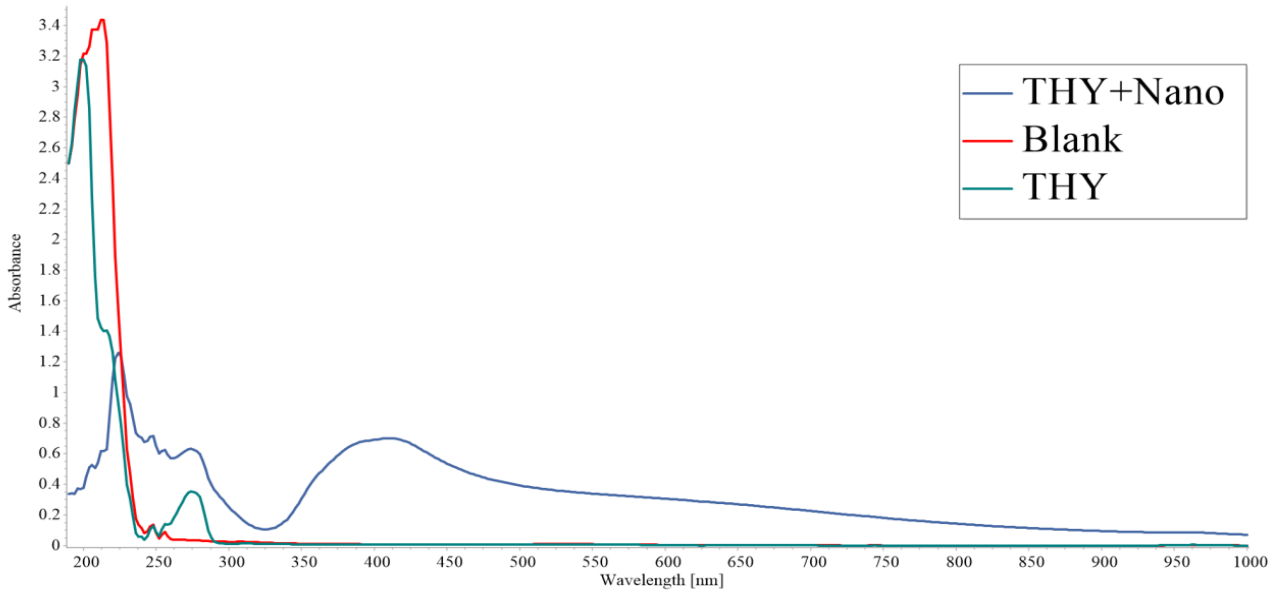


Figure 2. Sample (Sliver Nano withthymol antibiotic (A), Blank (All reagents without antibioticthymol (B), and thymol Pure (C) in the Absorption Spectrum.

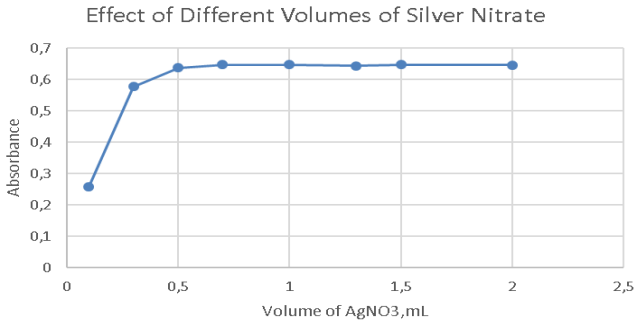


Figure 3. Effect of Silver nitrate

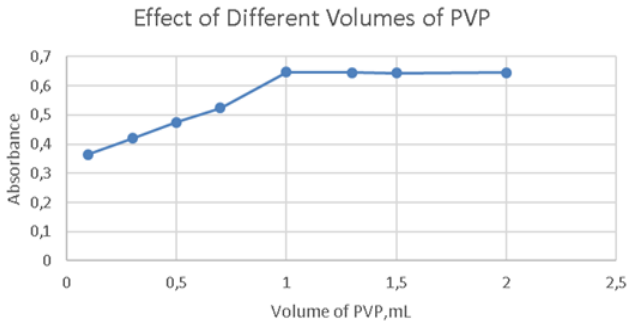


Figure 4. Effect of Polyvinylpyrrolidone

Table 2. The Sequence of Addition effect

Sequence	A+B+C+D+E	C+B+A+D+E	C+E+B+D+A	E+C+B+D+A
Absorbance	0.600	0.594	0.525	0.579

Effect of different volumes Base

The effects of different bases (NaOH, KOH, NH_4OH, and Na_2CO_3) on colour product formation were studied. 1 mL of (0.001 M) concentration was added to see how the bases affected the formation of the product thymol, NaOH was the perfect base for the color solution because it had a high absorbance. after determining the best volume of the base type Different volumes of NaOH were used to determine their effects on the formation of thymol: 0.1, 0.3, 0.5, 0.7, 1,..., and 3 mL, respectively, Figure 5, when 2 mL of sodium hydroxide (0.001 M) is added, the maximum absorbance is reached; therefore, 0.5 mL of sodium hydroxide (0.001 M) is used for this method, when the elimination of H+ may help in the formation of Ag-NPs, the effect of the solution's alkalinity on the reaction was investigated by varying the NaOH concentration, as seen, the peak strength of the silver nanoparticles signal increases by the increasing concentration of NaOH additionally then decreases, this decrease may be due to the Ag_2O formation. Consequently, a 0.2 mM NaOH concentration was identified as the optimal level for further studies (Nezhad et al. 2010).

Sequence of addition

The sequence in which the solutions are added in the reactions that produce the silver nanoparticles under investigation has a significant impact on the color intensity of the resulting compounds, therefore several experiments were conducted with a sequence of different additions and for all the studied interactions to choose the best addition sequence that gives the highest absorption of the resulting compounds as shown in Table 2.

It is found from Tables 3-7 that the order of addition of reagents is by mixing PVP, then Sodium hydroxide, then thymol, then dilute with distilled water to 8.7 mL, then Silver nitrate (A+ B + C +D + E) giving the highest absorbance, this sequence gives the best formation of the product.

The best addition sequence for all silver nanoparticle reactions was found to be (A+B+C+D+E) in Table 2, so it was used in subsequent experiments.

Temperature effect on colored product formed

The effect of temperature on the speed at which silver nanoparticles form was examined, with a temperature range of 25-75°C used, as shown in Figure 6, Stability in absorption has been found as absorbance increases with increasing temperature up to a 35°C. This can be due to the probability of stability in the formation of silver nanoparticles.

As a result, the preferred temperature for the formation of Silver nanoparticles was 35°C. In subsequent experiments with silver nanoparticle interactions, these temperatures were chosen.

Time effect on colored product formed

The effect of time on the formation of silver nanoparticles has been investigated, and under the best conditions that have been proven in previous experiments, for periods of time ranging from 10-120 minutes, with measurements taken every ten minutes, the resulting nanoparticle has high stability of more than one hour or more, allowing these interactions to be examined easily. Figure 7 indicates that, as a result, a development period of 40 minutes is chosen as the optimum in the general method.

The effect of time on the colored product formed after 72 hours

Time effect on the velocity of formation of nanoparticles was studied 72 hours after the velocity formation of nanoparticles was constant at 0.801 absorptions.

Calibration curve

The standard calibration curve for the colored product has been created under the optimum conditions discussed in Figure 8.

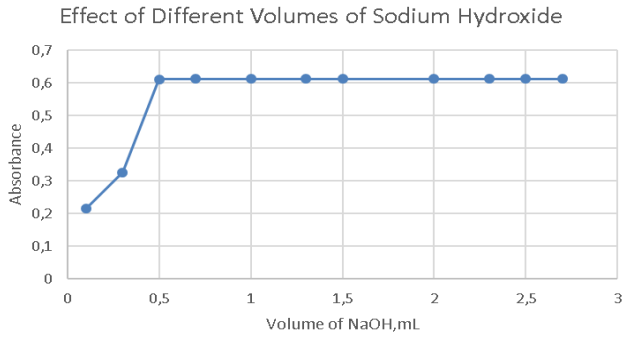


Figure 5. Effect of Different Volumes Sodium hydroxide

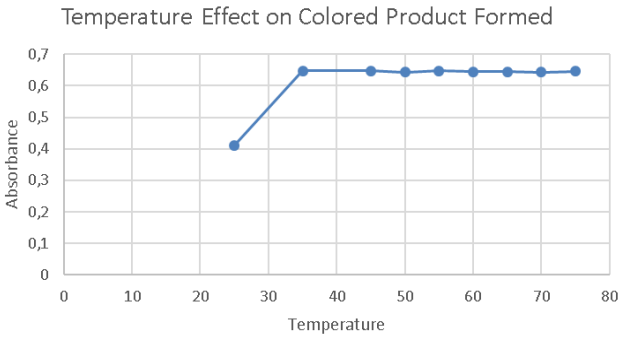


Figure 6. Effect of temperature on colored product

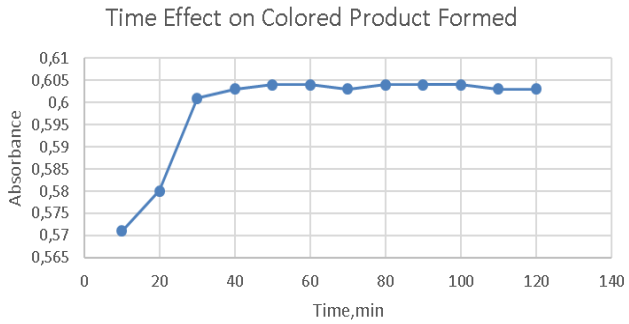


Figure 7. Time Effect on Colored Product Formed

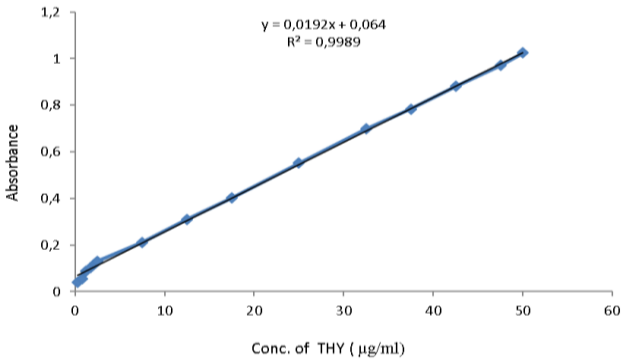


Figure 8. Calibration curve of thymol

Other analytical parameters are calculated, and the results are shown in Table 3 show that this analytical method performs well for determining thymol at low concentrations.

