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Mining and validating novel SSR markers based on coconut (*Cocos nucifera* L.) whole genome and their use for phylogenetic analysis

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Abstract. *Hatta ANNL, Sukma D, Maskromo I, Sudarsono. 2022. Mining and validating novel SSR markers based on coconut (*Cocos nucifera *L.) whole genome and their use for phylogenetic analysis. Biodiversitas 23: 5122-5131.* The coconut (*Cocos nucifera* L.) genome sequences were available at the chromosome level in 2019, and they can be used to develop the whole genome SSR markers. This study aimed to conduct in silico analysis of the whole coconut genome to mine SSR sequences, design SSR markers distributed across coconut chromosomes, validate the designed primers to generate SSR markers, and use them for the phylogenetic analysis of coconuts. The SSR loci were mined from publicly available coconut genomes. Targeted primers for amplifying 20 dinucleotide SSR loci were selected and used for phylogenetic analysis of coconuts. The SSR mining identified 543,774 dinucleotide repeat motifs across the coconut genome, and most of the dinucleotide SSRs are AG (46.18%) and AT (40.03%) repeats. Primer pairs targeted to amplify 876 SSR loci and distributed in 16 coconut chromosomes were successfully designed and deposited in a public data repository for public access. The 237 SSR primer loci were novel and could generate new SSR markers. Among 20 selected and tested primers, 18 produced SSR markers, and 15 pairs yielded polymorphic markers when used to evaluate 25 coconut accessions. The analysis showed that the designed SSR primers were informative for evaluating coconut genetic diversity and phylogenetic analysis.

Keywords: Chromosome, coconut, genetic diversity, SSR markers, whole genome

INTRODUCTION

Coconut (*Cocos nucifera* L.), known as the "tree of life," is capable of providing livelihood in many tropical countries (Oday and Marcone 2019). Enormous coconut genetic diversities exist worldwide because of various factors, such as local adaptation, mutation, and selection (Geethanjali et al. 2018). Natural genetic diversity may also occur because coconut is a cross-pollinated crop (Wu et al. 2019). In addition, mutations naturally caused by solar radiation have increased the genetic diversity of coconuts (Alinne et al. 2018). Recent data indicated that Indonesia has the most extensive coconut plantations (31.2% of the total ± 12 million ha worldwide) (Siregar et al. 2019). Moreover, enormous coconut genetic diversities exist in Indonesia, especially in the central and eastern parts of Indonesia (Pesik et al. 2016; Rahmawati et al. 2021).

Morphologically, coconuts are divided into typica (tall) and nana (dwarf) types. The tall coconuts usually have a large and tall trunk with bole at the base, which is naturally cross-pollinated, resulting in heterozygous and heterogeneous populations (Loiola et al. 2016). Meanwhile, dwarf coconuts have small and short stature without bole at the base, and its naturally self-pollinated, resulting in more homozygous and homogeneous populations (Kamaral et al. 2017). Recent research results have found bido coconut as typica coconut (Novarianto et al. 2022) and kopyor coconut as nana coconut (Rahayu et al. 2022). The presence of pollen transfer among dwarf, tall, and hybrid coconuts has been reported by Larekeng et al. (2018).

Conventional coconut plant breeding programs are generally carried out by crossing tall and dwarf coconut types (Kumar et al. 2018). The problems found in coconut plants have a relatively long juvenile period of coconut plants. Morphological differences between various kinds of coconut can only be observed after the plant has passed its juvenile stage (Perera 2020). Such characteristic makes it difficult for coconut breeders because it takes a long time to confirm the correctness of the crosses (Mahayu et al. 2022). Biotechnology is one of the most appropriate solutions to overcome those problems through molecular markers as the early detection of crosses more accurately. Molecular markers can also analyze coconuts' genetic diversity (Xiao et al. 2016).

Milestones for the use of molecular markers in coconut include RAPD (Rajesh et al. 2014), AFLP (Nartvaranant 2019), SNP (Muñoz-Pérez et al. 2022), and SSR (Caro et al. 2022). However, SSR (Simple Sequence Repeat) markers are the most recommended because they are highly polymorphic and co-dominant markers (Hao et al. 2020). The SSRs are DNA microsatellites with tandem repeats, widely distributed throughout both coding and non-coding

regions in the eukaryotic genome (Zhou et al. 2020a). The SSR markers could be used to find genetic variations through polymorphisms among tested individual samples (Tan et al. 2019).

Coconut SSR sequences have been published as DNA fragments in the NCBI DNA Database and the markers used for genetic diversity analysis (Perera et al. 2016; Indrachapa et al. 2019). Genome-wide coconut SSR markers have been developed (Lantican et al. 2019). However, the markers were derived from coconut genomic scaffold data instead of chromosomes. The complete genome data (i.e., 16 chromosomes) of the Hainan tall coconut cultivar was publicly available in the NCBI database in 2019. The availability of assembled coconut genomes at chromosome levels allows the development of markers associated with each coconut chromosome. Therefore, this study aimed to conduct in silico analysis of the whole coconut genome to mine SSR sequences, design SSR markers distributed across the 16 coconut chromosomes, validate the designed primers to generate SSR markers, and use them for the phylogenetic analysis of coconuts.

