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Coconut diversity based on chloroplast Single Nucleotide Amplified Polymorphism (SNAP) and Insertion-Deletion (InDel) markers

ANNISAA RAHMAWATI[1], DINY DINARTI[1], ISMAIL MASKROMO[2], HUGO ALFRIED VOLKAERT[3], SUDARSONO[1,♥]

[1]Plant Molecular Biology Laboratory, Department of Agronomy dan Horticulture, Faculty of Agriculture, Institut Pertanian Bogor. Jl. Meranti, IPB Dramaga Campus, Bogor 16680, West Java, Indonesia. Tel.: +62-251-8629350 ♥email: sudarsono_agh@apps.ipb.ac.id
[2]Indonesian Palms Research Institute, Agency for Agricultural Research and Development. Jl. Raya Mapanget, Mapanget, Talawaan, Manado 95001, North Sulawesi, Indonesia
[3]Center for Agricultural Biotechnology, Kasetsart University. Kamphaengsaen Campus, Kamphaengsaen 73140, Nakhonpathom, Thailand

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Abstract. *Rahmawati A, Dinarti D, Maskromo I, Volkaert HA, Sudarsono. 2022. Coconut diversity based on chloroplast Single Nucleotide Amplified Polymorphism (SNAP) and Insertion-Deletion (InDel) markers. Biodiversitas 23: 4073-4081.* Indonesia is known as a country rich in biodiversity, and a species with high diversity existing in Indonesia is coconut (*Cocos nucifera* L.). Although the high diversity of Indonesian coconut is recognized, information about its genetics is limited. The genetic relationship between Indonesian coconut and their maternal inheritance is important and has not been widely studied. This study aimed to analyze the genetic diversity of the Indonesian coconut using chloroplast SNAP and InDel markers, and evaluate their haplotype diversity. Sixty-four coconut accessions were evaluated using ten SNAP primers and five InDel primers based on cpDNA. All of the primers successfully amplified the cpDNA of most evaluated coconut accessions. Based on five InDel markers, six haplotypes were observed in *Cocos nucifera*. Phylogenetic analysis based on the combined SNAP and InDel markers divided the 64 coconut accessions into three clusters. The SNAP marker analysis results showed that they are less informative for evaluating coconut genetic diversity. On the other hand, the InDel markers are more informative and useful for coconut genetic diversity evaluation. Therefore, InDel markers based on chloroplast genomes has the potential for future coconut genetic variation analysis, evolutionary study, and DNA fingerprinting. The results should contribute to understanding the Indonesian coconut origins and evolution.

Keywords: Chloroplast DNA markers, coconuts cpDNA, haplotyping, InDel, SNAP

INTRODUCTION

Coconut is an essential perennial crop of the family Arecaceae (Xiao et al. 2017). The coconut palm (*Cocos nucifera*, Arecaceae), also called the tree of life, is a multipurpose crop because every part of it is usable either as a food source, handicraft, construction materials, and even a source of natural medicine (Lima et al. 2015; Rajesh et al. 2015; Aljohi et al. 2016; Perera et al. 2017).

Southeast Asia, particularly the island of Indonesia and the western Pacific rim are speculated to be the origin and the center of coconut diversity globally. The introduction of coconuts through trade routes connecting places, i.e. between Madagascar and Southeast Asia led to the dispersal of coconuts across the tropics (Gunn et al. 2011), a geographical distribution, and phenotypic diversity within specific regions (Harries and Clement 2014). The existing coconuts are grouped into two types, i.e., Tall and Dwarf types (Perera 2014; Boonkaew et al. 2018). However, natural or human-made hybridization between the two coconut types results in the third group (hybrid type).

Among the many Dwarf and Tall coconut types existing in Indonesia, at least two unique coconuts are Bido and Kopyor coconuts. The Bido coconuts are a local coconut variety showing early bearing, a low height increment, high yielding, large fruit size, and other beneficial traits (Tulalo et al. 2019). The kopyor coconuts are natural mutants with soft, fluffy and crumbly endosperm (Maskromo et al. 2013; Maskromo et al. 2016). Recessive genes control Kopyor characters (Novarianto et al. 2014; Setiawan et al. 2020).

Although the high phenotypic diversity of coconut accessions in Indonesia is recognized, information about their genetics is limited. Molecular markers have been used to assess genetic diversity and population differentiation in many plant species (e.g., Jo et al. 2017; Nadeem et al. 2018). Nuclear genetic markers are utilized to study genetic diversity and population differentiation. However, nuclear genome-derived markers sometimes are less suitable for finding a species' origin and evolution since they show a high substitution rate (Li et al. 2015).