Precision and accuracy

Precision first was measured using nine replicates at 2.5, 25, and 37.5 µg / mL thymol concentrations to check the precision and accuracy of the proposed method.

The accuracy of three specific thymol concentrations is calculated, and the results are shown in Table 4 indicate that the thymol determination method is accurate and satisfactory (Tawa and Shingo 1980).

Mechanism of the product

As shown in Figures 9 and 10, the mechanism of reaction may indicate a connection between the drugs under examination and the reagents used in their estimation.

Interferences effect

In order to ensure that method is selective, it was tested on a variety of samples, particularly pharmaceutical preparations containing the pharmaceutical thymol, the relationship of excipients (interferes) was studied , as it was achieved by conducting a spectral estimation of the estimated pharmacological compounds and adding these substances separately to the studied solutions, and these substances become ten times more concentrated than the studied drug compound, and using the same approach used in the calibration curve, PVP, then sodium hydroxide, then 1mL from (250 ppm) of the drug, then distilled water, then silver nitrate, then 1mL from concentration (2500 ppm) of each additive, applying the rest of the best conditions and measuring the absorbance of the product, calculating the error and recovery ratio, taking into consideration consider the dilution of the resulting solution to 25 mL with distilled water, the interference effects are acceptable if the error ratio does not exceed (2%). when compared to measurements without overlaps (Ahmed and Shahla 2019), (Each value is a three-reading average). We can see that the existence of additives has no effect on the thymol estimation methods by looking at the values of (percent Error) and (Recovery percent), the existence of additives had an effect on the absorption of the colored compound, as shown in Table 5, Notice the effect of such additives on the thymol estimation process by pursuing values of percent error and percent recovery.

Table 3. Analytical Parameter for Determining thymol

Parameter	Value
beer's law limit (ppm)	0.25-50
Molar Absorptivity (L / mol.cm)	2.2883 x 10^3
Correlation Coefficient	0.9989
Limit of Quantitation (LOQ) ppm	0.3682
Sandell's sensitivity(µg / cm2)	0.052
Limit of Detection (LOD) ppm	0.1104
Determination Coefficient	0.9994
Intercept (a)	0.0640
Slope (b)	0.0192

Table 4. Value Accuracy and Precision for the product compound of thymol

Concentration of thymol (ppm)		Relative % error	% Recovery	% R.S.D
Percent	Found			
2.5	2.440	-2.400	97.600	0.900
25	25.360	1.440	101.440	0.181
37.5	37.440	-0.160	99.840	0.127

Table 5. The effect of the presence of additives at a concentration of (25 ppm) on the absorbance of the compound thymol

Interference	% Error	% Recovery
lactose	-1.780	98.220
Talc	- 0.955	99.045
starch	0.995	100.995
Acacia	0.546	100.546
Sucrose	- 1.245	98.755
Glucose	1.274	101.274
magnesium citrate	- 0.170	99.830
Benzoic acid	- 0.887	99.113
aspartame	0.430	100.430
Mannitol	- 0.661	99.339
Cross povidone	0.740	100.740
Twin 80	0.395	100.395
Titanium dioxide	- 0.570	99.430
Microcrystal cellulose	- 1.150	98.850
Sucrose	0.120	100.120

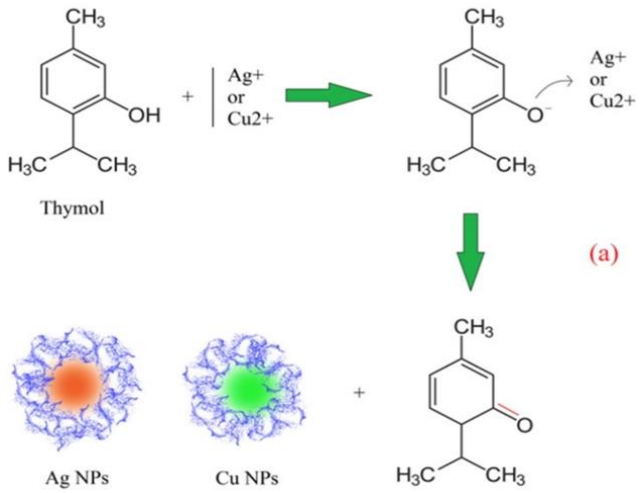


Figure 9. Probable mechanism of reduction in silver and ions by thymol (Alavi and Naser 2019).

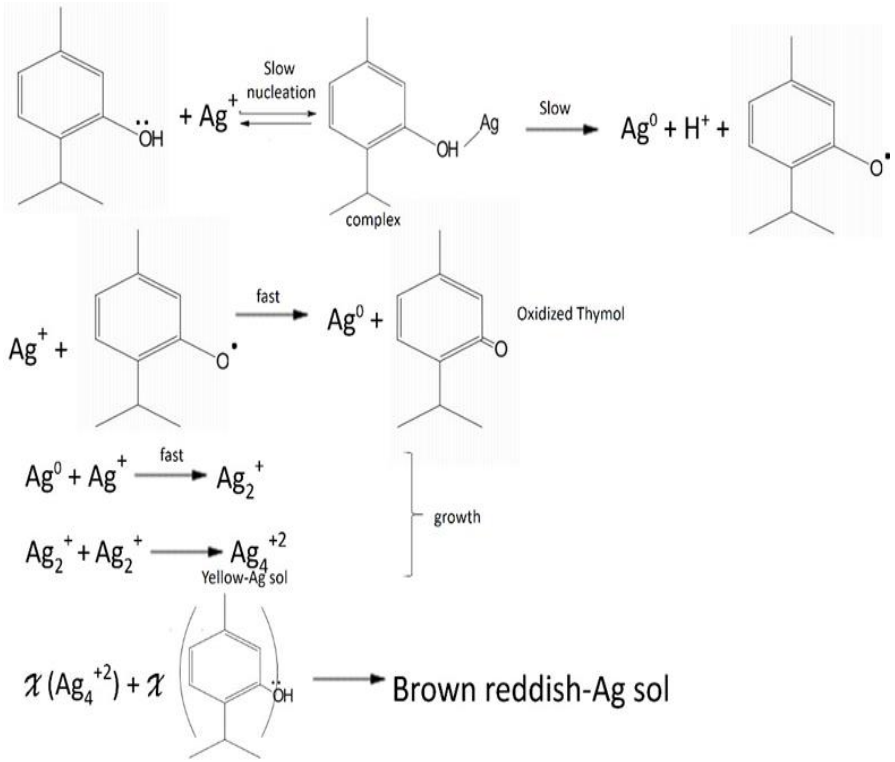


Figure 10. Mechanism of the generation of AgNPs at room temperature (Ganash 2019)

Application of the methods

To see if the methods proposed are effective, A number of pharmaceutical formulations containing thymol in pharmaceutical solutions had to be added according to the methods used, and a diluted solution had to be prepared (250 ppm), take three different volumes of each solution that was prepared, and apply the steps used to prepare the calibration curve, then calculate the accuracy of the analytical method used with these prepared solutions and all of the studied reactions is compatible with the results obtained for a variety of pharmaceutical preparations, show the effectiveness and success of the proposed method in applying to pharmaceutical preparations, as shown in Table 6. Each value in the table is the average of three readings, and to compare the effectiveness and success of proposed analytical methods with the results of a well-known and reliable method (found within the British and American pharmaceutical industries) of substance pure drug and its various forms of pharmaceutical preparations available on the market, the measured results for F and T are 9.28 and 2.45, respectively (Harvey 2000; Christian 2004; Moffat et al. 2011), we could see that the measured value is less than the theoretical value, indicating that the method is reliable.

Biological activity

Nanoparticles Antibacterial activity and antibiotics were tested against known human pathogens using a disk diffusion assay, and antibiotics with NPs showed a larger inhibition zone than antibiotics and NPs alone, this demonstrates the possibility of nanoparticles and antibiotics working together in a synergistic manner Bhosale et al. (2015) as in the following Table 7.

Silver nanoparticles were also studied on their own or in conjunction with antibiotics, with the findings indicating that they had antibacterial effects and synergistic activity (Hwang et al. 2012), the study centered on the susceptibility of microorganisms to silver nanoparticles, antibiotics, and their combined effects; when nanoparticles and antibiotics were given together, the diameter of the inhibition zone increased by a minimum of 2 to 4 mm (Geoprincy et al. 2014; Nikparast and Mahsa 2018) the dose-dependent capacity of AgNPs to inhibit the activity of biofilms produced by human pathogens identified under in vitro conditions is used to inhibit biofilm growth. According to these results, biologically synthesized AgNPs inhibited biofilm activity in all of the bacterial strains studied Gurunathan et al. (2014) as in the following Figures 11-14.