MATERIALS AND METHODS

Plant materials

The 25 coconut accessions from the Indonesian Palm Research Institute (IPRI), Manado, Indonesia, were used for SSR primer validation. The evaluated coconut accessions comprised six tall, seven dwarfs, and twelve hybrid coconuts (Table 1). Fresh leaf samples from each coconut accession were collected, wrapped in paper, and sent to Plant Molecular Biology Lab, Department of Agronomy and Horticulture, Institut Pertanian Bogor, Bogor, West Java, Indonesia, for DNA isolation.

Procedures

SSR mining across 16 coconut chromosomes

Assembled genomic sequences (16 chromosomes) of coconut cv. Hainan Tall (Acc. no. ASM812446v1), publicly available in the NCBI database (https://www.ncbi.nlm.nih.gov/assembly/GCA_008124465.1/) was used for in silico SSR loci mining. The downloaded genomic sequences were evaluated using Phobos version 3.3.12 (http://www.ruhr-uni-bochum.de/ecoevo/cm/cm_phobos.htm), and SSR loci associated with 16 coconut chromosomes were identified. The mined SSR loci included mono- to hexanucleotide repeats with a minimum number of repeats (n) of at least 20 units. Subsequently, 250 bp nucleotide flanking the SSR loci was also obtained. The frequency of SSR types and other related information in the coconut genomes were further analyzed using Microsoft Excel 2019.

SSR primer design

Primer pairs for amplifying each target locus were designed only from the dinucleotide SSR type that has at least 250 bp flanking nucleotide sequences. The SSR primers were designed using Primer3Plus online software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Primer3Plus software was set using the following criteria: 200-500 bp amplicon sizes, 18-28 bp primer length, 50°-63°C melting temperature (Tm), and 40-60% GC content. Each designed primer was aligned to the coconut chromosomes using the Geneious version 10.1.3 software to confirm that the generated primers were present in the desired coconut chromosome. The primers aligned once to the target and were not aligned to the others selected.

SSR map in 16 coconut chromosomes

The naming for the SSR primers identified in this study was standardized using *Cn* – chromosome number – and locus number (i.e., *Cn*-1-10 represents *C. nucifera* chromosome 1, SSR locus number 10). Linkage of the selected SSR loci with the appropriate primers identified in this study (876 SSR loci) and the 237 coconut SSR loci readily available in the NCBI. DNA Database was mapped and determined position across the 16 coconut chromosomes. The chromosomal position of the coconut SSR in the NCBI DNA Database was determined using BLAST to coconut genome (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_SPEC=Assembly&UID=104525614). Map positions of the identified SSR loci were conducted using Map Chart software version 2.32 (https://www.wur.nl/en/show/Mapchart.htm). The sequence sizes of 16 coconut chromosomes were used to determine the positions of selected SSR loci in the appropriate chromosomes.

Table 1. List of coconut accessions used for validation of the generated SSR markers

Accession name	Types	Abbreviation	Accession name	Types	Abbreviation
Tenga tall	Tall	TT	Khina-1	Hybrid	NYD × TT
Palu tall	Tall	PT	Khina-2	Hybrid	NYD × BT
Bali tall	Tall	BT	Khina-3	Hybrid	NYD × PT
Mapanget brown tall	Tall	MBT	Khina-5	Hybrid	BYD × MBT
MBT (four-generation selfing)	Tall	MBTS4	Hengniu-1	Hybrid	BYD × MGTS4
Mapanget green tall (four generation selfing)	Tall	MGTS4	Hengniu-2	Hybrid	Raja Dwarf × MBTS4
Pati yellow kopyor	Dwarf	YK	IMA-1	Hybrid	MRD × TT
Pati green kopyor	Dwarf	GK	IMA-2	Hybrid	MRD × BT
Pati brown kopyor	Dwarf	BK	IMA-3	Hybrid	MRD × PT
Malaysia yellow dwarf	Dwarf	MYD	IMA-4	Hybrid	MYD × TT
Malaysia red dwarf	Dwarf	MRD	IMA-5	Hybrid	MYD × BT
Nias yellow dwarf	Dwarf	NYD	IMA-6	Hybrid	MYD × PT
Bali yellow dwarf	Dwarf	BYD			

Primer validation using Indonesian coconuts

DNA isolation was carried out using young leaves of the coconut samples and the CTAB protocols routinely used in the Plant Molecular Biology Laboratory, Institut Pertanian Bogor (Larekeng et al. 2015). The isolated total nucleic acids concentration and quality were checked using 1% agarose gel electrophoresis in 1x sodium borate buffer. PCR amplification of the target SSR markers was done using total nucleic acid (50 ng) of each coconut sample (2 µL) as the template and the appropriate SSR primers (forward and reverse primers, 2 µL for each primer), MyTaq PCR mix (Bioline, 6.25 µL), and double distilled (dd) H_2O (0.25 µL) in a total volume of 12.5 µL.

The amplification was done in Takara PCR Thermal Cycler (Takara Bio Inc.) and using the following steps: one cycle of predenaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 15 secs, primer annealing at 48.5-52.7°C for 15 secs, depending on the SSR primer used, and primer extension at 72°C for 30 secs. A final cycle at 72°C for 5 mins was added for the final primer extension. The presence of PCR amplified fragments were checked using 1% agarose gel electrophoresis in 1x SB buffer at a constant voltage (100 V) for 60 mins. The DNA fragments were stained using Gelred™ (Biotium Inc.) at 0.2x per mL of agarose gel. The fractionated DNA fragments were visualized under a UV transilluminator, and the gels were photographed using a digital camera.