For the study of speciation and domestication history, the chloroplast genome (cpDNA) offers some advantages. The plant chloroplasts have their own genome (Allen 2015), which is highly conserved in their genome structure and gene content, has a lower substitution rate than the nuclear genome, and has a high copy numbers per cell (Liere and Borner 2013; Xu et al. 2015; Morley and Nielsen 2016). Another unique feature of cpDNA is its uniparental, and maternally inheritance. Hence, markers developed based on the cpDNA have been widely used in plant phylogenetics, phylogeography and evolutionary studies (e.g. Kim et al. 2017; Zhang et al. 2017; Nguyen et

al. 2020). Single nucleotide polymorphism (SNP) and insertion-deletion (InDel) are the most abundant genetic variation that exists in both the nuclear genome and cpDNA that can be used to develop DNA markers (Song et al. 2015).

In a previous study, Balladona et al. (2020) successfully designed primer pairs for single nucleotide amplified polymorphic (SNAP) and InDel markers based on detected coconut cpDNA nucleotide variations. This study aimed to analyze the genetic diversity of the Indonesian coconut using both SNAP and InDel cpDNA markers and evaluate their relationships based on the observed haplotypes. The results should contribute to a better understanding of coconut origins and evolution in Indonesia's many islands.

MATERIALS AND METHODS

Plant materials

A total of 64 coconut trees from various islands in Indonesia and neighboring countries were evaluated in this study (Table 1). The coconut accessions have been kept ex-situ at the Indonesian Palms Research Institute (IPRI), Manado, Indonesia. Collected samples were fresh leaves from mature palms. Subsequently, the collected leaf samples were sent by airmail courier from Manado to PMB Lab., Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University, Indonesia and used for total DNA extraction.

DNA extraction, PCR, and electrophoresis

Total DNA was extracted from ca. 0.5 g of fresh leaf using a cetyltrimethylammonium bromide (CTAB) protocol routinely used for isolating total DNA from leaves of various palm species (Allen et al. 2006; Pesik et al. 2017; Natawijaya et al. 2019; Purwoko et al. 2019; Budiman et al. 2020). The precipitated DNA was dissolved in 100-150 µL of TE buffer and stored at -20°C until used. DNA quality was evaluated qualitatively by agarose (1.0%) gel electrophoresis using sodium borate buffer, and NanoDrop 2000c spectrophotometer was used to estimate the DNA concentration.

Table 1. List of the evaluated coconut accessions and local or cultivar names, and the origin of the accessions

Coconut type	Code	Local name or cultivars	Accession origin	Individual numbers
Bido	BIDO	Bido	Morotai, North Maluku	3
Tall	LBT	Local Bido Tall	Morotai, North Maluku	2
Dwarf	LBD	Local Bido Dwarf	Morotai, North Maluku	2
Tall	IIT	Ilo-ilo Tall	Manado, North Sulawesi	2
Tall	KYT	Kalasey Tall	Minahasa, North Sulawesi	2
Tall	LPT	Lubuk Pakam Tall	Lubuk Pakam, North Sumatra	2
Tall	MWT	Marinsow Tall	Minahasa, North Sulawesi	2
Tall	PNT	Paslaten Tall	Tomohon, North Sulawesi	2
Tall	PUT	Palu Tall	Donggala, Central Sulawesi	2
Tall	SAT	Sawarna Tall	Lebak, West Jawa	2
Tall	CNT	Ceylon Tall	Sri Lanka	2
Tall	TAT	Tenga Tall	Minahasa, North Sulawesi	2
Tall	TET	Takome Tall	Temate Island, North Maluku	2
Tall	TST	Talise Tall	Palu, Central Sulawesi	2
Tall	TTT	Tontalete Tall	Minahasa, North Sulawesi	2
Tall kopyor	GKT	Green Kopyor Tall	Kalianda, South Lampung	1
Tall kopyor	BKT	Brown Kopyor Tall	Kalianda, South Lampung	1
Dwarf kopyor	YKD	Yellow Kopyor Dwarf	Pati, Central Java	2
Dwarf kopyor	BKD	Brown Kopyor Dwarf	Pati, Central Java	2
Dwarf kopyor	GKD	Green Kopyor Dwarf	Pati, Central Java	2
Dwarf	OKD	Orange Kopyor Dwarf	Pati, Central Java	1
Dwarf	AKD	Aromatic Dwarf	Philippines	2
Dwarf	GND	Green Nias Dwarf	Nias Island, North Sumatra	2
Dwarf	YBD	Yellow Bali Dwarf	Sangiang Plantation, Bali	2
Dwarf	YMD	Yellow Malaysia Dwarf	Malaysia	2
Dwarf	YND	Yellow Nias Dwarf	Nias Island, North Sumatra	2
Dwarf	RMD	Red Malaysia Dwarf	Malaysia	2
Dwarf	OSD	Orange Sagerat Dwarf	Minahasa, North Sulawesi	2
Dwarf	PWD	Pandan Wangi Dwarf	Malaysia	2
Dwarf	RAD	Raja Dwarf	Halmahera Island, North Maluku	2
Dwarf	SKD	Salak Dwarf	Pematang Panjang, South Kalimantan	2
Dwarf	TTD	Tebing Tinggi Dwarf	Tebing Tinggi, North Sumatra	2
Dwarf	WUD	Waingapu Dwarf	Sumba, East Nusa Tenggara	2