Table 6. F, t compare the accuracy and reliability of the proposed process with the standard nanoparticles composition reaction method Between thymol and silver ion nanoparticles (Moffat et al. 2011)

Preparation thymol Containing	Deliberated process				Official process			
	Conc. of thymol (ppm)		Re %	R.S.D%	Conc. of thymol (ppm)		Re%	R.S.D%
	Percent	Found			Percent	Found		
Fresh Burst wash mouth ADA,	2.5	2.390	95.600	0.909	2.5	2.520	100.800	0.819
American dental association	25	25.310	101.240	0.181	25	24.830	99.320	0.561
	37.5	37.390	99.710	0.127	37.5	37.820	100.720	0.427
Cool mint wash mouth ADA,	2.5	2.390	95.600	0.917	2.5	2.460	98.400	1.098
American dental association	25	25.050	100.20	0.173	25	24.910	99.640	0.570
	37.5	37.340	99.580	0.128	37.5	37.891	101.042	0.133
Mouth and Dental wash Zak	2.5	2.410	96.400	0.925	2.5	2.451	98.040	0.198
Egypt	25	24.940	99.760	0.184	25	25.340	101.360	0.358
	37.5	37.290	99.440	0.128	37.5	37.620	100.320	0.274
Pure	2.5	2.440	97.600	0.900	2.5	2.472	98.880	1.014
thymol	25	25.360	101.440	0.181	25	24.870	99.480	0.482
	37.5	37.440	99.840	0.127	37.5	37.555	100.146	0.344
F- value					0.102			
t-value					0.914			

Table 7. Antibacterial effects of silver nanoparticles against Gram-negative and Gram-positive pathogenic bacteria

Types of bacteria	Antibiotics	Silver nitrate (inhibition zone)	Silver nanoparticles (inhibition zone)	Antibiotics (inhibition zone)	Antibiotics with silver nanoparticles (inhibition zone)
Klebsiella pneumoniae	Thymol	7 m.m	12 m.m	11 m.m	15 m.m
Escherichia coli	Thymol	6 m.m	13.5 m.m	13 m.m	14 m.m
Staphyllococcus aureus	Thymol	6 m.m	8 m.m	0 m.m	9 m.m
Enterococcus faecalis	Thymol	5 m.m	6 m.m	0 m.m	7 m.m

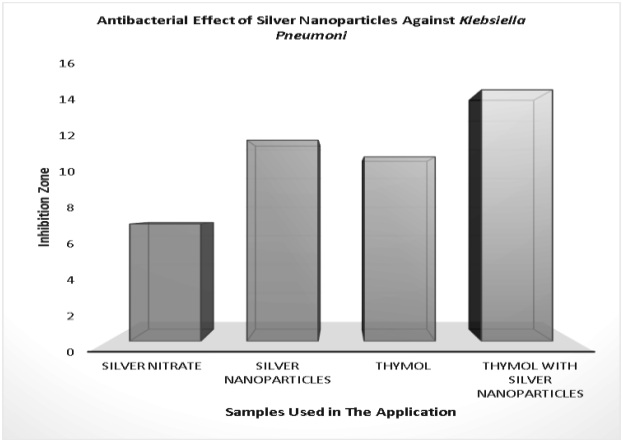


Figure 11. Effect of Antibacterial of Silver nanoparticles contra *Klebsiella pneumonia*

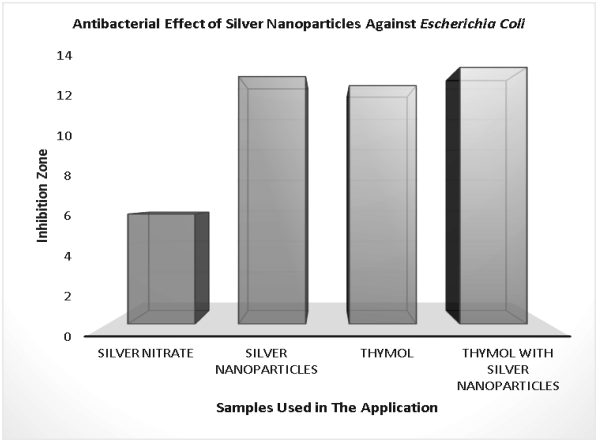


Figure 12. Effect of Antibacterial of Silver nanoparticles contra *Escherichia coli*

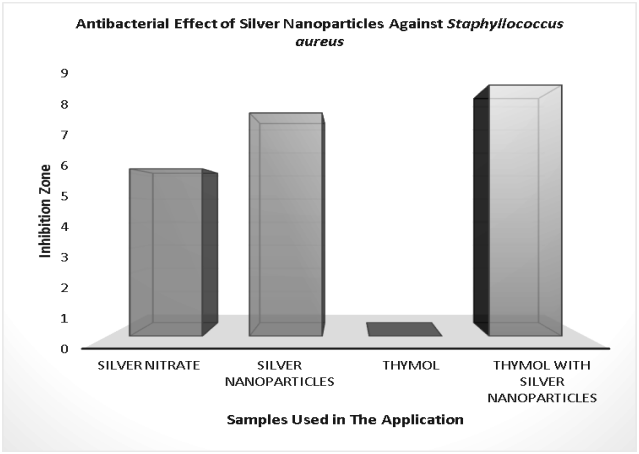


Figure 13. Effect of Antibacterial of Silver nanoparticles contra *Staphyllococcus aureus*

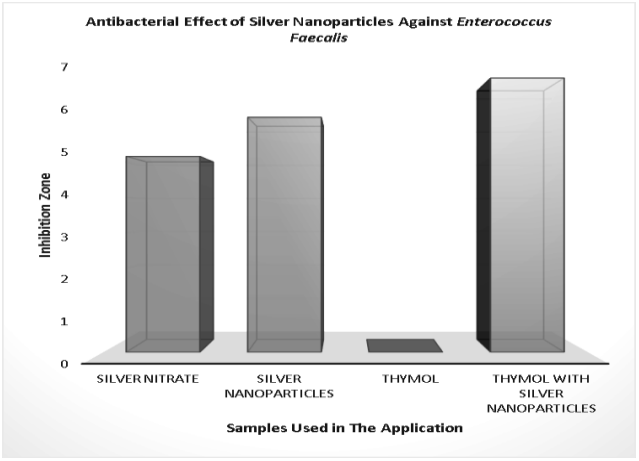


Figure 14. Effect of Antibacterial of Silver nanoparticles contra *Enterococcus faecalis*

In conclusion, simple and rapid quantitative spectrophotometric method based on direct assessment of thymol formed both in its pure form and in pharmaceutical preparations, based on modified nanoparticles as color sensors by the interaction of thymol oxidation and reduction with silver nitrate. The suggested spectral method for estimating thymol produced high sensitivity, low detection, and a good linear range. Colored products are characterized by their high stability in the water medium, and this method has good accuracy and precision. The method does not necessarily require any model pre-treatments or solvent extraction. The method was effective in estimating thymol in pharmaceutical preparations, and the results were in line with the original content. The statistical results t, F test of the proposed spectral method compared to the standard method revealed no significant differences in the accuracy and reliability of the method, as well as the validity of the analytical application of this method. The prepared sample was applied to some bacteria, as well as its efficacy in reducing the bacteria wall's resistance was clear.

REFERENCES

Aghamohseni B, Hassaninejad-Darzi SH, Asadollahi-Baboli M. 2019. A new sensitive voltammetric determination of thymol based on MnY nano zeolite modified carbon paste electrode using response surface methodology. Microchem J 145: 819-832. DOI: 10.1016/j.microc.2018.11.045.

Ahmed AK, Shahla JS. 2019. Determination of amoxicillin in pharmaceutical preparations by spectrophotometric and flow injection–activated chemiluminescence methods. Tikrit J Pure Sci 14: 63-79.

Alavi M, Naser K. 2019. Biosynthesis of Ag and Cu NPs by secondary metabolites of usnic acid and thymol with biological macromolecules aggregation and antibacterial activities against multi drug-resistant (MDR) bacteria. Int J Biol Macromol 128: 893-901. DOI: 10.1016/j.ijbiomac.2019.01.177.

Al-Ward HS, Al-Abachi MQ. 2012. Batch and flow-injection spectrophotometric determination of thymol using procaine hydrochloride as a new chromogenic reagent. Baghdad Sci J 9(2): 302-310. DOI: 10.21123/bsj.9.2.302-310.

Angelo T, Pires FQ, Gelfuso GM, da Silva JK, Gratieri T, Cunha-Filho M S. 2016. Development and validation of a selective HPLC-UV method for thymol determination in skin permeation experiments. J Chromatogr B Analyt Technol Biomed Life Sci 1022: 81-86. DOI: 10.1016/j.jchromb.2016.04.011.

Bhosale RS, Hajare KY, Mulay B, Mujumdar S, Kothawade M. 2015. Biosynthesis, characterization and study of antimicrobial effect of

silver nanoparticles by *Actinomycetes* spp. Int J Curr Microbiol Appl Sci 2: 144-151.

Christian GD. 2004. Analytical Chemistry, 6th ed. John Wiley and Sons, Inc.

