Development of Novel Coconut SSR Markers Derived From Genome-Wide Bioinformatics Prediction

Reina Esther S. Caro

University of the Philippines Los Banos

Jesmar Cagayan

University of the Philippines Los Banos

Roanne R. Gardoce

University of the Philippines Los Banos

Anand Noel C. Manohar (✉ acmanohar@up.edu.ph)

University of the Philippines Los Baños <https://orcid.org/0000-0003-2500-1539>

Alma O. Canama-Salinas

University of the Philippines Los Banos

Ramon L. Rivera

Philippine Coconut Authority

Darlon V. Lantican

University of the Philippines Los Banos

Hayde F. Galvez

University of the Philippines Los Banos

Consortia E. Reaño

University of the Philippines Los Banos

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Abstract

In the past, simple sequence repeat (SSR) marker development in coconut is achieved through microsatellite probing in bacterial artificial chromosome (BAC) clones or using previously developed SSR markers from closely related genomes. These coconut SSR markers are publicly available in published literatures and online databases; however, the number is quite limited. Here, we used a locally established, coconut genome-wide SSR prediction bioinformatics pipeline to generate a vast amount of coconut SSR markers. A total of 7,139 novel SSR markers were derived from the genome assembly of coconut 'Catigan Green Dwarf' (CATD). A subset of the markers, amounting to 131, were selected for synthesis based on motif filtering, contig distribution, product size exclusion, and success of *in silico* PCR in the CATD genome assembly. OligoAnalyzer-tool was also employed using the following desired parameters: %GC: 40–60%; minimum ΔG value for hairpin loop: -0.3 kcal/mol; minimum ΔG value for self-dimer: -0.9 kcal/mol; and minimum ΔG value for hetero-dimer: -0.9 kcal/mol. We have successfully synthesized, optimized, and amplified 131 novel SSR markers in coconut using 'Catigan Green Dwarf' (CATD), 'Laguna Tall' (LAGT), 'West African Tall' (WAT), and SYNVAR (LAGT x WAT) genotypes. Of the 131 SSR markers, 113 were polymorphic among the analyzed coconut genotypes. The development of novel SSR markers for coconut will serve as a valuable resource for mapping of quantitative trait loci (QTLs), assessment of genetic diversity and population structure, hybridity testing, and other marker-assisted plant breeding applications.

Declarations

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Conflicts of interests/competing interests

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Availability of data and material

Not applicable.

Code availability

Consent to participate

Not applicable.

Not applicable.

Ethics approval

Consent for publication

Not applicable.

Not applicable.

Introduction

Coconut (*Cocos nucifera* L.) is one of the most economically important crops in the Philippines. In 2017, the country produced 14.05 million metric tons of coconut and the value of production hit 120.3 million pesos (PSA 2018). The Philippines remained to be the top global supplier of coconut copra and desiccated coconut in both volume and total USD value as of 2010 (FAOSTAT 2013). Coconut oil, one of the many diversified products of coconut, ranked first among the top ten agricultural exports of the Philippines comprising 21.9 percent of the total agricultural exports in 2015 (PSA 2017).

Coconut is situated across the tropical and subtropical latitudes that are accessible to the equatorial Pacific Ocean current which possibly favored the evolution and dispersal of coconut. Coconut palms thrive well in humid coastal environments at about 18 degrees of latitude north or south of the equator where there is fertile soil, favorable temperature, and year-round rainfall (Foale 2003). Coconut belongs to the Indian center (II), and Indo-Malayan sub-center (II-A, where the Philippines belongs) in Vavilov's center of origin of cultivated plants (Vavilov 1926). It is generally classified into two types: tall and dwarf. The tall types are generally allogamous (heterozygous) or cross-pollinating, slow to mature; flower at 6-10 years with an economic life of 60-70 years. Dwarf types, on the other hand, are highly autogamous (homozygous), or mainly self-pollinating, early to flower at around 4-6 years after planting with a productive life of 30-40 years (Harries 1978; Meerow et al. 2012; Batugal et al. 2009).

