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PRIMER NOTE

DEVELOPMENT OF 12 POLYMORPHIC MICROSATELLITE LOCI FOR THE ENDANGERED SEYCHELLES PALM *LODOICEA MALDIVICA* (ARECACEAE)[1]

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- *Premise of the study:* The evolutionarily and ecologically distinct coco de mer palm *Lodoicea maldivica* (Arecaceae) is endemic to two islands in the Seychelles. Before colonization of the islands by man, the endangered palm formed large monodominant stands, but its natural range is now restricted to four main populations and several patches of isolated individuals. Microsatellite markers were designed to investigate the genetic structure of the remaining natural populations of *L. maldivica*.
- *Methods and Results:* We developed 12 polymorphic and three monomorphic microsatellite markers for this species, with a mean number of alleles per locus of 13.2 (range 5–21) and expected heterozygosity values ranging from 0.31–0.91 for the polymorphic loci.
- *Conclusions:* These markers enable us to study the patterns of genetic diversity, contemporary seed dispersal, and the fine-scale spatial genetic structure of this important conservation flagship species.

Key words: Arecaceae; coco de mer; *Lodoicea maldivica*; microsatellites; parentage analysis; Praslin.

Lodoicea maldivica (J. F. Gmel.) Pers. (Arecaceae; coco de mer) is an evolutionarily and ecologically distinct dioecious palm (Edwards et al., 2002, 2015) that holds several botanical records, among which are the largest female flowers in any palm and the largest seeds in the plant kingdom (Leishman et al., 2000). The species was once widespread across two Seychelles islands, Praslin and Curieuse (Malavois, 1787, quoted in Fauvel, 1909), but now persists in only four main semiconnected populations—at Vallée de Mai, Fond Peper, and Fond Ferdinand on Praslin, and also on Curieuse Island (Fleischer-Dogley et al., 2011).

The total *L. maldivica* population on Praslin and Curieuse was estimated at 24,376 individuals in 2004, but despite the relatively large population size, reproductive female trees make up only a small proportion (15.6%) of the population (Fleischer-Dogley, 2006). The recent population reduction is due to habitat degradation arising from several serious fires and lumber harvest (Bailey, 1942). Although *L. maldivica* nut kernel has been listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), which prohibits its exportation without a license, unsustainable harvesting and poaching of nuts continues to threaten the species, as natural regeneration is severely limited (Rist et al., 2010). Fleischer-Dogley et al. (2010) used amplified fragment length polymorphisms to assess genetic diversity in *L. maldivica*, but the dominant nature of the markers did not permit detailed genetic analyses. By developing microsatellite markers, we provide the foundation for in-depth molecular research on the ecology and population genetics of the species, and a tool for the conservation and sustainable production of *L. maldivica* nontimber products. This study reports the isolation and characterization of 12 polymorphic and three monomorphic microsatellite loci in *L. maldivica.*

METHODS AND RESULTS

Size-selected fragments from genomic DNA were enriched for simple sequence repeat (SSR) content using magnetic streptavidin beads and biotin-labeled CT and GT repeat oligonucleotides. The SSR-enriched library was made by the company ecogenics (Balgach, Switzerland) and analyzed on a Roche 454 platform using the GS FLX Titanium reagents (454 Life Sciences, a Roche Company, Branford, Connecticut, USA). The 6607 reads had an average length of 143 base pairs. Of these, 617 contained a microsatellite insert with a tetra- or a trinucleotide of at least six repeat units or a dinucleotide of at least 10 repeat units. Primer design was done using the Primer3 core (Rozen and Skaletsky, 1999). Suitable primer design was possible in 212 reads. Seventy-eight primer pairs were tested, and the most reliable polymorphic candidates were optimized. Genomic DNA was extracted from silica gel–dried *L. maldivica* leaf or