Deepthi RS, Narasimha RG. 2013. Antimicrobial activity of mangrove plant *Avicennia officinalis* (Lam. Briqvet) on selected pathogens. Res J Pharm Biol Chem Sci 4: 335-341.

Dhahir SA, Hussein HJ. 2012. Spectrophotometric determination of thymol in pure and Listerine mouth washing. Tikrit J Pure Sci 17: 97-102.

Fiori GML, Bonato PS, Pereira MPM, Contini SHT, Pereira AMS. 2013. Determination of thymol and carvacrol in plasma and milk of dairy cows using solid-phase microextraction. J Braz Chem Soc 24(5): 837-846. DOI: 10.5935/0103-5053.20130109.

Ganash AA 2019. Electrochemical properties and mechanistic study of the green synthesis of silver nanoparticles using Bardaqush extract solution. Mater Res Express 6: 065024. DOI: 10.1088/2053-1591/ab0d40.

Gao H, Cao W, Liang Y, Cheng N, Wang BN, Zheng JB. 2010. Determination of thymol and phenol in honey by LC with electrochemical detection. Chromatographia 72: 361-363. DOI: 10.1365/s10337-010-1628-4.

Geoprincy G, Saravanan P, Gandhi NN, Renganathan S. 2014. A novel approach for studying the combined antimicrobial effects of silver nanoparticles and antibiotics through agar overlayer method and disk diffusion method. Dig J Nanomater Biostructures 6: 1557-1565.

Gurunathan S, Han JW, Kwon DN, Kim JH. 2014. Enhanced antibacterial and anti-biofilm activities of silver nanoparticles against Gram-negative and Gram-positive bacteria. Nanoscale Res Lett 9(1): 1-17. DOI: 10.1186/1556-276X-9-373.

Hajimehdipoor H, Shekarchi M, Khanavi M, Adib N, Amri M. 2010. A validated high-performance liquid chromatography method for the analysis of thymol and carvacrol in *Thymus vulgaris* L. volatile oil. Pharmacogn Mag 6: 154-158. DOI: 10.4103/0973-1296.66927.

Harvey D. 2000. Modern Analytical Chemistry, 1st ed. McGraw-Hill Higher Education.

Hwang IS, Hwang JH, Choi H, Kim KJ, Lee DG. 2012. Synergistic effects between silver nanoparticles and antibiotics and the mechanisms involved. J Med Microbiol 61(12): 1719-1726. DOI: 10.1099/jmm.0.047100-0.

Jiménez-Salcedo M, Tena MT. 2017. Determination of cinnamaldehyde, carvacrol and thymol in feedstuff additives by pressurized liquid extraction followed by gas chromatography-mass spectrometry. J Chromatogr A 1487 14-21. DOI: 10.1016/j.chroma.2017.01.042.

Kavitha R, Francisca P, Auxilia A. 2019. Biosynthesis, characterization and antibacterial effect of plant-mediated silver nanoparticles from *Adenanthera pavoninal*. JETIR 6: 2349-5162

MacFaddin JF. 2009. Biochemical Tests for Identification of Medical Bacteria. Williams and Wilkins, Philadelphia.

Meeran MFN, Javed H, Al Taee H, Azimullah S, Ojha SK. 2017. Pharmacological properties and molecular mechanisms of thymol: Prospects for its therapeutic potential and pharmaceutical development. Front Pharmacol 8: 1-34. DOI: 10.3389/fphar.2017.00380.

Moffat AC, Osselton MD, Widdop B, Watts J. 2011. Clarke's analysis of drugs and poisons, 4ed. Pharmaceutical Press, London.

Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken HR. 1995. Manual of clinical microbiology, 6th Ed. ASM Press, Washington DC.

Nezhad MRH, Tashkhourian J, Khodaveisi J. 2010. Sensitive spectrophotometric detection of dopamine, levodopa and adrenaline using surface plasmon resonance band of silver nanoparticles. J Iran Chem Soc 7: S83-S91. DOI: 10.1007/BF03246187. DOI: 10.1007/BF03246187.

Nikparast Y, Mahsa S. 2018. Synergistic effect between phyto-synthesized silver nanoparticles and ciprofloxacin antibiotic on some pathogenic bacterial strains. J Med Bacteriol 7: 36-43.

Olurinola PF. 1996. A laboratory manual of pharmaceutical microbiology. Idu, Abuja, Nigeria.

Ramalivhana JN, Obi CL, Samie A, Iweriebor BC, Uaboi-Egbenni P, Idiaghe JE, Momba MNB. 2014. Antibacterial activity of honey and medicinal plant extracts against Gram-negative microorganisms. Afr J Biotechnol 13: 616-625. DOI: 10.5897/AJB11.892.

Roosta M, Ghaedi M, Daneshfar A, Sahraei R. 2015. Ultrasound-assisted microextraction-nano material solid-phase dispersion for extraction and determination of thymol and carvacrol in pharmaceutical samples: Experimental design methodology. J Chromatogr B Biomed Appl 975: 34-39. DOI: 10.1016/j.jchromb.2014.10.035.

Tawa R, Shingo H. 1980. Differential kinetic determination of mixtures of aniline and its derivatives using n, n-dimethyl-p-phenylenediamine. Chem Pharm Bull 28(7): 2136-2143. DOI: 10.1248/cpb.28.2136.

Vu XH, Duong TTT, Pham TTH, Trinh DK, Nguyen XH, Dang VS. 2018. Synthesis and study of silver nanoparticles for antibacterial activity against Escherichia coli and Staphylococcus aureus. Adv Nat Sci-Nanosci Nanotechnol 9(2): 025019. DOI: 10.1088/2043-6254/aac58f.

Ziyatdinova G, Ziganshina E, Cong PN, Budnikov H. 2017. Voltammetric determination of thymol in oregano using CeO 2-modified electrode in Brij® 35 micellar medium. Food Anal Methods 10: 129-136. DOI: 10.1007/s12161-016-0562-y.

NUSANTARA BIOSCIENCE
Vol. 13, No. 1, pp. 138-145
May 2021

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n130118

Characterization and evaluation of the variability of dragon fruit accessions in Dairi District, North Sumatra Province, Indonesia

LUTHFI AZIZ MAHMUD SIREGAR♥, NINA UNZILA ANGKAT, REVANDY ISKANDAR DAMANIK
Department of Agro-technology, Faculty of Agriculture, Universitas Sumatra Utara. Jl. Prof A. Sofyan No. 3 Padang Bulan, Medan 20155, North Sumatra, Indonesia. Tel./fax. +62-61-8213236, ♥email: luthfi1@usu.ac.id

Manuscript received: 31 January 2021. Revision accepted: 31 May 2021.

Abstract. *Siregar LAM, Angkat NU, Damanik RI. 2021. Characterization and evaluation of the variability of dragon fruit accessions in Dairi District, North Sumatra Province, Indonesia. Nusantara Bioscience 13: 138-145.* Many types of dragon fruits were planted in Dairi District of North Sumatra Province without much information of their importance in economic values. Therefore, this study was carried out to evaluate the variability of the dragon fruit population in this region to assist the local farmers in selecting the best species for planting. This study used a morphological observation method based on dragon fruit descriptor employing a purposive sampling technique. A total of 56 dragon fruit accessions collected from Sidikalang, Sitinjo, and Siempat Nempuhulu Sub-districts were studied and characterized based on the morphological characteristics of stems, flowers, and fruit. The results showed some variations in the stem, flower, and fruit morphology among the studied population. The dragon fruit plant that is widely cultivated by farmers in three sub-districts of Dairi District is a species of *Hylocereus polyrhizus*. Phenotypic diversity analysis showed seven quantitative characters with broad criteria of eighteen characters observed, namely stem length, pericarpel length, number of lobes, flower shoot length, number of bractea, bractea peak length, and fruit weight. However, phenotypic diversity analysis indicates they are closely related to each other except for accession D17 the red dragon fruit or red pitaya (*Hylocereus spp*.), and it could be the exotic species for nation-wide planting in Indonesia.

Keywords: accession, Dairi District, dragon fruit, morphological characteristic, phylogenetic

INTRODUCTION

Dragon fruit (*Hylocereus* spp.), also known as pitaya or pitahaya, belongs to the Cactaceae family. The genus Hylocereus recently was included in Selenicereus group (Korotkova, et al. 2017). The plant is native to Central America and it was spread from Florida to Brazil. There are about 14 major Hylocereus species in America but the popularly cultivated worldwide species are *Hylocereus undatus, H. monachantus,* and *H. megalanthu*s (Hernandez and Salazar 2012).

Dragon fruit plants were introduced into Indonesia around year 2000 from Thailand. They were later cultivated as an agricultural crop in some areas such as Yogyakarta, Malang, Mojokerto, Bogor, and Jember (Purba 2012). At this time, dragon fruit has been widely known in various regions of Indonesia. Initially, this fruit was difficult to find in traditional markets and could only be found in certain supermarkets, but with the increasing area and spread of planting areas, dragon in fruit marketing has spread to various circles of society including in traditional markets. However, it is still a relatively new crop, hence the dragon fruit in Indonesia still has a low diversity (Santoso 2013; Winarsih 2010). Until now, the cultivation of dragon fruit in Indonesia is still concentrated in a few areas such as the islands of Java, Sumatera, and Kalimantan.