Coconut is a diploid with 32 chromosomes ($2n = 2X = 32$). It belongs to the family Arecaceae (Palmaceae) in the subfamily Cocoideae and is the lone species of genus *Cocos* (Perera et al. 2007). The estimated genome size of coconut is approximately 2.6 Gbp comprising of 50-70% repetitive sequences (Alsaihati et al. 2014). Lantican et al. (2019) reported the estimated genome size of 'CATD' to be 2.14 Gbp. The abundance of repeat contents in the coconut genome becomes advantageous in the assessment and characterization of coconut varieties/populations using molecular marker techniques. The use of molecular tools offers a more accurate assessment than the conventional way of characterizing coconut which is through morphological and agronomical traits that are mostly influenced by many environmental factors (Perera et al. 2003).

Molecular markers have established its importance as a modern breeding tool for crop improvement (Xu and Crouch 2008; Kesawat and Das 2009; Sindhumole and Ambili 2011). The use of molecular tools can significantly accelerate the overall duration of breeding programs for coconut improvement. One of the

extensively used markers in molecular breeding and genetic diversity analyses is the simple sequence repeats (SSR). SSRs are short tandem repeats that have repeating units of di-, tri-, tetra- and pentanucleotides (Powell et al. 1996). They are approximately 1-8 bp long, abundant, and well distributed throughout the genome on which repeat units can vary between genotypes/individuals which make it a very useful tool in fingerprinting, genotyping and genetic diversity analyses (Sharma et al. 2008).

In the past, SSR marker development in coconut was achieved through microsatellite probing in bacterial artificial chromosome (BAC) clones or using previously developed SSR markers from closely related genomes (Rivera et al. 1999; Perera et al. 2003). These coconut SSR markers are publicly available; however, the number and distribution across chromosomes is quite limited for quantitative trait loci (QTL) mapping and genetic diversity studies. Fortunately, with the current advancements in next-generation sequencing (NGS) technologies, it has now become possible to mine SSRs across the entire genome. By using genome-wide bioinformatics prediction, we can generate a vast amount of SSR markers efficiently.

This study aims to provide a valuable resource of SSR markers for potential use in marker assisted selection breeding for coconut.

Materials And Methods

Plant materials and leaf collections

Leaf samples of the coconut parental genotypes ‘Catigan Green Dwarf’ (CATD), ‘Laguna Tall’ (LAGT), ‘West African Tall’ (WAT) and a synthetic variety denoted as SYNVAR (LAGT × WAT) used in this study were obtained from the Philippine Coconut Authority–Zamboanga Research Center (PCA–ZRC) in San Ramon, Zamboanga City, Philippines. Coconut leaflets coming from the youngest frond or the “first leaf” and are free from any pest damage were carefully chosen as samples. Three (3) leaflets were gathered from each of the left and right portions of the midrib near the base of the frond. The samples were transported to the Genetics Laboratory at the Institute of Plant Breeding - University of the Philippines Los Baños (IPB-UPLB), Laguna, Philippines, for DNA extraction.

Table 1. Coconut genotypes used in the study for screening the SSR markers.

Tree Number	Coconut Cultivars	Code	Palm Number	Origin
1	Catigan Green Dwarf	CATD	1715	Davao City
2	West African Tall	WAT	0519	Ivory Coast
3	West African Tall	WAT	0610	Ivory Coast
4	West African Tall	WAT	0704	Ivory Coast
5	West African Tall	WAT	0720	Ivory Coast
6	Laguna Tall	LAGT	0107	Davao City
7	Laguna Tall	LAGT	0508	Davao City
8	SYNVAR (LAGT × WAT)	AN17	4017	Zamboanga City

Genomic DNA extraction of Coconut Parental Genotypes

Genomic DNA extraction. A total of eight (8) individuals/palms of the coconut genotypes (Table 1) were collected and extracted with genomic DNA following the procedure adapted from Doyle and Doyle (1990) with modifications. DNA quality and yield were determined by electrophoresis in 1% UltraPure™ agarose (Invitrogen Corp., Carlsbad, California, USA) in 1× Tris-borate EDTA (TBE) running buffer at 100 V for 40 min, 0.5 µg mL⁻¹ ethidium bromide staining, and UV illumination at 300 nm using the Enduro GDS Touch Imaging System (Labnet International, Inc, Edison, New Jersey, USA). DNA concentration was estimated by visual comparison of the intensity of fluorescence with known concentrations of lambda (λ) DNA molecular weight standards (Sigma-Aldrich Inc., St. Louis, Missouri, USA).