Fifteen primer combinations (10 SNAP primer and 5 InDel primer pairs) developed previously by Balladona et al. (2020) (Tables 2 and 3) were used to amplify specific loci from the coconut cpDNA. The polymerase chain reactions (PCR) were carried out in a 12.5 µL reaction mixture having 6.25 µL MyTaq HS Red Mix PCR Kit, 0.375 µL (10 µM) of each primer, 2 µL genomic DNA (20 ng of DNA template) and added with ddH_2O up to 12.5 µL. The PCR reaction was performed in a Bio-rad™ thermal cycler using the following cycling parameters: initial denaturation at 95°C for 3 minutes; followed by 35 amplification cycles of denaturation at 95°C for 15 seconds, annealing at 50-60°C for 30 seconds, elongation at 72°C for 30 seconds; and a final extension at 72°C for 7 minutes. The PCR products were electrophoresed through 2.0% agarose gels in the sodium borate buffer and stained with 0.5 µL Gelred™. The electrophoresis was run at a constant voltage of 50 volts for 85 minutes. A 100 bp DNA ladder (Vivantis) was loaded in each agarose gel as a size standard to estimate the PCR product's size. The gel photographs were taken to record the results of agarose electrophoresis. The generated alleles were scored based on the presence and size variations of the generated amplicons (Balladona et al. 2020).

Table 2. A list of the single nucleotide amplified polymorphism (SNAP) primer pairs developed based on chloroplast nucleotide sequence variations of coconut used in this study

Primer IDE	Primer sequence	Tm (ºC)	Primer length	PCR product size (bp)
CT_SNP1_REF	CACATGAGTGGATATATAGGAATCA	53	25	176
CT_SNP1_REV	ATGTCTCCTACGTTACCCGTAATA	55	24	
CT_SNP1_ALT	CACATGAGTGGATATATAGGATTCC	54	25	176
CT_SNP2_REF	ATGCATAAGGATGTTGTGGTCT	54	25	190
CT_SNP2_REV	ACTTAGTTTCCGCCTGGGT	55	19	
CT_SNP2_ALT	GCATAAGGATGTTGTGGTCC	54	20	188
CT_SNP3_REF	ACGAAACACTTGGTTTCGATC	55	21	175
CT_SNP3_REV	GCTATCGGCCCAGTGAATA	54	19	
CT_SNP3_ALT	CCACGAAACACTTGGTTTCTATT	56	23	177
CT_SNP4_REF	GCGAGAATTAATTATTGGGCAC	56	22	161
CT_SNP4_REV	CCTCGTTCCTGAAAAGTAGTCA	54	22	
CT_SNP4_ALT	CAGCGAGAATTAATTATTGGTGAT	55	24	161
CT_SNP5_REF	GAGTCATGGATACAGGAGCCT	54	21	185
CT_SNP5_REV	TAAAGATCCTCATTGGTGCG	54	20	
CT_SNP5_ALT	AGTCATGGATACAGGAGCCC	55	20	184
CT_SNP6_REF	TCAGTGATCAAATCATTCATACCA	55	24	182
CT_SNP6_REV	TTTGTTGGGGATAGAGGGAC	55	20	
CT_SNP6_ALT	CAGTGATCAAATCATTCATACCC	54	23	181
CT_SNP7_REF	CATTTCGTGACTTATTGGTAAATTT	54	24	189
CT_SNP7_REV	TTTATCGATATGAGTGTTCTATATCA	51	20	
CT_SNP7_ALT	CATTTCGTGACTTATTGGTAAATTG	55	23	189
CT_SNP8_REF	CCAGAAAGAATTCAGTTCAGAAGTA	55	25	180
CT_SNP8_REV	CTTTTCCTTCTTCTTGTTGCTG	54	22	
CT_SNP8_ALT	CAGAAAGAATTCAGTTCAGAGGTC	55	24	179
CT_SNP9_REF	CGGAACAAGTAAACACATTTTCAA	55	25	178
CT_SNP9_REV	GAAATCTCATTCGTACTATAACTCA	54	26	
CT_SNP9_ALT	CCGGAACAAGTAAACACTATTTACAG	56	26	179
CT_SNP10_REF	AAGGTATGGAACCCGAGTAAG	53	21	177
CT_SNP10_REV	CCAATACATCGCAGGGTTC	56	19	
CT_SNP10_ALT	CAAGGTATGGAACCCGAGATAC	55	22	178

Table 3. The list of the insertion-deletion (InDel) primer pairs developed based on chloroplast nucleotide sequence variations of coconut and used in this study