There are two peel types of dragon fruits that have been released in Indonesia. They are the white flesh and the red flesh dragon fruits (Kristanto 2008). Phenotypic and genotypic characterization of dragon fruits would be useful for the dragon fruit plant breeding program. However, the characterization based on morphological and cytological characters is still limited for the dragon fruits until today. The availability of this information would be of great assistance in developing an appropriate method for the cultivation of a particular species (Setyowati 2008). Studies have found that high variation in characteristics of agronomic importance, even within the same species of Hylocereus is considered valuable for future breeding studies (Tran and Yen 2014).

Several studies related to the diversity of dragon fruit have been reported. Rahmawati and Mahajoeno (2009) reported a study of variations of dragon fruit based on morphology, isozyme, and vitamin C content in the area of Pasuruan (East Java), Sukoharjo, and Klaten (Central Java), and Bantul sub-districts (Yogyakarta). Tran and Yen (2014) reported a study on pollination methods on fruit set and fruit characters in several Pitaya clones, which aimed to improve pollination efficiency, fruit quality, and yield by determining pollination agro-management requirements.

Cultivation of dragon fruits has been undertaken in Dairi District, North Sumatra, for more than five years but on a small scale. Due to the lack of information on the origin and scientific information about the genotypes cultivated, this research was conducted to study the morphological and related characters of dragon fruit in the highlands of Dairi District, North Sumatra. This study can

be an initial reference and source of germplasm to carry out further studies in the field of plant breeding utilizing genotypes or accessions of dragon fruit cultivated in North Sumatra.

MATERIALS AND METHODS

This study was conducted from April to June 2017 at the dragon fruit farms in the Sitinjo, Sidikalang, and Siempat Nempuhulu Sub-districts in Dairi District of North Sumatra.

The research location was determined by first obtaining information on the number of dragon fruit farmers through the local government in 3 sub-districts in Dairi District, North Sumatra, namely Sidikalang Sub-district, Sitinjo Sub-district, and Siempat Nempuhulu Sub-district. Based on this information, 14 farmers have been practicing dragon fruit cultivation for more than three years. For every one farmer's land, 4 (four) plant accessions were taken as sample plants, therefore 56 (fifty-six) sample plants were obtained which were used in this study. The study samples used were healthy disease-free dragon fruits at flowering and fruiting stages. A purposive sampling method was used on accessions in each study area. Data collection was conducted by observing morphological characters qualitatively and quantitatively (Table 1) based descriptor guidebook International Union for The Protection of New Varieties of Plant (UPOV) for dragon fruit plants.

The qualitative and quantitative data were standardized and the phylogenetic was analyzed by using IBM SPSS program (Statistical Package for the Social Sciences) version 21 with cluster analysis to determine the degree of relationship between the accession of each sample. Cluster analysis was used to visualize the multivariant data (of the measured parameter) survey. Cluster analysis resulted in a dendrogram used to assess patterns of diversity of the survey data. Agglomerative Hierarchical Clustering with average linkage (between-group) method was used according to Mongi (2015) as stated below.

$$di,j = \sqrt{\sum_{k=1}^{p}(x_{ik} - x_{jk})^2}$$ with:

Where:

di, j : the distance between the object i with the object k

xik : object value i at the variable to-k

xjk : object value j at the variable to-k

p : number of variables cluster

In addition, the phenotypic diversity value was calculated according to the following equation (Steel et al. 1997):

$$\sigma^2 p = \frac{\Sigma(x_i - x)^2}{N}$$

Where: $\sigma^2 p$ is the diversity of phenotypes, x_i is the sample values of –i, and x is the value of the sample average of –i.

The standard deviation of phenotypic diversity was calculated as:

$$Sd\sigma^2 p = \sqrt{\sigma^2 p}$$

$Sd\sigma^2 p$ is the standard deviation of phenotypic diversity

The criteria for assessing the breadth and depth of phenotypic diversity are determined based on the following conditions (Mansyah et al. 2003):

- If $\sigma^2 p > 2Sd\sigma^2 p$, it means that the diversity is wide.
- If $\sigma^2 p > 2Sd\sigma^2 p$, it means that the diversity is narrow.

Tabel 1. The qualitative and quantitative characters of dragon fruit observed in this study

Organ	Characters		
	Qualitative	Quantitative	
Stem	Stem surface texture	Stem length (cm)	
	Color of thorns	Stem width (cm)	
	Bone edges shape	Distance between areoles (cm)	
	The intensity of gray on areoles	Arch height (mm)	
		Long thorns (mm)	
Flower	Flower bud shape	Flower bud: length of the pericarpel (cm)	
	Flower bud shape of the apex	Flower bud: width of the pericarpel (cm)	
	Flower bud color	Flower bud: length of the perianth (cm)	
	The intensity of red color of bract	Length of style (cm)	
	Sepal main color	Number of stigma lobes	
	Sepal: pattern of secondary color		
	Cleavage of the pistil lobe		
	Color of stigma lobes		
Fruit	Position of bracts towards the peel	Fruit length (cm)	Width of the base of the bracts (cm)
	The main color of middle bracts	Fruit width (cm)	
	Color of peel (exclude bracts)	Fruit: ratio length/width	Thickness of peel (mm)
	Color of flesh	Fruit: number of bracts	Sweetness
		Length apical of bracts (cm)	Fruit weight (g)

RESULTS AND DISCUSSIONS

Morphological diversity of dragon fruits in Dairi District

Results obtained from the 56 accessions of dragon fruit planted from three sub-districts in Dairi District, North Sumatra showed that there were differences in several morphological characters of stems, fruits, and buds. However, there were also similarities in terms of flower crown color, lobe color, and spine color among the study's accessions.

The stem morphology used for the characterization study was stem length, stem width, arch height, the texture of the stem surface, and the shape of the bone edges of the stem (Figure 1). Most of the dragon fruit plants have a triangular stem with smooth surface textures. Some stem is rectangular in shapes such as G1 and G19, while some have a rough texture like those in G3. In addition, the character of the bone edges found is convex, but in G17 accession is flat.

According to plantation owners, the G1 accession is an unknown variety, while G19 accession is the Super-red hybrid variety. According to Rahmawati and Mahadjoeno (2009), the dragon fruit plants planted in Pasuruan, Sukaharjo and Bantul have significant differences in stem morphology between the different species/varieties. The variations in stem morphology such as curvature of the stem, margin hardness (presence of sclerenchyma), distance between areoles, number of spines, rib height, rib thickness, length, color of the stem, and are considered important for species differentiation (Mejia et al. 2013).

The results of descriptive observations on the morphology of flowers showed that the length of the pericarpel, the length of the flower shoots, the length of the stigma, and the number of lobes were different in some of the observed accessions. Floral morphology such as flower bud shape, the color of flower bud, and petal pattern were found to be different in several accessions. The flower buds of dragon fruit plants are generally oval in shape (narrow ellipse and medium ellipse), but accessions G1, G11, G38, G43, G48, G49, G50, G52, and G53 have intense red round flower buds. All have similar white flower crowns. However, the pattern of flower petals found is a pattern of petals on the edge and has no pattern (Figure 2).

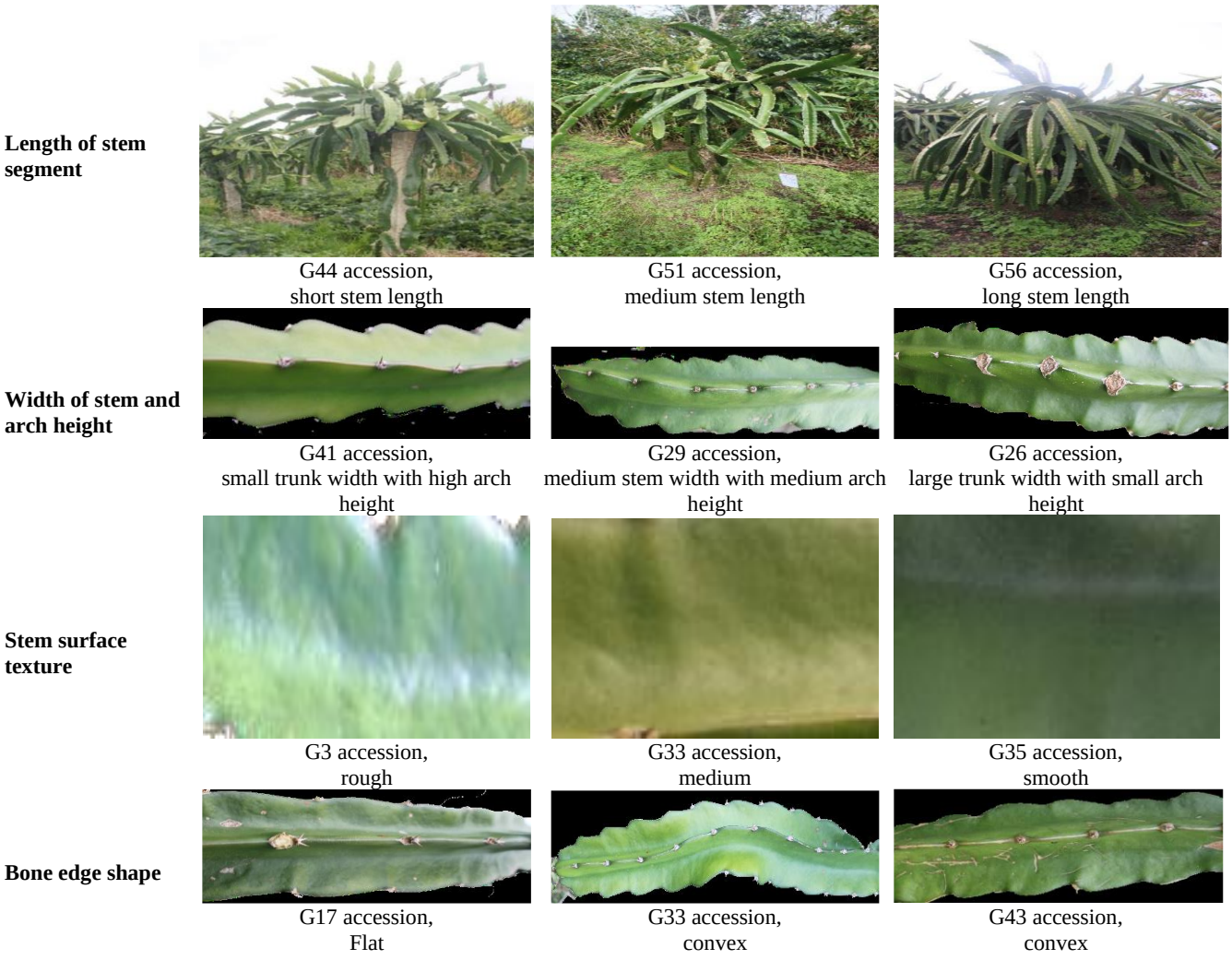


Figure 1. The morphological characters of the stems showing a variety of phenotypes