Development of SSR markers using the genome assembly of coconut ‘Catigan Green Dwarf’ (CATD)

A set of 7,139 novel SSR markers was previously automatically generated based on the SSR loci annotation of the genome assembly of coconut ‘Catigan Green Dwarf’ (CATD) using GMATA software package (Wang and Wang 2016; Lantican et al. 2019). Given the vast amount of the predicted SSR markers, selection criteria were employed to obtain high-quality markers for eventual use in coconut genotyping. Motif filtering, contig distribution, and product size exclusion were used to further filter the predicted markers by manual checking. Markers with AT/AT and TA/TA repeat motifs were excluded in the selection. *In silico* PCR in the ‘CATD’ genome assembly (Lantican et al. 2019) was then performed to ensure *in vitro* SSR amplification prior to synthesis (Rotmistrovsky et al. 2004). OligoAnalyzer-tool (Integrated DNA Technologies, Inc., Coralville, Iowa) was also employed using the following desired parameters: %GC: 40-60%; minimum ΔG value for hairpin loop: -0.3 kcal/mol; minimum ΔG value for self-dimer: -0.9 kcal/mol; and minimum ΔG value for hetero-dimer: -0.9 kcal/mol for further filtering of the SSR markers.

PCR analysis

PCR was carried out with 10- μ L reaction volume (15 ng genomic DNA, 1 $\times$ PCR buffer (10 mM Tris pH 9.1 at 20 °C, 50 mM KCl, 0.01% Triton™ X-100; Vivantis Technologies, Malaysia), 1.5 mM MgCl₂, 0.2 mM dNTPs (Promega Corporation, Madison, Wisconsin, USA), 0.2 μ M forward and reverse primer (Integrated DNA Technologies Pte. Ltd., Singapore) and *Taq* DNA polymerase (Vivantis Technologies, Malaysia). The temperature profile used is as follows: initial denaturation at 95 °C for 3 min, 30 cycles of denaturation (95 °C, 30 sec), annealing (45-60 °C depending on the primer pair, 45 sec), extension (72 °C, 1 min), and final extension at 72 °C for 5 min. Amplifications were carried out in the Applied Biosystems Veriti™ 96-well Thermal Cycler (Thermo Fisher Scientific, Madison, Wisconsin, USA). PCR products were resolved with electrophoresis using 8% non-denaturing polyacrylamide gel in 1 $\times$ Tris-Borate EDTA buffer at 100 V for 60-75 min in the C.B.S. Scientific Triple Wide Mini-Vertical System™ (C.B.S. Scientific Company San Diego, California, USA), and visualized using 0.5 μ g mL⁻¹ ethidium bromide staining and UV illumination using the Enduro GDS Touch Imaging System (Labnet International, Inc, Edison, New Jersey, USA). Gels were scored manually for the presence or absence of bands.

Results And Discussion

Local bioinformatics pipeline produces high-quality SSR markers

A vast amount of coconut SSR markers amounting to 7,139 was previously generated using a locally established bioinformatics pipeline (Lantican et al. 2019). A subset of these were pre-selected based on the characteristics of a good primer which includes sequence length (18-25 bp), GC content (40-60%), absence of runs of 4 or more of one base, absence of repetitive sequences, low dimerization capability and low hairpin loop formation (Zhao and Stodolsky 2004). Markers with AT/AT and TA/TA repeat motifs were excluded in the selection since these are the most common type of repeats found in the coconut/palm genome (Palliyarakkal et al. 2011; Xia et al. 2014; and Lantican et al. 2019) on which the high repeat content may hinder specificity of the markers and/or may result to non-specific amplification of products. Markers were also selected based on the distribution in the contig to cover the entire coconut genome. *In silico* PCR in the CATD genome assembly was performed. This allows checking of contig specificity of the marker and ensures *in vitro* SSR amplification (Rotmistrovsky et al. 2004). Product size range of the markers was also limit to 80-400bp for easy visualization in gel and OligoAnalyzer-tool was used to check dimerization capability and formation of hairpin-loop of the primers to produce high-quality markers.