Primer ID	Primer sequence	Tm (ºC)	Primer length	PCR product size (bp)
CT_InDels1_F	TTCCATAATCTCATTGTTTTT	51.7	21	410
CT_InDels1_R	ACTGTTTGGATCTGTGTGA	51.8	19	410
CT_InDels2_F	GAAAGAGACTTTCATTTCCAGTC	56.3	23	410
CT_InDels2_R	CCAAGGGCTATAGTCATAGT	56.5	23	410
CT_InDels3_F	AAACCTTCTATCAACAGGAT	50.4	20	887
CT_InDels3_R	AAATAGAGGGTAAGTTGAGATCTGT	56	25	887
CT_InDels4_F	AAGATTTTGTTCAGCATGTTCT	55.7	22	234
CT_InDels4_R	AAAAAGGGCGTGGAAACAC	60	19	234
CT_InDels5_F	AGACGAAGAGAAAGGTCTATCC	55.8	22	234
CT_InDels5_R	TCAAAACACTATGTATGGATGA	53.2	22	234

Data analysis

The generated alleles for both the SNAP and InDel markers were scored as binary data (1 for the presence of a particular DNA band and 0 for the absence). The joint haplotype data were used to construct the phylogenetic tree and the haplotype networks. The phylogenetic trees were constructed using Weighted Neighbor-Joining, and the bootstrap was conducted using 1,000 iterations. The phylogenetic analysis was done using Dissimilarity Analysis and Representation for Windows (DARWin) software version 6.0.8 (Perrier and Jacquemoud-Collet 2006). Haplotype networks are used to identify genetic linkage and mutation patterns between individuals. Reconstruction of the haplotype network was carried out using the Median-Joining method with the Network software version 5.0.1.1 (Bandelt et al. 1999).

RESULTS AND DISCUSSION

Coconut germplasm variability is essential for coconut genetic improvement since it is the breeding program's starting material. Therefore, a high diversity of coconut germplasm should be collected and kept for supporting sustainable coconut breeding programs. Unfortunately, keeping ex-situ coconut germplasm collections is not easy since we need to keep the palm trees in the field, which needs intensive resources.

As many as 1621 coconut accessions have been found and collected worldwide (Perera et al. 2017). Therefore, coconut genetic diversity evaluations are necessary to use resources efficiently. Moreover, breeding activities for new coconut varieties should be the following targets after germplasm characterization.

The SNAP and InDel primer effectiveness testing

The SNAP primers were designed based on the single nucleotide polymorphism (SNP) occurring in the cpDNA of many palm species (Balladona et al. 2020). Ten SNAP primer pairs amplified PCR fragments from DNA of 64 coconut accessions. For the haploid chloroplast genome, it would be expected that all of the evaluated accessions yield a single DNA amplification product for either the reference (REF) or alternate (ALT) primer in combination with the proper reverse (REV) (Jansen and Ruhlman 2012; Morley and Nielsen 2016; Balladona et al. 2020). However, reports have shown the presence of sequence duplication within certain parts of the cpDNA, such as the inverted repeat region (Asaf et al. 2020) and certain chloroplast genes (Lidholm et al. 1991; Martinez-Alberola et al. 2013; Choi et al. 2015; Bennett et al. 2017).

However, PCR amplification using DNA of coconut and the evaluated SNAP primers almost without exception yielded a band for both the REF and the ALT primers in combination with the REV primer (Figure 1). Such amplification showed that in general the SNAP marker is not informative enough to detect the single nucleotide polymorphism in the coconut chloroplast genome.

In the plant chloroplast genome, the protein-coding gene duplication is less frequent than those in the non-coding regions (Xiong et al. 2009; Martinez-Alberola et al. 2013; Bi et al. 2018; Li et al. 2019; Asaf et al. 2020). Most gene duplication in the chloroplast genome occurred in the IR regions (Martinez-Alberola et al. 2013; Bi et al. 2018; Li et al. 2019; Asaf et al. 2020). Moreover, the gene duplication in single-copy coding regions was usually due to expanding the IR regions (Xiong et al. 2009).

Such data showed that the evaluated SNAP marker loci are in the duplicated segments of the chloroplast genome (IR regions). This finding confirmed preliminary reports about the chloroplast-specific SNAP marker analysis results in coconuts (Balladona et al. 2020).

Many PCR-based markers have been developed and used to reveal coconut genetic diversity (Pandin 2010; Rajesh et al. 2015), the genetic relationship among individuals in a population (Geethanjali et al. 2018), and other purposes (Zhou et al. 2020). Simple sequence repeat (SSR) is the most common marker used for genetic diversity analysis (Natawijaya et al. 2019; Purwoko et al. 2019; Budiman et al. 2020), finding parents to identify certain progenies (Pesik et al. 2015) and evaluating the combining ability for the endosperm quantity characters (Rahayu et al. 2019).

Other increasingly essential markers include single nucleotide polymorphism (SNP) based markers, which are very robust since there is a single SNP in every 130 base pairs (bp) of the plant genome (Schneider et al. 2001) and have also been used in coconut genetic studies (Sukendah et al. 2009; Larekeng et al. 2015; Pesik et al. 2017). The abundance of SNP in the genome may be used to develop SNP-based markers, and the SNP-based markers in coconuts have been used for genotyping (Sukendah et al. 2009; Pesik et al. 2017) and pollen dispersal analysis (Larekeng et al. 2015) of Kopyor coconuts and evaluate population structure (Singh et al. 2013). However, those studies used nuclear genome-based markers, which were less helpful in studying the evolutionary process.