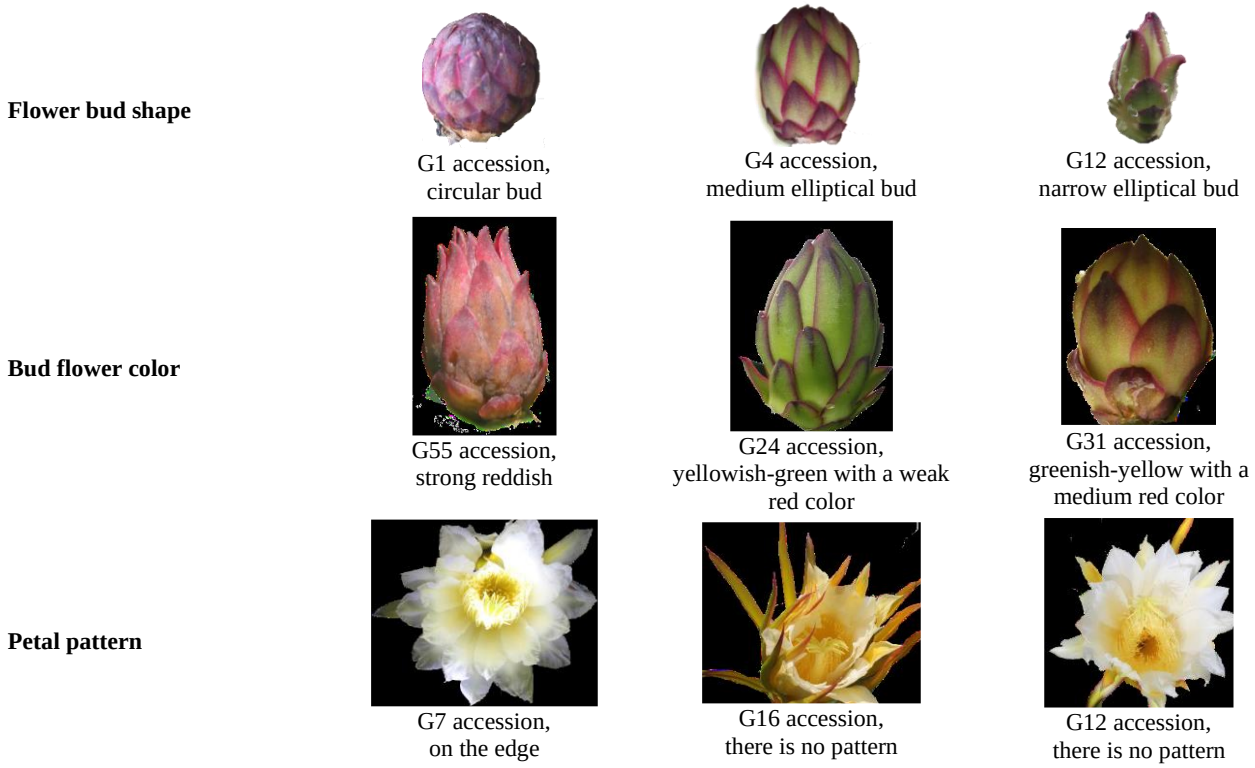


Figure 2. Phenotypic diversity in flower morphology

Tabel 2. Stem characteristics of 56 accessions of dragon fruit plants showing broad phenotype diversity in Dairi Sub-district, North Sumatra Province

Value	Morphological of stem characteristic				
	Length of stem segment (cm)	**Width of stem (cm)**	**Arch height (mm)**	**Distance between areoles (cm)**	**Length of spine (mm)**
$\bar{X}$	81.75	6.24	4.76	4.59	3.14
σ^2p	1243.8	1.85	2.39	0.96	0.91
Sd σ^2p	35.2	1.36	1.54	0.98	0.95
2Sd σ^2p	70.4	2.72	3.09	1.96	1,90
Criteria	Broad	Narrow	Narrow	Narrow	Narrow

Note: $\bar{X}$: mean value; σ^2p: diversity of phenotypes; Sd σ^2p is standard deviation of phenotypic diversity

Castillo et al. (2005) found that the major differences between accessions were presented in the characteristics of reproductive structures. Tran and Yen (2014) reported that three similarities in all clones were confirmed, including the spherical button shape of the medium elliptical, the petal color of white, and the stigma lobe color of cream. In part, the differences were recorded at the dimension of mature bud and part structures of flowers.

The fruit morphology in dragon fruit is important for characterization study to differentiate one species from another. It was found that dragon fruits in Dairi District have different fruit morphological in terms of fruit shape, number of bractea, and the position of bractea. The shape of the fruit could be categorized as medium (as in G9), elongated (as in G13), and compressed (as in G41). The number of bractea correlates with the base width of the bractea, where dragon fruit with a large number of bractea has a narrow base width. The position of the bractea that was found was upright and attached (adpressed) (as in G20), slightly held out (as in G6), and strongly held out (as in G22) (Figure 3).

Based on the character of pink to red flesh color and red fruit skin, it can be concluded that the dragon fruit that farmers in Dairi District widely cultivate is a species of *H. polyrhizus*. Sudarjat et al. (2019) reported that four dragon fruit accessions have different characterizations, including growth and the ability to adapt to certain environments. There are three types of dragon fruit: *Hylocereus undatus*, pink skin with white flesh; *H. polyrhizus*, red flesh with pink skin; *H. costaricencis*, violet red flesh with pink skin and *Hylocereus* (*Selenicerus*) *megalanthus*, white flesh with yellow skin (Le Bellec et al. 2006). Fruit set, fruit weight, and total soluble solid content of four pitaya clones were differently influenced by pollination types (Tran and Yen 2014).

Phenotype diversity

Based on the analysis of phenotypic diversity by comparing the standard deviation values, it can be seen that in the morphological characterization of the stem there are (one character with broad phenotypic diversity criteria, namely length of the stem, and four narrow phenotypic diversity, namely width of stem, arches high, the distance between areoles and the length of the spines (Table 1). According to De Dios (2005), most species of Hylocereus were similar in stem and flower morphology.