Marker polymorphisms analysis using PAGE

Allele sizing of amplified PCR fragments from 25 coconut samples and the 18 SSR marker loci was conducted in 6% polyacrylamide gel electrophoresis (PAGE) using 1x SB Buffer (Brody and Kern 2004) in the Cole-Parmer® Dedicated Height Sequencers. The evaluated PCR amplified sample products (3 µL) were mixed with formamide loading dye (2 µL), denatured at 95°C for 10 minutes, and placed in ice water. The run PAGE for allele sizing was carried out at 60 W for 120 mins, and fractionated DNA samples were stained using silver staining as described by Vieira et al. (2016).

Data analysis

The stained acrylamide gels were dried at room temperature overnight and documented using a scanner. Scoring the SSR alleles was done directly in the stained acrylamide gels and indirectly in the scanned acrylamide gels. The marker scores for 25 Indonesian coconut accessions for 18 SSR loci were tabulated in Microsoft Excel to calculate pairwise genetic distances. Subsequently, calculated genetic distances were used to construct a phylogenetic tree employing the weighted Neighbor-Joining method. Calculation of the pairwise genetic distance (dissimilarity matric) and construction of phylogenetic tree was done using Dissimilarity Analysis and Representation for Windows (DARwin) version 6.0.14 (https://darwin.cirad.fr/).

RESULTS AND DISCUSSION

Simple Sequence Repeat (SSR) motifs

Analysis results revealed the broad distribution of the SSRs of mono-, di-, tri-, penta-, and hexanucleotide motifs in the 16 coconut chromosomes. We found as many as 9,521,002 total SSR loci from the *C. nucifera* genome data in a total of 2,202,455,121 bp of 16 chromosomes of the coconut genomes (Table 2). The density of the SSR loci found in this study was 4,322.9 SSR/Mb. The density of SSR loci in this study was higher than that of sago palm (*Metroxylon sagu* Rottb.), with an SSR density of 132.57 SSR/Mb (Purwoko et al. 2019). The higher SSR density in the coconut genome is due to the more complete genomic data in the chromosomal format compared to others that are only in the form of genomic scaffold data (Riangwong et al. 2020; Caro et al. 2022).

Each coconut chromosome sequence data was analyzed using Phobos to mine SSR sequences from mononucleotide motifs to hexanucleotides and find the flanking sequences. The results showed differences in the mononucleotides to hexanucleotides SSR motif distribution among the coconut chromosomes. The highest frequency was tetranucleotide repeats (i.e., 4,560,207), while the lowest was dinucleotide repeats (i.e., 543,774). The frequency of the tri-, penta-, mono-, and hexanucleotide repeats were 1,997,501; 1,765,658; 703,266; and 671,862 respectively (Table 2).

A total of 22,748 SSRs loci consisting of di-, tri-, tetra-, penta-, and hexanucleotide of five or more repeats were identified using a partial genome of 38 coconut accessions (Riangwong et al. 2020). On the other hand, 7,139 SSR loci were found using the Catigan Green scaffold genome (Caro et al. 2022). In the 38 coconut accessions from Thailand, the total SSR loci consisted of 15,165 dinucleotides (66.67%), 6,570 trinucleotides (28.88%), 659 tetranucleotides (2.90%), 116 pentanucleotides (0.51%), and 238 hexanucleotides (1.05%) repeats were found (Riangwong et al. 2020). The total number of identified SSR loci (di- to hexanucleotide motifs) in oil palm is 245,654 (Babu et al. 2019). The dinucleotide repeats are the most common repeat motif found in all oil palm (*Elaeis guineensis* Jacq.) chromosomes. Dinucleotide repeats were also identified as the coconut's most abundant microsatellite class (Riangwong et al. 2020). Meanwhile, many mononucleotide motifs were found in date palm (*Phoenix dactylifera* L.) genomes (He et al. 2017).

The development of SSR primers in this study focused on dinucleotide motifs because there were no other repeat sequences in the flanking sequences of the dinucleotide repeats. The other SSR motifs showed the presence of other repeats in the flanking sequences. Huo et al. (2021) stated that other types of repeat sequences in the flanking region could become a significant constraint in the primer design process. Moreover, the use of SSR primers, designed using the polymorphic dinucleotide SSR motifs was reported as effective in the date palm (Naeem et al. 2018), oil palm (Zhou et al. 2020b), chilli (Zhong et al. 2021), tomato (Castellana et al. 2020), rice (Tripathi et al. 2020), and wheat (Haque et al. 2021). Therefore, only dinucleotide repeats were further evaluated in this study.