A total of 131 SSR markers were synthesized and 98% of these were comprised by dinucleotide repeats (or 2-mer) while the remaining 1 and 1% were composed of trinucleotide and tetranucleotide repeats, respectively, as shown in Figure 1. The predominance of dinucleotide repeats in coconut and other related species is supported by previous works of Rivera et al. (1999), Palliyarakkal et al. (2011), Xia et al. (2014), and Lantican et al. (2019). AG and GA motifs are the most abundant dinucleotide repeats found in the 131 SSR markers, with 29 and 18.3%, respectively. These are followed by CT (14.5%), TG (13.7%), TC

(11.5%), AC (7.6%), and GT (3.8%) repeats, while 1 and 1% of the markers contained a trinucleotide repeat of AAG and tetranucleotide repeat of ACAT, respectively. This result coincides with studies of Palliyarakkal et al. (2011) and Xia et al. (2014) on which AG/GA/TC/CT motifs were also the most common dinucleotide repeats found in coconut/palm genome.

The development of SSR markers using advanced bioinformatics tools in this study has become very efficient in generating high number of markers in coconut. The generated SSRs here are expected to contribute to the pool of available molecular markers (Lebrun et al. 1998; Perera et al. 1998; Xiao et al. 2013; Xia et al. 2014; Wu et al. 2019) for fingerprinting, genetic diversity analysis and QTL mapping as well as other relevant studies in coconut.

SSR markers exhibit polymorphism in test coconut varieties

All SSR markers showed successful amplification in coconut genomic DNA. Of the 131 SSR markers, 113 (86%) were polymorphic among the test coconut varieties while the remaining 18 (14%) were monomorphic. An average of 2.70 alleles per locus was observed across test varieties, implying a high degree of polymorphism of the selected SSR markers. The results obtained here are consistent with previous studies on which high levels of polymorphism are likely attributed to phenotypic variation and differences in the breeding behaviors of the dwarf and tall varieties which are said to be generally autogamous (self-pollinating) and allogamous (cross-pollinating), respectively (Perera et al. 1999; Rivera et al. 1999; Teulat et al. 2000). Representative gels of polymorphic SSR markers optimized among coconut genotypes are presented in Figure 2 on which distinct and good amplification patterns were observed. Also, majority of the polymorphic markers have AG repeats (29%) and GA repeat motif (19%) as shown in Figure 3. The product size of these markers ranged from 130 to 690 bp. The summary of the characteristics of the selected SSR markers are presented in Table 2 which includes the name of marker, annealing temperature, repeat motif, contig distribution, product size range and number of alleles.

Table 2. Characteristics of the selected coconut SSR markers with name, annealing temperature, repeat motif, contig number, product size range, and number of alleles.

er ID	Tm (°C)	Optimized Ta (°C)	Predicted Length (bp)	Motif	Contig	Observed Size range (bp)	Polymorphisms	Number of alleles per locus
7	55.6	56	299	AG	0	242-260; 404-480	P	2
172	53.55	57	280	TG	1	280-320	P	3
329	55.3	55	342	CT	1	320-360	P	2
653	54.15	57	240	GA	4	130-170; 170-230	P	3
765	55	53	211	GA	5	210-265	P	4
995	56.55	54	196	AG	6	190-242	P	2
1095	56.25	54	229	CT	7	230-250	P	4
3414	56.85	50	289	AC	35	290-380	P	2
3683	55.65	54	360	TG	40	320-370	P	2
4036	56.8	55	254	GA	45	290-320	P	3
4153	55.2	53	388	TC	46	380-400	P	2
4627	55.25	51	237	AG	56	220-245	P	4
4772	53.5	59	351	GT	59	380-450	P	3
4830	54.75	53	341	AG	60	320-400	P	2
4976	55.65	58	199	AC	64	170-200; 215-250	P	6
5103	56.15	54	307	CT	65	300-350	P	3
5211	54.75	51	388	CT	68	190-210; 242-310	P	6
5746	55.45	50	152	CT	80	380-400	P	4
5910	53.25	53	338	AG	82	330	M	1
6063	54.7	53	394	AC	86	390-450	P	2
6206	55.4	53	321	GT	89	300-340	M	2
6376	55.6	53	368	CT	92	380-450	P	3
6463	57	57	304	GA	96	230-240; 300-320	P	4
6507	53.4	51	336	GA	98	150-160; 180-200	P	5
6571	54.15	62	398	CT	99	400-450	M	1
6672	56.05	56	363	GA	102	330-370	P	4
7007	54.3	57	381	TG	109	350-380	P	2
7162	55.65	56	259	GT	113	250-320; 400-420	P	3
7449	56.3	54	246	TC	119	240-260	P	4
7553	54.5	50	267	AG	121	240-260	P	3
7710	56	54	301	GA	127	220-230	P	3
7859	55.65	54	355	AG	132	300-310	P	2
8015	54.9	55	391	TG	135	250-320; 400-480	P	2
8444	55.5	54	295	GT	152	200; 250- 320	M	1
8741	54.35	57	248	TC	166	320	M	1
9091	55.1	58	373	AG	185	320-380	P	2