Among the five InDel primer pairs, two (CT_InDels2 and CT_InDels4) yielded a single monomorphic PCR product in all evaluated Indonesian coconuts (Figure 2 - upper panel), and thus were not informative for assessing cpDNA diversity. The three other InDel primers (CT_InDels1, CT_InDels3, and CT_InDels5) yielded allele variations to amplify InDel markers using the Indonesian coconut DNA (Figure 2 - lower panel). The allele variation was represented by the presence and absence of the target band in the PCR product due to a polymorphism interfering with the proper annealing of one of the primers and preventing PCR amplification.

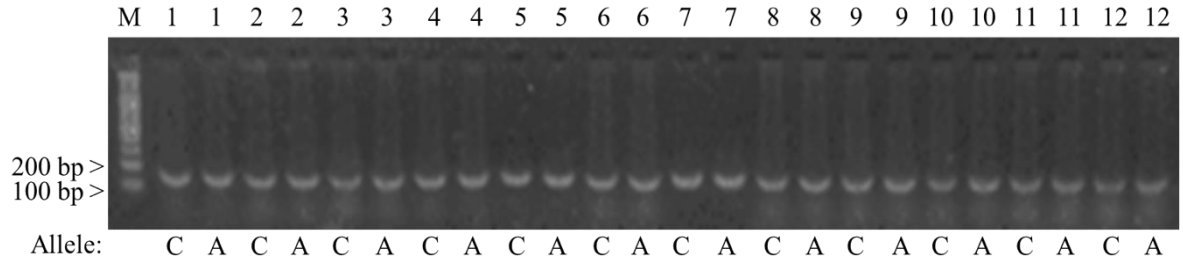

Figure 1. Amplified allele profile of SNAP markers generated using CT_SNP8 primers. The PCR products were electrophoresed through 1.0% agarose gels in the sodium borate buffer and stained with 0.5 µL Gelred™. The electrophoresis was run at a constant voltage of 50 volts for 85 minutes. Column 1-3: Bido coconut, 4-6: Kopyor coconut, 7-9: Dwarf coconut, and 10-12: Tall coconut accessions. A: Reference allele, C: alternative allele, M: 100 bp DNA marker

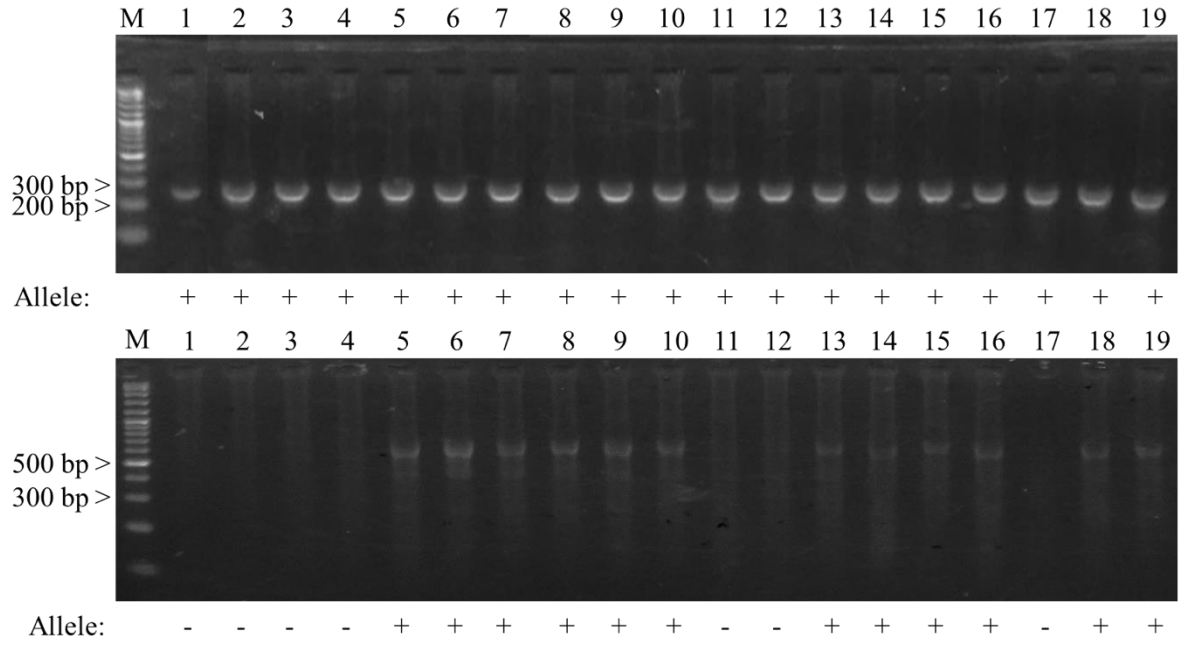

Figure 2. Amplified allele profile of the insertion-deletion (InDel) markers generated using CT_InDels2 (top) and CT_InDels3 primers (bottom). The PCR products were electrophoresed through 1.0% agarose gels in the sodium borate buffer and stained with 0.5 µL Gelred™. The electrophoresis was run at a constant voltage of 50 volts for 85 minutes. Column 1-4: Tall coconut, 5-7: Bido coconut, 8-10: Kopyor coconut, and 11-19: Dwarf coconut accessions. Alleles (+): amplified PCR product present and (-): amplified PCR product absent. M: 100 bp DNA marker