The total number of dinucleotide SSR motifs in the 16 coconut chromosomes is 543,774, and based on their frequencies, they were evenly distributed among the 16 chromosomes (Table 3). Chromosome number 1 had the highest frequency of the dinucleotide repeat motif (55,196), and chromosome number 15 had the least (13,412). The selection of dinucleotide repeats was based on at least 20 repeat units. The selection of at least 20 repeats is intended to produce visible SSR markers using agarose gel electrophoresis. Our selection results showed that 4,556 dinucleotides SSRs could be used for primer design. The primer design using Primer3plus resulted in 876 primers that can generate SSR markers distributed in 16 coconut chromosomes. The designed primers were less than the available SSR because of the rigid criteria listed in the materials and methods. The designed 876 SSR primers are deposited in the IPB University official repository website (https://repository.ipb.ac.id/handle/123456789/107693) for public use.

Of the 7,139 SSR loci using the Catigan Green scaffold genome have found 131 novel SSR markers (Caro et al. 2022). Of the 131 SSR markers, 113 were polymorphic among the analyzed coconut genotypes (Caro et al. 2022). Meanwhile, based on silico analysis, Riangwong et al. (2020) found that 2,451 SSR marker loci were polymorphic in coconuts. Further analysis showed that 315 loci were suitable for the SSR marker development, and 100 were selected and used for the SSR primer design (Riangwong et al. 2020). This study identified that coconut SSR primers are higher than those found in other palm species, such as oil palm and sago palm. Zhou et al. (2020b) reported that 331 SSR primers were successfully designed from 1,020 dinucleotide SSR loci in oil palm. Purwoko et al. (2019) reported that 93 primers were successfully designed from 500 perfect SSR loci in sago palm.

Chromosome number 6 has the highest potential SSR loci (435 loci) for SSR primer design (Table 3). However, there were only 64 SSR primers obtained from chromosome number 6. On the other hand, chromosome number 7 has only 311 potential SSR loci. Nevertheless, there were 91 SSR primers obtained from chromosome number 7. The least number of SSR primers were obtained from chromosome 11 (21 SSR primers), with the potential SSR of 276 loci (Table 3).

In the Hainan Tall coconut, the dominant motives identified from the dinucleotide motifs were AG (251.126; 46.18%), followed by AT (217.665; 40.03%), AC (72.297; 13.30%), and CG (2.686; 0.49%) (Figure 1). In the Catigan Green genome, the AG and GA dinucleotide motifs were the most abundant compared to other dinucleotide repeats. The next dinucleotide repeat ranks were CT, TG, TC, AC, and GT motif repeats (Caro et al. 2022). Meanwhile, the AG/CT motif was the most abundant among the dinucleotide repeat motifs in oil palm, followed by the AT repeat motif (Bai et al. 2017). In date palm, AG/TC is 50.50%, followed by AT/TA of 35.50%, AC/TG of 13.25%, and CG/GC of 0.75% (Kadri et al. 2019). The AT/TA motif was the most common in cocoa (*Theobroma cacao* L.), followed by AG/TC (Xia et al. 2017).

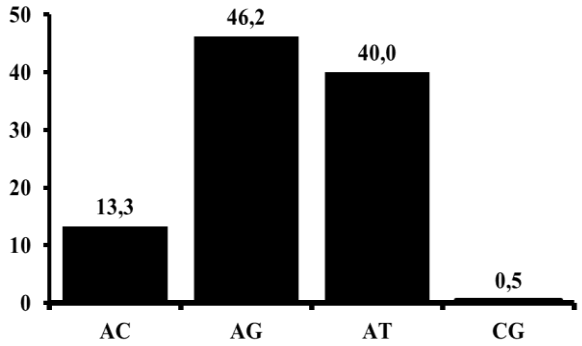

Figure 1. Frequency distribution of dinucleotide repeats based on motif types

Table 2. Summary of SSRs identified in 16 coconut chromosome sequences

Calculated information	Numbers or frequency
The total length of all chromosomes (bp)	2,202,455,121
Total number of identified SSRs	9,521,002
The density of SSR loci (SSR/Mb)	4,322.9
SSR motif types: Mononucleotide	703,266
Dinucleotide	543,774
Trinucleotide	1,979,501
Tetranucleotide	4,560,207
Pentanucleotide	1,765,658
Hexanucleotide	671,862

Table 3. Summary of dinucleotide repeats from 16 chromosomes and the generated primer

Chromosome number	Total SSR loci identified	SSR loci selected (with > 20)	Designed SSR primer
1	55,196	419	69
2	48,424	431	60
3	45,635	316	63
4	36,863	328	67
5	32,287	279	59
6	47,134	435	64
7	38,210	311	91
8	31,630	288	20
9	29,379	192	48
10	30,172	269	43
11	34,039	276	21
12	21,547	186	57
13	29,481	208	50
14	30,871	311	70
15	13,412	119	44
16	19,494	188	50
Total	543,774	4,556	876

SSR validation

Different product sizes of the reference SSR marker fragment sizes range from 194 bp to 498 bp (Table 4). Most of the SSR primer pairs have PCR amplified product sizes of between 200 to 300 bp, such as Cn-16-1 (248 bp), Cn-14-1 (284 bp), and Cn-12-1 (243 bp) primers, Cn-10-1

primers (244 bp), Cn-9-1 primer (219 bp), Cn-8-1 primer (298 bp), Cn-6-1 primer (240 bp), Cn-4-1 primer (243 bp) and Cn-3-1 primer (282 bp). The primer sizes ranged from 19 bp to 23 bp, and the recommended Tm of primers varies from 53°C to 57°C. Moreover, all primer pairs were distributed in the 16 coconut chromosomes, and their relative positions were presented in Figure 2. The development of SSR markers generated from coconut and other palms has been reported by Kamaral et al. (2016), Preethi et al. (2020), and Wang et al. (2020), but only stands for gene-generated or gene-related SSR. This study provides SSR markers generated from the whole coconut genome.