9331	54.8	45	374	GA	196	350-380	P	2
9514	54.95	50	324	TG	200	150; 220- 240	P	3
9655	54.55	50	381	AG	207	242-280; 370-400	P	2
9918	55.5	56	254	GT	224	230-242	P	2
10005	54.35	54	251	CT	229	242-260; 320-350	P	2
10146	54.9	58	208	GA	237	210-225; 240-250	P	3
10298	54.2	54	237	AG	245	230-245	P	2
10608	53.85	53	389	GA	265	350-380	P	2
10723	55.55	56	324	TC	274	280-350	P	3
10821	56	59	326	AG	280	310-350	P	3
11095	55.4	55	361	AG	295	330-360	P	2
11349	52.55	62	280	AC	308	200; 300- 350	P	3
16404	55.6	54	334	AG	1122	300-400	P	3
16553	53.45	48	339	GA	1168	200; 320- 340	P	3
16634	55.95	54	357	AG	1203	400-500	P	2
17050	54.85	52	397	CT	1370	400-470	P	2
17101	54.9	50	299	AG	1401	250-290	P	3
17156	54.7	53	293	GA	1418	240-265	P	3
17229	55.75	56	400	AC	1462	280-320	P	2
17487	54.25	52	288	CT	1550	310-350	P	3
17639	55.85	54	303	TC	1621	280-340	P	4
17725	55.1	55	234	AG	1655	170-190; 220-250	P	6
17797	55.25	53	370	GA	1688	315-350	M	2
17875	55.1	50	397	AG	1750	300-400	P	2
18331	55.45	55	368	AG	1987	320-500	M	1
18501	55.25	55	380	TC	2084	350-400	P	2
18573	55.45	57	383	TG	2130	400-440	P	2
18799	56.25	56	255	AG	2306	240-320	P	2
18903	56.2	56	396	AG	2370	350-400	P	2
18972	56.6	59	358	TC	2409	230-260; 330-400	P	5
19118	54.1	52	393	CT	2535	230-320	P	4
19193	55.75	54	398	TC	2585	360-400; 690	P	3
19386	55.25	52	354	AG	2748	300-330; 400-420	P	3
19611	54.2	50	364	AC	2872	300-340	P	3
19799	55.2	55	337	TC	2988	320-410	P	3
20018	54.5	53	362	AG	3251	300-330	P	3
20227	55.55	54	278	TG	3455	320-400	M	1
20739	54.6	58	270	AG	4154	250-330	P	3
21015	54.05	57	333	CT	4606	130-160; 310-500	P	2