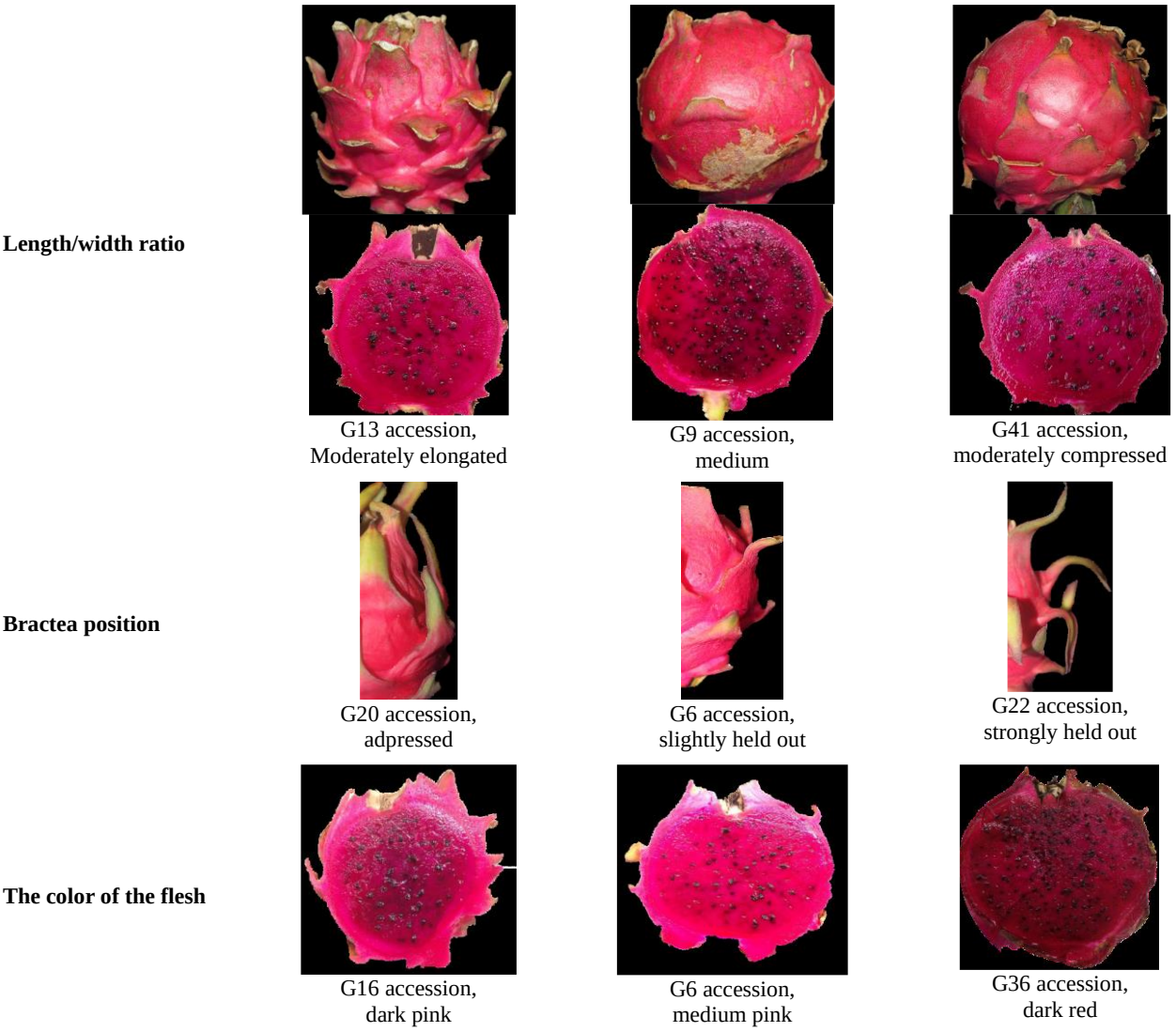


Figure 3. Fruit morphological characters showing phenotypic diversity

Table 3. Flower characteristics of 56 accessions of dragon fruit plants showing broad phenotype diversity in Dairi Sub-district, North Sumatra Province, Indonesia

Value	Morphological of flower characteristic					
	Length of pericarpel (cm)	Width of pericarpel (cm)	Lenght of stigma (cm)	Number of stigma lobes (unit)	Length of the top flower (cm)	Length of pericarpel (cm)
$\bar{X}$	15.86	4.07	15.3	26.71	16.29	15.86
σ^2p	5.81	0.38	2.16	5.75	5.57	5.81
Sd σ^2p	2.41	0.61	1.47	2.40	2.36	2.41
2Sd σ^2p	4.82	1.22	2.94	4.79	4.72	4.82
Criteria	Broad	Narrow	Narrow	Broad	Broad	Broad

In flower morphological characterization, there are three characters with wide phenotypic diversity, namely length of pericarpel, number of lobes, and length of top flower; and two characters with narrow phenotypic diversity, namely width of pericarpel and length of stigmas (Table 3). Whereas for fruit morphological characterization, there were three characters with wide phenotypic diversity: number of bractea, length of apex bractea, fruit weight. Five characters with narrow phenotypic diversity were fruit length, fruit width, length of base bracts, the thickness of peel, and the sweetness of the fruit (Table 4). Apart from differences in species or accessions, differences in fruit morphology can be related to changes in the physiological level of dragon fruit at various stages of fruit development (Kammapana et al. 2013). The main differences among several Hylocereus species were the size and color of the fruit and the number and form of the spines (De Dios 2005). There are several

species of dragon fruit that were cultivated in Indonesia, they are white dragon fruit (*H. undatus*); red dragon fruit (*H. polyrhizus*); purplish-red dragon fruit (*H. costaricensis*); and yellow dragonfruit (*H. megalanthus*) (Andoko and Nurrasyid 2012).

Phylogenetic of dragon fruits in Dairi District

The relationship between 56 accessions of dragon fruit plants planted in Dairi District could be evaluated based on the dissimilarity value between accessions. The smaller the dissimilarity value between one variable and another, the closer the relationship between variables is (the more similar), on the contrary, the greater the dissimilarity value, the higher the dissimilarity between variables. The lowest dissimilarity value (3.790) of dragon fruit plants was accession G19 and G24, identified in Dairi planted in Sidikalang Sub-district, Sitinjo Village 2. The close relationship between G24 and G19 was indicated by the existence of six different characters (bractea position on fruit, fruit flesh color, spines length, pericarpel width, fruit width, fruit sweetness) out of the 35 observed characters. The highest dissimilarity value (12.773) was obtained in G1 accession found in Sidikalang Sub-district, Batang Beruh Village, and accession G17 found in Sidikalang Sub-district, Sitinjo Village 2, with 19 character differences from the studied 35 characters. These 19 different characters were stem texture, bone edge shape, red-colored flower buds. flower bud shape and, flower bud color, cleavage on the lobe, length/width ratio, fruit skin color, pulp color, the main color of bractea, distance between areoles, length of thorn, length of bractea peak, skin thickness, base width bractea, fruit sweetness, fruit weight, and fruit length. Results showed dissimilarity values of other accessions in Dairi District as in Table 5.

Based on dendrogram grouping (Figure 4) there are two, three, and four groupings at 25, 20, 17 euclidean distance scales, respectively. The small *euclidean distance* scale indicates the studies' accessions are closely related and have more similar characters.

On 25 euclidean distance scale, the accessions we classified into two groups. The first group consisted of 55 accessions of dragon fruit plants, namely G1, G2, G3, G4, G5, G6, G7, G8, G9, G10, G11, G12, G13, G14, 15, G16, G18, G19, G20, G21, G22, G23, G24, G25, G26, G27, G28, G29, G30, G31, G32, G33, G34, G35, G36, G37, G38, G39, G40, G41, G42, G43, G44, G45, 46, G47, 48, 49, G50, G51, G52, G53, G54, G55, G56. This group is united by a special character, namely the convex edge of the stem bone. The second group consisted only of G17 accessions, which did not form groups with other accessions. A special character that separates G17 from other accessions is the flat edge of the stem bone.

On a scale of 20, there are three groupings of phylogenetic relationships. The first group consisted of 52 accessions of dragon fruit plants, namely G2, G3, G4, G5, G6, G7, G8, G9, G10, G11, G12, G13, G14, 15, G18, G19, G20, G21, G22, G23, G24, G25, G26, G27, G28, G29, G30, G31, G32, G33, G34, G35, G36, G37, G38, G39, G40, G41, G42, G43, G44, G45, G46, G47, 48, 49, G50, G51, G52, G53, G54, G56. This group is connected by a special character that is an edge of the stem bone which is convex and the yellowish-green flower buds. The second group consisted of G1, G16, and G55 accession. This group is related to special red flower buds. The third group consisted of a single accession that is G17 with a special character that is a flat edge of the stem bone.

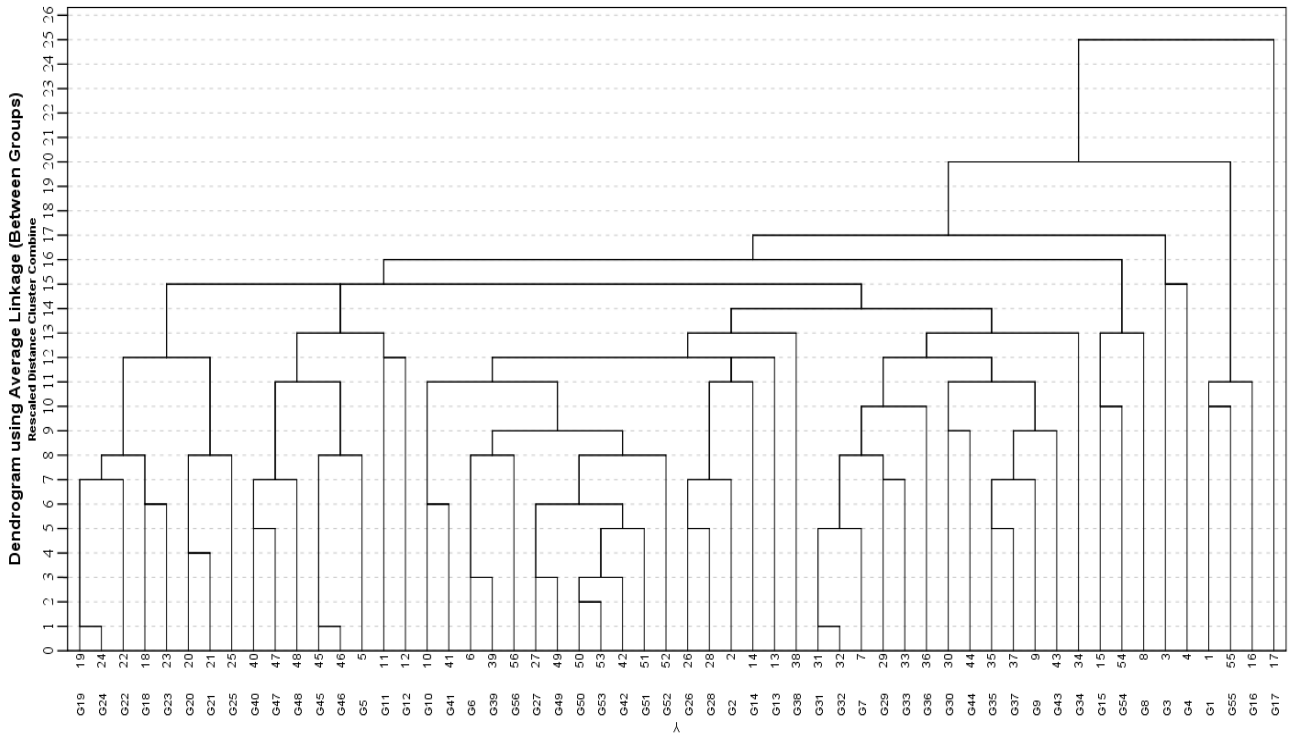


Figure 4. Dendrogram grouping based on quantitative and qualitative characters of dragon fruit accession obtained from Dairi District, North Sumatra Province, Indonesia