We selected 20 primer pairs based on the designed 876 SSR primers for SSR marker validation. Of the 20 primers selected, 12 pairs with AT motif, six primer pairs with AG motif, and two primer pairs with AC motif (Table 4). The CG motifs were also identified, but most of them showed other repeat motifs in the flanking sequences, and they were not used for primer design. The CG motif was also found in date palms but rarely generated good SSR primer pairs (Khouane et al. 2020). Gradient PCR was carried out to find the best annealing temperatures for the designed primers and the optimum primer annealing temperature for each primer pairs were 48.5^0 C (primer Cn-16-1 and Cn-9-1), 50°C (primer Cn-14-1, Cn-12-2, Cn-5-1, Cn-2-1 and Cn-1-1), 50.1°C (primer Cn-12-1 and Cn-6-1), 52°C (primer Cn-16-2, Cn-16-1, Cn-14-1, Cn-11-1, Cn-10-1, Cn-8-2, Cn-8-1, Cn-7-1, Cn-4-2 and Cn-4-1), and 52.7°C (primer Cn-3-1).

Table 4. Primer list used for SSR marker validation

Locus code	Repeat motif	Primer sequences	Tm (ºC)	Predicted product size (bp)
Cn-16-2	$(AT)_{23.5}$	F: TCAGAGGTAATTACATAGCTCCA	55.8	385
		R: GCAAGGCATACTTGAATCAC	55.8	
Cn-16-1	$(AC)_{21}$	F: GTGACACATGGATGGAATGA	57.6	248
		R: AAGCCTTGAGATGAGCAGAT	56.7	
Cn-16-1	$(AT)_{23}$	F: CTGAGTCTGATGCTCCATTG	56.9	427
		R: TTACAGCTGAACTCCTGCAC	56.6	
Cn-14-1	$(AT)_{81}$	F: TGGATATTAGATACTGTTCCGATT	56.1	284
		R: CTTGTCTGATGGCCAATCTT	57.7	
Cn-14-1	$(AG)_{23.5}$	F: AATCAGGAACTAGAAGAAGAACG	56.1	383
		R: GGCATCTTCGGTATCCTAAC	56.3	
Cn-12-2	$(AT)_{27}$	F: GATCAGCAAGGAATCTATCG	55.0	400
		R: GCATCATGATATGCTCTTGTG	56.8	
Cn-12-1	$(AG)_{26.5}$	F: GCTCAGAACAAGCTCCAGA	56.7	243
		R: CCTTCCTTCCAGGATTCTTA	56.0	
Cn-11-1	$(AT)_{21.5}$	F: TATGATGGCCTCCGGACCT	62.7	383
		R: CATCTTCTCCAGCTCCAACT	57.0	
Cn-10-1	$(AT)_{24}$	F: ATGAGCGTGCATCTCCAA	58.8	244
		R: ATGGCTTGGATCATTGATGT	57.8	
Cn-9-1	$(AT)_{21.5}$	F: CCACAACTTATGCAATCCAA	57.1	219
		R: CTCTGTTGGAAGCTTGATCC	57.5	
Cn-8-2	$(AC)_{24.5}$	F: TCCTGCTTACTCCATGACAA	56.8	498
		R: CAGTTCCAGGTTCAACCAAT	57.5	
Cn-8-1	$(AT)_{23.5}$	F: GTATAGCCACAGATCCAACG	55.7	298
		R: ACGTCTATGAGACTCCGAAGA	56.6	
Cn-7-1	$(AG)_{29}$	F: TACCAACCTTGGAGGACCA	58.9	380
		R: GGAGGTATACAGGACATAAGTGG	56.7	
Cn-6-1	$(AG)_{26.5}$	F: TGTCAGTTAGCATAACATGAAGT	54.3	240
		R: CAATGACAGAATGGAACAACA	57.0	
Cn-5-1	$(AT)_{38.5}$	F: TCAATGGTCCACTAATCCAA	56.4	196
		R: ACCGACTCCACACGTAACTT	57.2	
Cn-4-2	$(AG)_{24}$	F: GACTTCGAATTCTCGCCATA	57.9	329
		R: GGATGTTCTTCCTCCAACC	56.9	
Cn-4-1	$(AT)_{21}$	F: GATCAGATTCTGCGTCAACA	57.3	243
		R: TCAGCTGCATTAGGATAAGC	55.8	
Cn-3-1	$(AG)_{20.5}$	F: AGAGATCGAAGTGTCGGAAG	57.0	282
		R: CTCGTCACAAGGTCGGTAG	56,8	
Cn-2-1	$(AT)_{23}$	F: CTGTTGTTCTGCCGTGTTAT	56.3	300
		R: TGCAATGGAAGGTAACTGAA	56.8	
Cn-1-1	$(AT)_{27}$	F: CTTCGTCTTATGTATTGATGCT	53.9	353
		R: TGAGGTATATTCTTCATGGATTCT	56.1	