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TABLE 1. Characteristics of the 12 polymorphic and three monomorphic microsatellite loci in *Lodoicea maldivica*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)[b]	Fluorescent dye	Multiplex[c]	Conc. (μM)	GenBank accession no.
Multiplex PCR							
Lm4716	F: TGGAGAGTACAATAGATGAAATGCC	$(CA)_{12}$	128–140	YY	MP1	0.30	KT897315
	R: AACGGAGTTATCATGCTTGC					0.30	
Lm2630	F: AAATAAGAGCAACCAGAGAAGTC	$(GA)_{16}$	121–157	ATTO565	MP1	0.30	KT897316
	R: GCAGGTGTCTCAATCAAGGC					0.30	
Lm8853	F: CTATGGTCTAGGTGGACGCC	$(ATGT)_9$	193–231	ATTO550	MP1	0.20	KT897317
	R: GGCTGGACATGCGTTCTATG					0.20	
Lm5648	F: CCAAGACTGTAACTTGTTCCCC	$(TATC)_{12}$	235–285	FAM	MP1	0.20	KT897318
	R: AGGCTTAGTGTTCAGGACCG					0.20	
Lm6782	F: GGTCTAAAACTATTGGAGCAAATCAA	$(TATG)_{12}$	252–334	ATTO565	MP2	0.30	KT897319
	R: AGACTCTTAAGTGGGCGAAC					0.30	
Lm1153	F: TTGGGATACATGAGAGCGGG	$(GA)_{13}$	120–166	FAM	MP2	0.30	KT897320
	R: AGATCAGTTGACTATTTGTTACTCTC					0.30	
Singleplex PCR[d]							
Lm4293	F: TCACCTTAGAGATGGTGCAGG	$(GTAT)_7$	138–200	FAM	1	0.08	KT897321
	R: TGCACTTGAAGGTTACGTATG					0.32	
Lm1750	F: AGTACTTAGGCATAGGCCAGC	$(TACA)_{10}$	218–234	ATTO565	1	0.08	KT897322
	R: ATGACATGGCCTGGAAGAGC					0.32	
Lm2407	F: GGGATCCTCATCCCATGCTC	$(ACAT)_9$	84–112	FAM	1	0.06	KT897323
	R: TCGTACCGCCTAAGCCTAAC					0.24	
Lm6026	F: AGAGCACTTTTTGCCAACCC	$(TATG)_8$	147–225	YY	1	0.06	KT897324
	R: ACATCTCATGTGAGGGCATTC					0.24	
Lm0144	F: GCGCGTGCACACATAGATAG	$(TAGA)_8$	244–280	ATTO550	1	0.06	KT897325
	R: CATGCTCTCCGCTAAAACCC					0.24	
Lm2071	F: CCATCTCCGCCATTTTTCCC	$(GA)_{13}$	104–138	FAM	2	0.08	KT897326
	R: TACGCACCTACGTTCCTTCC					0.32	
Lm7170	F: ACGCATGGGAAGGATCTCAC	$(ATAC)_9$	213[e]	FAM	2	0.08	KT962232
	R: ATGGGGGCTTGTCCATTAGG					0.32	
Lm1012	F: GTCGATGGTGCTTCTAGCTG	$(TACA)_7$	251[e]	ATTO565	2	0.08	KT962233
	R: CCTGCTTACCATGAAAGGTCG					0.32	
Lm5950	F: ACCGAATGGAACAAAGTCACAC	$(TATC)_7$	180[e]	ATTO565	2	0.08	KT962234
	R: CGTTAGAAACATAGGAAACAGCC					0.32	

[a] Values based on samples collected from the four populations across the natural range on Praslin and Curieuse (1252 samples for the polymorphic markers and 64 samples for the monomorphic markers).

[b] Range of allele sizes includes M13 tail (5′-TGTAAAACGACGGCCAGT-3′) attached to the forward primer.

[c] Mix for multiplex PCR (MP1 and MP2) or pseudo-multiplex mix (1 and 2) for fragment analysis (using singleplex PCR products).

[d] The singleplex PCRs used forward primers labeled with M13 tails (5′-TGTAAAACGACGGCCAGT-3′) at the 5′-ends (as described by Schuelke, 2000) and reverse primers and M13-primer universal tails labeled with either FAM, ATTO565, ATTO550, or YY (Microsynth).

[e] Monomorphic microsatellite marker.

TABLE 2. Genetic properties of 12 de novo microsatellite markers in the four extant *Lodoicea maldivica* populations.[a,b]