Polymorphisms of InDel in the chloroplast genome have been found among species (Kim et al. 2015). InDel markers could better detect genetic variation in the chloroplast genome than SNP markers. The SNP markers showed much lower polymorphisms than the InDel markers, making determining the coconut population's haplotype difficult. It was due to InDel having a slightly higher mutation rate than substitution nucleotides in cpDNA, especially between closed related species, and repetitive motives of InDel having the highest rate of evolution (Ingvarsson et al. 2003; Jansen and Ruhlman 2012; Jiang et al. 2017). A comparison between the SNP and SSR markers based chloroplast genome of *Theobroma cacao* showed that SSR was more variable than SNP. The SNP markers grouped the cacao population into three haplotypes, whereas the SSR markers were classified into eight haplotypes (Yang et al. 2013). Besides, the polymorphism level of InDel markers was higher than SSR markers and slightly higher than SNP markers, especially in the genotyping perspective, and InDel markers were relatively cheaper for genotyping than SNP (Jain et al. 2019). InDel in the chloroplast genome is very common and has provided an informative result of phylogenetic relationships between closely related taxa (Morton and Clegg 1993).

This study determined genotypic variations using SNP and InDel polymorphisms in the chloroplast genome. Because of substitution or insertion-deletion (InDel)

mutations, genetic variations may become helpful tools for evaluating relationships among individuals, the origin of a species, and haplotype distribution (Yue et al. 2018). A single nucleotide difference could be an insertion or deletion, transition, and transversion between individuals of intra- or interspecies or alleles in the paired sequences (Kucukkal et al. 2014).

The chloroplast genome has highly conserved sequences and a lower mutation rate than the nuclear genome (Androsiuk et al. 2020). However, some deviations from sequence conservation in the chloroplast genome have also been reported, such as the presence of genes duplication in *Pinus* (Lidholm et al. 1991), *Campanulaceae* (Cosner et al. 1997), *Arabidopsis* (Koch et al. 2005), *Elaeagnaceae* (Choi et al. 2015), *Euglena archaeoplastidiata* (Bennett et al. 2017) and *Delphinieae* (Park et al. 2020). The duplication events in the chloroplast genome may have an essential role in evolutionary processes and novel genetic functions (Panchy et al. 2016).

Haplotype diversity analysis

Changes in amplified products generated from InDel marker loci indicated the presence of mutation either in the primer annealing sites or within the amplified products. A haplotype could be constructed based on the amplification profiles using InDel primers and the individual plant DNA, and a haplotype network was used to compare similarity and difference among haplotypes of evaluated individuals.

Haplotype network analysis based on the polymorphic InDel markers indicated the presence of six haplotypes among 64 coconut accessions (Figure 3A). The haplotype 1 (HAP-1) was the most common (Figure 3A), detected among 28 accessions, including 18 accessions of Dwarf coconuts, seven Dwarf Kopyor coconuts, two Tall Kopyor coconuts, and one Bido coconut. Two loci differences separated HAP-1 from HAP-4 or HAP-5 (Figure 3A). Eight coconut accessions belonged to the HAP-4, including five Tall coconut types (two TST, one PNT, one TET, and one MWT coconuts), one LBT, and two Bido coconuts, while six coconut accessions belonged to the HAP-5, including two TTD, two PWD, and two RAD. The HAP-2 can be derived from HAP-4 or HAP-5 through two paths (Figure 3A), each standing for a single mutation. Six coconut accessions belonged to the HAP-2, including three Tall coconut accessions (two KYT and one TTT), two LBD, and one LBT coconuts.

The HAP-3 originated from the HAP-4 through a single mutation (Figure 3A). Four coconut accessions belonged to the HAP-3, including one LPT, one SNT, one TAT, and one TTT coconuts. Finally, the HAP-6 might be derived from either HAP-2 or HAP-3 through another mutation (Figure 3A). Eleven coconuts accessions belonged to the HAP-6, including two IIT, two SAT, one TAT, one MWT, one PNT, two PUT, one SNT, and one TET coconuts.

A haplotype (a haploid genotype) combines alleles along one chromosome. The alleles are inherited as a single block of genetic materials (Bromham 2016). The haplotype network is an analysis to comprehend the pattern of nucleotide mutations among combination alleles and visualize the relationships among the nucleotide mutations in a population (Yue et al. 2018). The highest frequency haplotypes are the most ancient ones (Chen et al. 2019). Moreover, the most common haplotypes and the most widely distributed haplotypes among the population are assumed to be ancestor haplotypes. Hence, the derived haplotypes would be less frequent (López et al. 2016). The sago palm is another palm species showing polymorphic haplotypes of their chloroplast genome among different sago accessions (Abbas et al. 2010).