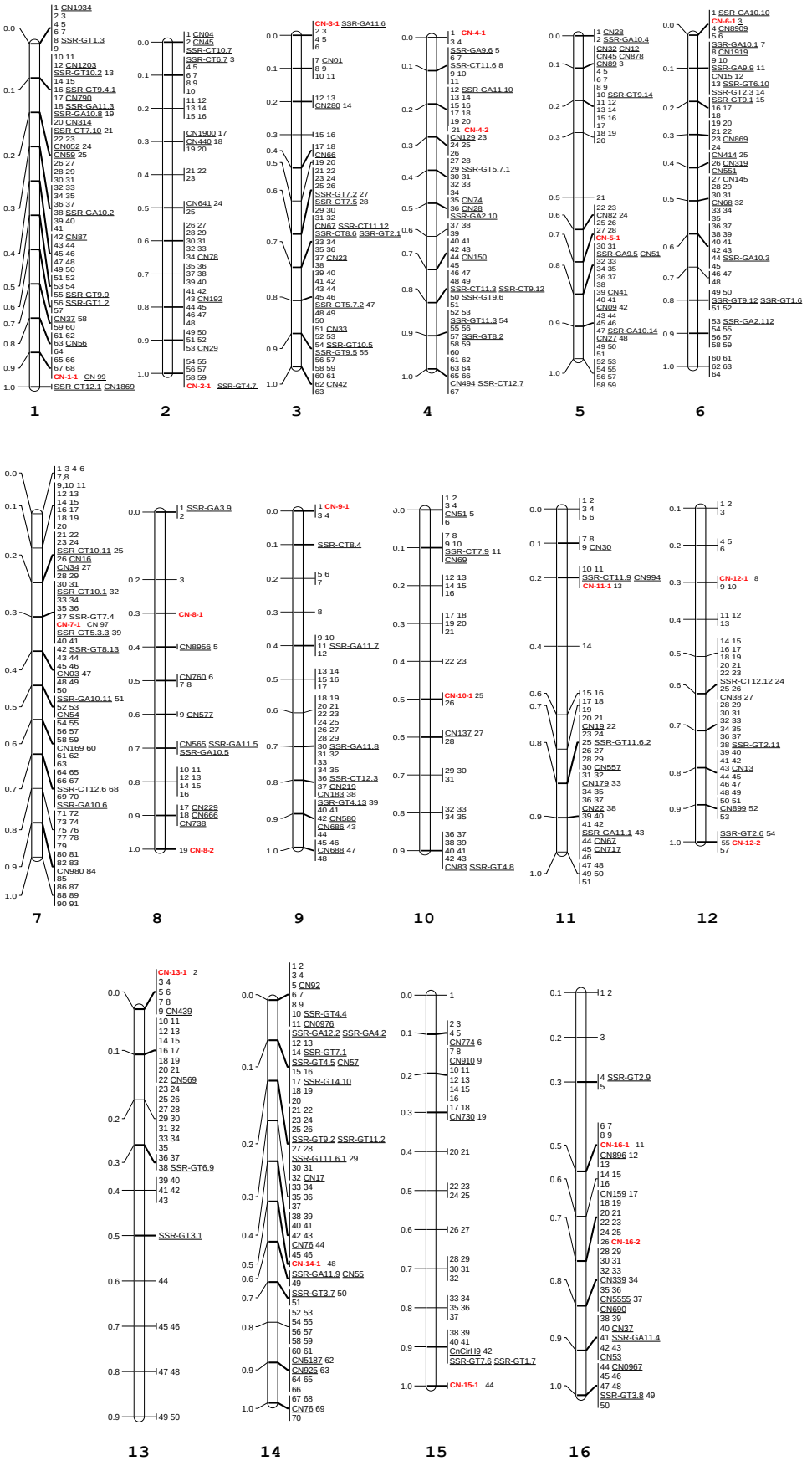

Figure 2. This study identified a linkage map of 876 SSR loci and 198 SSR loci previously deposited in the NCBI DNA Database in the 16 coconut chromosomes. The underlined SSR loci were those found in the NCBI DNA Database, and the red labelled SSR loci were the 20 loci used for SSR marker validation

Of the 20 tested primers, 18 generated amplicons for evaluated Indonesian coconut accessions. The two primer pairs that did not generate amplicon were Cn-14-1 and Cn-2-1 (Data not presented). As many as 237 coconut SSR loci were deposited in the NCBI DNA database. Sixteen accessions out of 237 were artificial oligonucleotides, and 221 were genomic fragments containing SSR loci. Twenty-three out of the 237 loci deposited in the NCBI DNA database were not mappable in the 16 coconut chromosomes. The 198 SSR loci from NCBI DNA Database and the 876 SSR loci found in this study were mappable to the 16 coconut chromosomes (Figure 2).