	Vallée de Mai (n = 482)				Fond Peper (n = 293)				Fond Ferdinand (n = 265)				Curieuse (n = 212)			
Locus	A	H_o	H_e	HWE[c]	A	H_o	H_e	HWE[c]	A	H_o	H_e	HWE[c]	A	H_o	H_e	HWE[c]
Lm4716	4	0.525	0.514	3.243 ns	5	0.455	0.467	5.893 ns	3	0.457	0.500	3.749 ns	7	0.476	0.548	518.001***
Lm2630	17	0.570	0.879	1824.687***	17	0.543	0.909	1103.038***	18	0.598	0.895	807.148***	16	0.612	0.880	384.399***
Lm8853	6	0.454	0.540	32.452**	5	0.579	0.586	19.938*	6	0.481	0.563	46.053***	5	0.566	0.608	23.262**
Lm5648	13	0.797	0.857	99.149 ns	12	0.806	0.834	67.738 ns	12	0.820	0.841	54.122 ns	11	0.768	0.820	159.926***
Lm6782	15	0.429	0.747	1213.166***	13	0.463	0.716	536.756***	13	0.398	0.699	623.970***	17	0.401	0.743	738.903***
Lm1153	18	0.482	0.831	1148.177***	16	0.569	0.802	711.396***	16	0.537	0.823	1013.648***	15	0.398	0.846	720.193***
Lm4293	9	0.155	0.437	1791.297***	7	0.310	0.519	294.419***	9	0.191	0.415	1046.787***	14	0.194	0.602	1163.556***
Lm1750	5	0.573	0.649	53.724***	5	0.657	0.675	15.885 ns	4	0.564	0.633	14.284*	5	0.524	0.632	24.213**
Lm2407	6	0.258	0.309	66.480***	6	0.337	0.369	21.129 ns	6	0.354	0.406	35.106**	7	0.448	0.597	97.316***
Lm6026	11	0.412	0.791	1526.267***	11	0.444	0.758	824.227***	10	0.361	0.791	704.062***	8	0.341	0.754	432.905***
Lm0144	8	0.374	0.603	343.146***	8	0.425	0.646	689.482***	6	0.395	0.648	176.941***	9	0.320	0.706	370.609***
Lm2071	15	0.838	0.852	161.679***	15	0.771	0.823	110.342 ns	13	0.817	0.853	94.963 ns	13	0.830	0.830	77.941 ns

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; n = number of individuals sampled.

[a] Geographic coordinates for the populations are: Vallée de Mai = 4°19′43″S, 55°44′11″E; Fond Peper = 4°20′01″S, 55°44′17″E; Fond Ferdinand = 4°21′02″S, 55°45′39″E; and Curieuse = 4°16′45″S, 55°43′25″E.

[b] Sixteen individuals were tested from each population using the three monomorphic loci.

[c] Deviations from HWE using χ^2 tests: *$P \le 0.05$, **$P \le 0.01$, ***$P \le 0.001$; ns = not significant.

flower tissue (n = 1252) following the DNeasy 96 Plant Kit (QIAGEN, Hombrechtikon, Switzerland) manufacturer's protocol, except that grinding was carried out at four cycles of 30 s at 30 Hz, and the first incubation step was extended to 1 h at 65°C. Leaf tissue samples from *L. maldivica* individuals from each population are located at the Tissue Collection of the Royal Botanic Gardens, Kew, Richmond, Surrey, United Kingdom (Appendix 1).

Two methods were used for PCR reactions: two multiplex PCRs were used to amplify six primers, and the remainder of the primers were amplified in singleplex. Multiplex PCRs (MP1 and MP2) were carried out using primers labeled with either FAM, ATTO565, ATTO550, or Yakima Yellow (YY) (Microsynth, Balgach, Switzerland) (Table 1). PCR amplifications were carried out in 10.3-µL reactions containing 1× PCR Buffer (colorless Flexi GoTaq PCR buffer), 0.2 mM dNTPs, 3.1 mM $MgCl_2$, 0.05 U/µL *Taq* Polymerase (all Promega Corporation, Zürich, Switzerland), 0.18 µg/µL bovine serum albumin (BSA; BioConcept, Allschwil, Switzerland), 1.3 µL DNA, labeled forward primers, and unlabeled forward and reverse primers (for primer concentrations see Table 1).

Touchdown PCRs were carried out on a Bio-Rad Dyad Cycler (Bio-Rad Laboratories, Hercules, California, USA) with the following conditions: initial denaturation 95°C/4 min; 12× (denaturation 95°C/30 s, starting annealing temperature 62°C/30 s, decreasing by 0.5°C/cycle, extension 72°C/30 s); 29× (MP1)/28× (MP2) (denaturation 95°C/30 s, annealing 56°C/45 s, extension 72°C/30 s); and final extension 72°C/30 min and storage at 10°C. PCR product (2.5 µL) was added to 10 µL of HIDI formamide and 0.25 µL GeneScan 500 LIZ Size Standard (Applied Biosystems, Waltham, Massachusetts, USA).