Table 5. The relationship of dragon fruit accessions in Dairi District, North Sumatra, based dissimilarity matrix

Number	Phylogenetic		Dissimilarity
1	G19	G24	3.790
2	G45	G46	3.881
3	G31	G32	4.040
4	G41	G50	4.727
5	G20	G21	4.831
6	G26	G28	4.961
7	G53	G7	4.964
8	G6	G56	5.049
9	G35	G37	5.063
10	G27	G51	5.198
11	G18	G23	5.226
12	G2	G26	5.238
13	G10	G49	5.332
14	G39	G42	5.466
15	G5	G6	5.528
16	G47	G48	5.532
17	G33	G36	5.834
18	G30	G40	5.876
19	G27	G38	6.997
20	G38	G50	7.003
21	G39	G52	7.018
22	G37	G19	7.035
23	G25	G52	7.036
24	G54	G45	10.162
25	G18	G16	10.250
26	G8	G34	10.401
27	G16	G22	10.495
28	G17	G1	12.773

On a scale of 17, four distinct groups were observed. The first group consisted of 50 accessions of plants G2, G4, G5, G6, G7, G8, G9, G10, G11, G12, G13, G15, G18, G19, G20, G21, G22, G23, G24, G25, G26, G27, G28, G29, G30, G31, G32, G33, G34, G35, G36, G37, G38, G39, G40, G41, G42, G43, G44, G45, 46, G47, 48, 49, G50, G51, G52, G53, G54, G56. The second group consisted of G3 and G14 which are related to 11 similar characters The third group consisted of G1, G16, and G55. The fourth group is only G17 which segregates itself.

Results obtained showed that the relationship between the dragon fruit accessions understudied could be categorized as relatively close. Cahyarini et al. (2004) stated that two or more accessions/accessions can be said to be similar if the similarity distance or similarity level is not less than 0.60 or 60%. Hence it can be said that the fifty-six accessions observed had close relationships with each other. According to the dragon fruit plant owners, the dragon fruit plants are reproduced by stem cuttings and thus have a high level of genetic similarity. Dragon fruit plants are found scattered in some areas, there is the possibility that they come from a single genetic source with different genetic types and then dispersed to various places with the help of humans. This is also consistent with research conducted by Rahmawati and Mahajoeno (2009) and Grimaldo-Juarez et al. (2007) that purplish-red and red dragon fruit were in the same group, hence they had a close relationship with each other. Although the G17 accession is in a separate group because it is the shape of the flat bone edges. Morillo et al. (2016) stated that several morphological characteristics can be described to distinguish various types of dragon fruit species. One of the most important characteristics of Hylocereus to distinguish it is the morphology of the stem (Grimaldo-Juarez et al. 2007).

Studies in other countries have found high variation in characteristics of agronomic importance, even within the same species of *H. undatus* which is considered favorable for future breeding studies (Tel-zur et al. 2011). Several accessions of dragon fruit plants have distinctive morphological characters and can be used as a good material source breeding programs or as propagation materials due to several characters, such as high total soluble sugar characters as found in G51, G53 G1, G8, G12, G27 (19.4-20.0 Brix), the large fruit size of G36, the thin thickness of fruit peel in G17 and G25. Such characteristics are attractions for dragon fruit marketing.

In conclusion, stem, flower, and fruit morphological characters could be used for the characterization of dragon fruit variants in Dairy District, Indonesia. Characteristics of pink to red flesh and red fruit skins indicate that the dragon fruit plant that farmers in Dairi District widely cultivate is a species of *H. polyrhizus*. Based on the analysis of phenotypic diversity, quantitative characters with broad criteria, namely stem length for stem morphology; the length of pericarpel, number of lobes, length of top flower for flower morphology; and number of bractea, length of apex bractea, fruit weight for fruit morphology. Based on phenotypic diversity, most of the accessions planted in this area are closely related.

REFERENCES

Andoko A, Nurrasyid H. 2012. Five Successful Moves to Produce Prime Quality Dragon Fruit. PT AgroMedia Pustaka, Jakarta. [Indonesian]

Cahyarini RD, Yunus A, Purwanto E. 2004. Identification of the genetic diversity of some local varieties of soybean in Java based on isozyme analysis. Agrosains 6: 79-83. [Indonesian]

Castillo R, Livera M, Márquez GJ. 2005. Caracterización morfológica y compatibilidad sexual de cinco genotipos de pitahaya (*Hylocereus undatus*). Agrociencia 39: 183-194.

De Dios HC. 2005. A new subspecies of *Hylocereus undatus* (Cactaceae) from Southeastern México. Haseltonia 11: 11-17. DOI: 10.2985/1070-0048(2005)11[11:ANSOHU]2.0.CO;2

Grimaldo-Juarez O, Terrazas T, Garcia-Velasquez A, Cruz-Villagas M, Ponce-Medina JF. 2007. Morphometric analysis of 21 pitahaya (*Hylocereus undatus*) genotypes. J Prof Assoc Cactus Dev 9: 99-117.

Hernandez YDO, Salazar JAC. 2012. Pitahaya *(Hylocereus* spp.): A short review. Comunicata Scientiae 3: 220-237.

Kammapana L, Buanong M, Techavuthiporn C, Jitareerat P, Wongs-Aree C, Yamauchi N, Srilaong V. 2013. SEM studies on the morphology of 'white pulp' dragon fruit. Acta Hortic 989: 85-90. DOI: 10.17660/ActaHortic.2013.989.8

Korotkova N, Borsch T and Arias S. 2017. A phylogenetic framework for the Hylocereeae (Cactaceae) and implications for the circumscription of the genera. Phytotaxa 327 (1): 1-46. DOI: 10.11646/phytotaxa.327.1.1

Kristanto D. 2008. Cultivating Dragon Fruit in Pots and in Gardens. Penebar Swadaya, Jakarta. [Indonesian]

Le Bellec F, Vaillant F, Imbert E. 2006. Pitahaya (*Hylocereus spp*.), a new fruit crop, a market with a future. Fruits 61: 237-250. DOI: 10.1051/fruits:2006021

Mansyah E, Baihaki A, Setiamihardja R, Darsa JS, Sobir, Poerwanto R. 2003. Phenotypic variability of mangosteen in several production centers in Java. J Hortic 13: 147-156. [Indonesian]

Mejia HA, Ruiz SBM, Montoya CA, Sequeda CR. 2013. In situ morphological characterization of *Hylocereus spp*. (Fam. Cactaceae) genotypes from Antioquia and Córdoba (Colombia). Rev Fac Nal Agric Medellin 66: 6845-6854.

Mongi, CE. 2015. Usage Two-Step Cluster Analysis for Mixed Data. JDC, Vol 4: 1. Sam Ratulangi University, Manado.

Morillo AC, Tovar YP, Morillo Y. 2016. Morphological characterization of *Selenicereus megalanthus* (K. Schum. ex Vaupel) moran in the province of Lengupa. Ciencia en Desarrollo 7: 23-33. DOI: 10.19053/01217488.v7.n2.2016.4072

Purba R. 2012. 21 Popular Tabulamplot Types. Agromedia, Jakarta. [Indonesian]

Rahmawati B, Mahadjoeno E. 2009. Variation of morphology, isozymic, and vitamin C content of dragon fruit varieties. Nus Biosci 1 (3): 133-137. DOI: 10.13057/nusbiosci/n010305

Santoso PJ. 2013. Cultivation of organic dragon fruit in the yard, based on the experience of farmers in Malang District. Iptek Hortikultura 9: 26-31. [Indonesian]

Setyowati A. 2008. Morphological and Cytological Analysis of Yellow Skin Dragon Fruit Plants (*Selenecerus megalanthus*). Thesis. Universitas Sebelas Maret, Surakarta. [Indnesian]

Sudjana. 2008. Statistical Method. PT Tarsito, Bandung. [Indonesian]

Steel RGD, Torrie JH, Dicke DA. 1997. Principles and Procedures of Statistics: A Biometrical Approach. 3rd Edition, McGraw Hill, Inc. Book Co., New York, Winarsih S. 2010. Recognizing and Cultivating Dragon Fruit. Aneka Ilmu, Semarang. [Indonesian]

Sudarjat, Leovika A, Suminar E, Isnaniawardhani V, Qanit MAH, Fauzi AA, Mubarok S. 2019. Morphological characterization and adaptation of four dragon fruit genotypes in Pangandaran District of Indonesia. Asian J Plant Sci 18: 21-25. DOI: 10.3923/ajps.2019.21.25

Tel-Zur N, Mizrahi Y, Cisneros A, Mouyal J, Schneider B, Doyle JJ. 2011. Phenotypic and genomic characterización of vine cactus collection (Cactaceae). Genet Resour Crop Evol 58: 1075-1085. DOI: 10.1007/s10722-010-9643-8

Tran DH, Yen CR. 2014. Morphological characteristics and pollination requirement in red pitaya (*Hylocereus spp*.). Int J Agric Biosys Eng 8: 268-272.