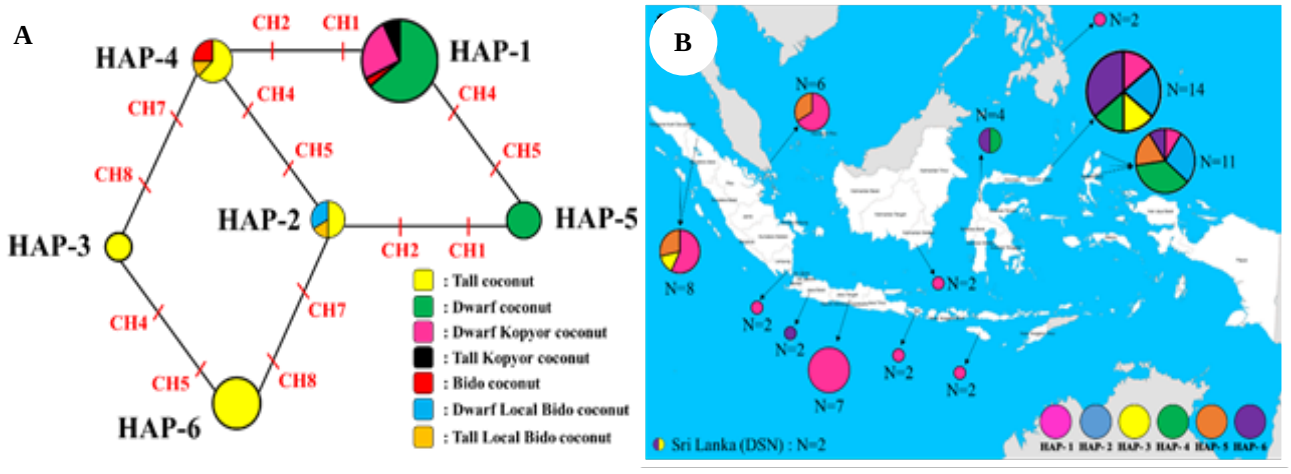

Figure 3. A. Reconstruction of the haplotype networks of 64 coconut individuals based on InDel markers. There are six haplotypes (HAP-1 - HAP-6), and The red lines indicate the number of insertion or deletion (indel) mutations. B. Distribution of the identified haplotypes among collected coconuts from various locations in Indonesia. CH - the mutation frequency to show the changes from one haplotype to the other, i.e. compare to HAP-1, two changes (CH4 and CH5) occurred in chloroplast DNA to become HAP-5. The colors show the haplotype group of the evaluated coconut accessions. The diameters of the circles are proportional to the sample numbers (Ns)

Based on the coconut haplotype distribution in Indonesia, North Maluku and North Sulawesi showed the highest frequency of chloroplast haplotype variations than other regions. The haplotype frequency among coconuts from different regions in Indonesia also showed that the chloroplast haplotype variations are primarily seen in those two locations. Therefore, future exploration of coconut germplasm in Indonesia should focus on those two regions (North Maluku and North Sulawesi).

Haplotype diversity of the chloroplast genome is valuable data for high-resolution phylogenetic analysis and evolutionary studies because it is highly conserved and has low recombination (López et al. 2016; Park et al. 2019). Unfortunately, SNP markers' phylogenetic analysis showed very low genetic variation, as Lee et al. (2017) reported. Lee et al. (2017) conducted a phylogenetic analysis using the gene-based SNP markers (matK) on 12 *Cudrania tricuspidata* Bureau ecotypes, including nine from Korea and three ecotypes from China. Lee et al. (2017) study indicated that the 12 ecotypes were composed of two clusters. Cluster I consisted of 9 ecotypes from Korea, and cluster II consisted of 3 ecotypes from China. Among 12 ecotypes, the nine ecotypes from Korea showed the highest homology (100%) among the Korean ecotypes, whereas the three ecotypes from China showed only 58% homology between Chinese ecotypes. Those results indicate that the same ecotypes and the same location origin do not always mean they must have close genetic similarities.

Genetic distances and phylogenetic analysis

Summary of pairwise genetic distance generated based on dissimilarity among the evaluated coconut accessions was presented in Figure 4. The results showed that the majority of the pairwise dissimilarity distance values ranged from 0 to 0.2 (Figure 4). Only a few dissimilarity distance values are larger than 0.2 (between 0.2 to 0.6) (Figure 4). Such results showed that most of the evaluated SNAP and InDel markers derived from coconut chloroplast genome generate similar allele profile and only a few show allele variations. These data are in line with the general understanding that the nucleotide sequences of the chloroplast genome are conserved among accessions within the same species.