The singleplex PCRs used forward primers labeled with M13 tails (5′-TGTAAAACGACGGCCAGT-3′) at the 5′ ends (as described by Schuelke, 2000) (Table 1). PCRs occurred in 11-µL reaction volumes containing 1× PCR Buffer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.025 U/µL *Taq* Polymerase, 0.18 µg/µL BSA, 1.0 µL DNA, forward primers with M13 tails, reverse primers and M13-primer universal tails labeled with either FAM, ATTO565, ATTO550, or YY (Microsynth) (for primer concentrations see Table 1). Cycling for singleplex PCRs was as follows: initial denaturation 95°C/5 min; 12× (denaturation 95°C/30 s, starting annealing temperature 62°C/30 s, decreasing by 0.5°C/cycle, extension 72°C/30 s); 25× (denaturation 95°C/30 s, annealing 56°C/45 s, extension 72°C/30 s); 8× (denaturation 95°C/30 s, annealing 53°C/45 s, extension 72°C/45 s); and final extension 72°C/30 min and storage at 10°C. PCR products were combined to create two pseudo-multiplex mixes (Table 1). For each PCR product (Lm4293, Lm2407, Lm6026, and Lm0144 were diluted 20× first), 1.2 µL were added to 10 µL of HIDI formamide and 0.15 µL of GeneScan 500 LIZ Size Standard (Applied Biosystems). Singleplex and multiplex products were denatured for 3 min at 92°C and run on an ABI 3730xl automatic capillary sequencer (Applied Biosystems). Electropherograms were scored with GeneMarker 2.6.0 (SoftGenetics, State College, Pennsylvania, USA).

The number of alleles, deviations from Hardy–Weinberg equilibrium (HWE), and observed and expected heterozygosity values were calculated (Table 2) using GenAlEx 6.5 (Peakall and Smouse, 2006). Linkage disequilibrium was tested in GENEPOP (Raymond and Rousset, 1995). The 12 polymorphic loci revealed between five and 21 alleles, with a total of 158 alleles across all *L. maldivica* individuals (Table 2). Significant deviation from HWE was seen in the majority of loci in all populations (Table 2). Expected heterozygosity values ranged from 0.399–0.896 (mean ± SE: 0.687 ± 0.048) for the polymorphic markers. No significant linkage disequilibrium was detected between loci pairs after sequential Bonferroni correction (α = 0.05) (Holm, 1979). The putative presence of null alleles in 11 loci (all except the monomorphic loci and Lm4716) was detected using MICRO-CHECKER 2.2.3 (van Oosterhout et al., 2004); however, these are unlikely to affect HWE at such low frequencies (Dakin and Avise, 2004). There was no evidence for large allele dropout.

CONCLUSIONS

We developed 12 highly polymorphic and three monomorphic loci for *L. maldivica*, with allele numbers ranging from five to 21 for the polymorphic loci. The pattern of homozygote excess can be observed across almost all loci in all populations. This can likely be explained by high inbreeding levels due to the very clustered growth patterns observed in the species. These markers will provide a useful tool in investigating the natural population structure, seed dispersal patterns, and fine-scale genetic structure of this highly charismatic and important endemic palm species (Morgan et al., in prep.).

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Appendix 1. Locations and DNA bank information for populations of *Lodoicea maldivica* used in this study.[a]

Population	Cohort	UTM coordinates[b]	Tissue collection no.
Vallée de Mai, Praslin	Adult male	359634.8mE, 9521289.06mN	6091
Vallée de Mai, Praslin	Adult male	359660.3mE, 9521279.96mN	6092
Fond Peper, Praslin	Adult male	359871.3mE, 9520653.71mN	6093
Fond Peper, Praslin	Juvenile	359634.8mE, 9520672.20mN	6094
Fond Ferdinand, Praslin	Adult female	361575.2mE, 9518670.34mN	6095
Fond Ferdinand, Praslin	Juvenile	361494.4mE, 9518728.30mN	6096
Curieuse Island	Juvenile	358386.5mE, 9526223.40mN	6097
Curieuse Island	Immature	358391.0mE, 9526213.75mN	6098

[a] Silica gel–dried leaf samples deposited at the Tissue Collection of the Royal Botanic Gardens, Kew, Richmond, Surrey, United Kingdom.
[b] Universal Transverse Mercator coordinates: WGS 84, UTM Zone 40S.