21174	55.65	62	392	AG	4907	380-400	P	4
21493	55	55	315	TC	5710	220-230; 320-400	P	2
318	55.6	60	358	TG	1	310-330	P	2
425	55.9	59	217	TG	2	130-160; 180-200	P	3
808	56.1	55	225	GA	5	280-320; 400-430	P	3
3765	54.7	55	372	TC	40	290-380	P	3
4127	54.65	58	274	TG	46	160; 350- 390	M	1
5054	54.1	57	391	TG	64	400-440	P	2
5329	55.05	53	320	ACAT	70	300-320; 360-410	P	2
5632	56.4	56	180	TG	78	180-200	P	5
6746	56.05	59	382	TG	103	380-400	P	2
6908	53.05	58	344	CT	107	200-300	P	3
7627	54.45	54	356	TG	124	240-350	M	1
7985	54.4	57	323	AC	135	320-340	P	2
8083	55.15	58	247	GA	137	200-250	P	3
8371	56.2	54	310	AG	148	310-400	P	3
8904	55.85	56	364	GA	178	320-350	P	2
9440	53.9	54	333	GA	199	300-340	P	2
9988	51.45	49	383	GA	228	230-320	P	4
10263	54.5	55	313	CT	244	290-320; 400-500	P	5
10632	54.25	52	376	AC	268	150	M	1
10681	55.85	57	337	TG	269	320-400	P	3
11807	55	55	305	CT	346	242-330	P	3
12241	56.9	55	315	AG	385	240-330	P	4
12746	54.3	54	383	TG	441	380-450	P	2
13043	55.25	55	383	AG	472	320-340	P	3
13232	54.7	58	165	TC	495	170-230	P	3
13632	57.2	57	379	TG	561	300-330	P	3
13852	53.55	52	184	AG	595	390-450	M	2
13946	56.9	57	363	TC	605	330-400	P	3
14272	54.55	53	371	AG	643	320-410	P	2
14692	56.85	53	370	GA	700	330-380	P	2
15137	56.1	56	337	TC	796	330	M	1
15508	56.75	57	374	AG	868	200-220; 320-400	P	3
15694	55.15	55	353	AG	908	320-390	P	3
15970	55.15	53	294	TG	994	220-280	P	3
5852	57.4	60	310	AG	82	310-400	P	3
17532	54.4	55	388	AG	1570	350-500	P	2
17684	53.3	53	176	CT	1636	170	P	2
18364	55.45	58	347	GA	2021	260-320; 470	P	4
19333	55.15	53	304	AC	2726	280-420	P	4

19929	56	56	321	AG	3153	260-330	P	3
20075	54.95	55	280	GA	3333	300-320	P	4
1433	55.85	50	183	GA	10	150-170	M	1
1524	55.3	55	266	GA	11	230-250	M	1
1644	56.8	57	205	TC	12	190-210; 270	P	4
1809	56.6	57	386	AG	14	350-500	M	1
2363	55.85	56	400	AG	22	350-380	M	1
2470	54.6	55	369	CT	23	250-300	M	1
2651	56.05	54	306	GA	25	280-320	P	3
2960	55.05	55	300	CT	30	260-300	P	3
3730	54.95	53	296	AAG	40	250-280	P	3
3865	57.05	57	323	AG	43	300-320	P	2
4080	57.9	53	327	AG	45	300-320	P	4
4336	55.35	55	229	AC	49	230-300	P	4
5287	55.4	53	205	CT	69	230-340	P	4
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	2.70

Note: M – monomorphic; P – polymorphic; N/A – not applicable

Microsatellites or SSR markers are a very useful molecular tool for studying genetic diversity and genotyping of coconut (Lebrun et al. 1998; Perera et al. 1998; Perera et al. 2003; Konan et al. 2007; Xiao et al. 2013). It has been extensively used in these analyses since SSR markers are abundant and well distributed throughout the genome, multi-allelic, co-dominant, highly polymorphic, and highly reproducible (Powell 1996; Mason 2015). Previous studies like Rivera et al. (1999), Perera et al. (2003), Xiao et al. (2013), and Wu et al. (2019) have already developed SSR markers in coconut for genetic diversity studies and these markers showed high levels of polymorphism as well.

Here, we demonstrated that a locally established bioinformatics pipeline can mine SSR markers from NGS data with actual utility in terms of amplification and distinguishing power across several varieties of coconut. The advantage of using a genome-wide bioinformatics prediction approach in marker development is its relatively fast and cost-effective way of generating vast amounts of markers (Anderson and Lubberstedt 2003; Gupta et al. 2013). SSRs and SNPs can be easily generated automatically in the genome sequences with the use of these programs or pipelines (Ching et al. 2002; Lantican et al. 2019).

Polymorphic markers in this study will be further used to genotype the coconut mapping population generated from a three-way cross of ‘Pacific’ LAGT and CATD, and ‘Indo-Atlantic’ WAT coconut for QTL mapping analysis. The development of novel SSR markers for coconut will serve as a valuable resource for mapping QTLs, assessment of genetic diversity and population structure, hybridity testing, and other marker-assisted plant breeding applications.

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Figures

Figure 1

Percentage of repeat motifs of the selected SSR markers.

Figure 2

Representative gels of polymorphic SSR markers optimized among coconut genotypes.

Figure 3

Percentage of polymorphic SSR markers per motif.