The linkage map of all SSR loci showed no overlap between SSR loci found in this study and those previously deposited in NCBI DNA Database. All the SSR primers validated to generate SSR markers showed different loci positions in each coconut chromosome. Four validated SSR primers are mapped in the upper side of the chromosomes, such as the Cn-3-1, Cn-4-1, Cn-6-1, Cn-9-1, and Cn-13-1. Nine validated SSR loci are mapped in the middle side of the coconut chromosomes, such as the Cn-4-2, Cn-5-1, Cn-7-1, Cn-8-1, Cn-10-1, Cn-11-1, Cn-12-4, Cn-16-1, and Cn-16-2. In contrast, five validated SSR primers are mapped in the lower side of the coconut chromosome, such as the Cn-1-1, Cn-2-1, Cn-8-2, Cn-12-2, and Cn-15-1 (Figure 2).

Polymorphic amplicons were seen among the 15 validated SSR loci across 25 coconut accessions evaluated. On the other hand, the Cn-1-1 and the Cn-4-1 generated a monomorphic SSR marker. The Cn-5-1 SSR primers did not yield amplicon when evaluated in the acrylamide gel electrophoresis. Using molecular markers such as SSR will only be effective when the evaluated locus generates polymorphic markers (Okoye et al. 2020). The number of alleles yielded per SSR locus across 25 coconut accessions evaluated ranged from 2 to 4. An example of polymorphic alleles generated using the Cn-6-1 SSR primers is presented in Figure 3.

The SSR marker profiles of individuals having their genotype as either homozygous or heterozygous are presented in Figure 3. The homozygous individuals are characterized by the appearance of one band, while the heterozygous ones show two alleles (Larekeng et al. 2015, 2018).

Phylogenetic analysis

Genetic diversity analysis was then carried out on 25 Indonesian coconut accessions based on the 15 polymorphic SSR loci. Results of the phylogenetic analysis using DARwin software version 6.0.14 showed that the 25 Indonesian coconut accessions evaluated were divided into three major clusters, and Cluster separation did not always follow the coconut types, such as dwarf, tall, and hybrid coconuts (Figure 4). Cluster I consisted of 9 accessions, namely Khina-1, Khina-2, Khina-3, Khina-5, Hengniu-1, Hengniu-2, IMA-1 hybrids, one dwarf accession (BYD), and one tall accession (TT). Cluster II comprised 11 accessions, namely dwarf kopyor accessions (BK, GK, and YK), three dwarf coconuts (MRD, MYD, and NYD), and four hybrids (IMA-2, IMA-3, IMA-4, IMA-5, and IMA-6). Cluster III consisted of the five tall accessions: MBT, MGT S4, MBT S4, BT, and PT.

Cluster I is dominated by coconut hybrids, such as the Khina and Hengniu hybrid coconuts generated by IPRI coconut breeders (Indonesian Agriculture Ministry 2019). In addition, in the cluster I also include IMA hybrids, BYD (dwarf type), and TT (tall type). Two hybrid accessions had BYD as the female parent in cluster I, namely the Hengniu-1 hybrid (BYD x MGT S4) and the Khina-5 hybrid (BYD x MBT). Two coconut hybrids were developed using TT as their male parent, such as the Khina-1 hybrid (NYD x TT) and the IMA-1 hybrid (MRD x TT). Both Khina-1 and IMA-1 hybrids were developed using the same TT male parent. Wu et al. (2019) reported that hybrid accessions and the male parent belonged to the same cluster in phylogenetic analysis.

Dwarf accessions and some coconut hybrids dominated cluster II. All Dwarf accessions evaluated in this study except for BYD were clustered together in cluster II. Almost all IMA hybrid accessions, except for IMA-1, were included in cluster II. Kopyor dwarf accessions (i.e., YK, GK, and BK) also belonged to cluster II. IMA hybrids' female parents were either MYD or MRD, which also belonged to cluster II. This result is in line with Kandoliya et al. (2018), which show that the female parent and its hybrid progenies belonged to the same cluster.

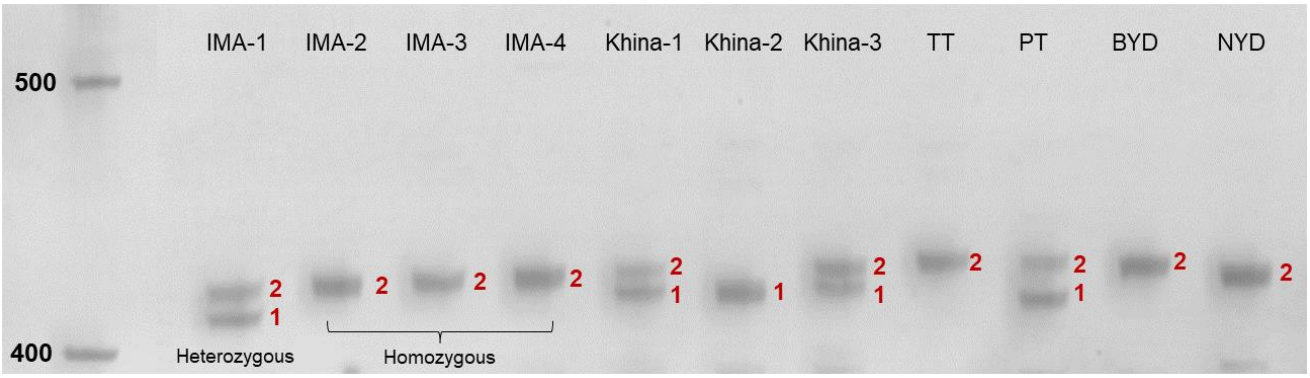

Figure 3. Polymorphic SSR alleles are generated using the Cn-6-1 SSR primers, and the predicted genotypes of evaluated individuals as homozygous and heterozygous based on the SSR marker allele profiles. The individuals with the 1 or the 2 scores predicted their genotypes as homozygous 11 or 22. The individual having the 1 and 2 scores, their genotypes were predicted as heterozygous