The phylogenetic tree constructed based on dissimilarity distances among the evaluated coconut accessions was presented in Figure 5. Phylogenetic analysis results showed that the evaluated coconut accessions are clustered into three clusters (Cluster I, II and III, Figure 5). Most of the coconut individuals belong to cluster II (36 coconut accessions). Meanwhile, 17 coconut accessions belong to the cluster I and 11 accessions belong to cluster III. The cluster I (Figure 5) consisted of eight Tall coconuts (LBT1, LPT3, MWT4,5, TST6,7, TTT4,5), two Tall Kopyor coconuts (KKT1,3), and seven Dwarf Kopyor coconuts (BKD-A, K, GKD-A, K, OKD-A, YKD-A,K). The cluster II.A. consisted of eleven Dwarf, five Tall, and three Semi Tall coconuts accessions (Figure 5), while cluster II.B. consisted of eleven Dwarf and six Tall coconut accessions (Figure 5). The cluster III.A. consisted of one Dwarf (OSD2), five Tall (IIT2,4; IPT8; SAT2,3), and one Kopyor Tall coconut accessions (KKT2, Figure 5), while cluster III.B. consisted of three Dwarf (OSD1; PWD1,2) and one Tall coconut accessions (PNT4, Figure 5).

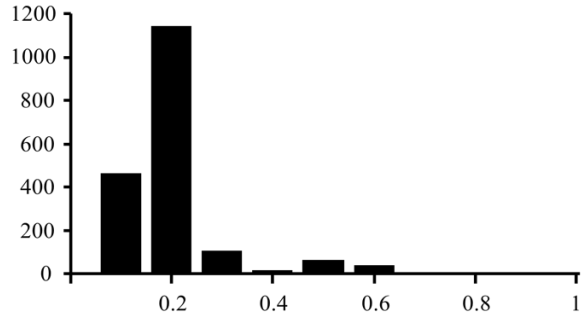

Figure 4. Frequency distribution of the pairwise genetic distance values generated based on dissimilarity among coconut accessions evaluated in this study. The pairwise dissimilarity distance was calculated using DARWin software and using SNAP and InDel marker data of the chloroplast genome

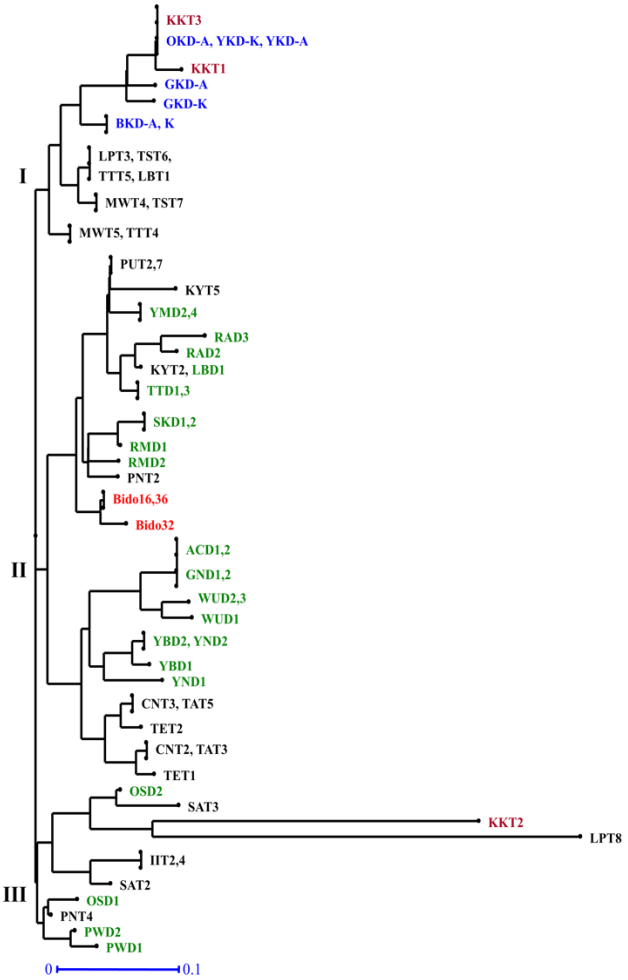

Figure 5. Phylogenetic tree using Weighted Neighbor-Joining method based on ten SNAP and five InDel markers of the chloroplast DNA. The tree was constructed using dissimilarity based genetic distances calculated with DARWin Software. Accession name in black is Tall, green is Dwarf, red is Bido Semi-Tall, brown is Kopyor Tall, and blue is Kopyor Dwarf coconut

Based on this study's results, it could be concluded that ten pairs of SNAP primers and five pairs of InDel primers successfully amplified most of the cpDNA of the evaluated coconuts. However, the two marker systems (SNAP and InDel markers) cannot be used to distinguish specific coconut variety or type. The evaluated SNAP markers gave a low polymorphism and less informative. Meanwhile, the InDel markers were more informative and resulted in a better phylogenetic and haplotype clustering than the SNAP marker. The evaluation of the chloroplast genome diversity may be estimated using the evaluated SNAP and INDEL markers, and the markers can be used to do *C. nucifera* accessions genetic analysis in the future.

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