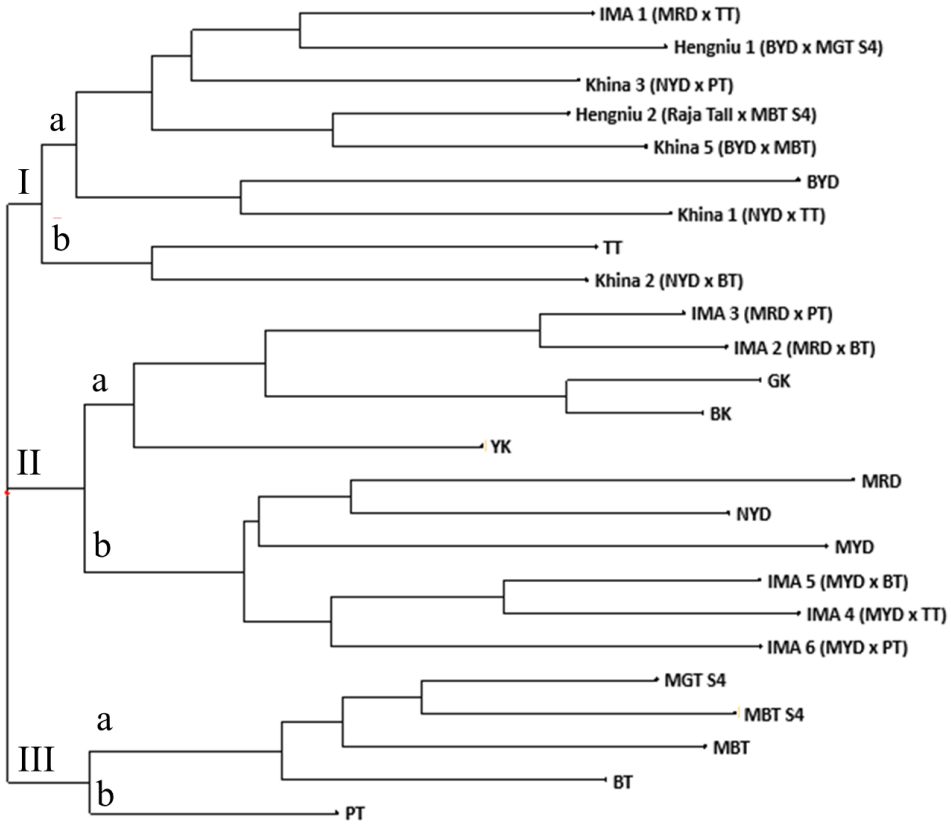

Figure 4. Phylogenetic tree of 25 Indonesian coconut accessions based on 15 polymorphic SSR markers

Cluster III consisted of almost all Tall accessions evaluated in this study, such as MBT, MGT S4, MBT S4, BT, and PT. According to Geethanjali et al. (2018), the tall coconut accessions mostly belonged to the same cluster in phylogenetic analysis. Indonesian coconuts' genetic diversity analysis results using SSR markers did not always show clustering based on the coconut types (i.e., dwarf, tall, and hybrid). This finding was in line with Preethi et al. (2020). Although there are differences in plant morphology and flowering characteristics between the three types of coconuts (Caro et al. 2022), the clustering using the evaluated SSR markers may not always be in line with the phenotypic differences.

Genetic diversity analysis DARWin software has also successfully been carried out in oil palm (Nugroho et al. 2019), sugar palm (Rinawati et al. 2021), and date palm (Huda et al. 2019). Clustering using DARwin software was commonly used to evaluate the genetic diversity of the evaluated samples based on dominant and co-dominant data (Saha et al. 2019). The advantage of using DARWin software is that the bootstrap analysis can be done to validate the clustering analysis (Hegde et al. 2018). Clustering analysis could also be done using the R Studio pheatmap cluster (Mangiola and Pafenfus 2020); however, clustering analysis in this study was only done using DARWin software.

In conclusion, tetranucleotides had the highest number of SSR repeat motifs, while the least frequent motifs were dinucleotides. The AG, AT, AC, and CG repeats were present within the dinucleotides repeat motif. Based on the dinucleotide repeats, as many as 876 SSR primers were designed, and 20 primers were validated for generating polymorphic SSR markers. The evaluated primers were 12 pairs from AT motif SSR, six pairs of AG, and two pairs of AC. The 18 SSR primers yielded amplicons, while the two did not (i.e., Cn-16-1 and Cn-6-1). The 15 out of 18 tested SSR primers yielded polymorphic SSR markers across 25 Indonesian coconut accessions. After using 15 SSR loci to estimate the genetic diversity of the 25 Indonesian coconut accessions, the accessions were clustered into three major clusters. Results of this study showed that the 876 designed SSR primers should help support the breeding of Indonesian coconuts in the future and were publicly available for coconut breeders.

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