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* (Arecales) 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:** Arecales; coco de mer; *Lodoicea maldivica*; microsatellites; parentage analysis; Praslin.

*Lodoicea maldivica* (J. F. Gmel.) Pers. (Arecales; 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: TGGAGACTACAATGATGAAATGCC   | (CA)\(_{12}\) | 128–140                      | YY              | MP1             | 0.30      | KT897315              |
|           | R: AACGGAGTTATGATGTTG       |              |                              |                 |                 | 0.30      |                       |
| Lm2630    | F: AAATAGACGCAACCAGAAGTGC  | (GA)\(_{6}\) | 121–157                      | ATTO565         | MP1             | 0.30      | KT897316              |
|           | R: GCAGGTGTGCTACAAGGCC      |              |                              |                 |                 | 0.30      |                       |
| Lm8853    | F: CATAGGTGATGATGAAATGCC    | (ATGT)\(_{6}\) | 193–231                      | ATTO550         | MP1             | 0.20      | KT897317              |
|           | R: GGCTGAAATGATGACCTAG      |              |                              |                 |                 | 0.20      |                       |
| Lm5648    | F: CCAAGCTGATACACTGTGCTCC  | (TATC)\(_{12}\) | 235–285                      | FAM             | MP1             | 0.20      | KT897318              |
|           | R: AGCTGTTAGTTGAGGACCC      |              |                              |                 |                 | 0.20      |                       |
| Lm6782    | F: GGTCTAAAACATTTGGAGGAAATCAA | (TATG)\(_{12}\) | 252–334                      | ATTO565         | MP2             | 0.30      | KT897319              |
|           | R: AGACTCTTAATGGGAGGAC       |              |                              |                 |                 | 0.30      |                       |
| Lm1153    | F: TTGGGATACTAGGAGGAC       | (GA)\(_{13}\) | 120–166                      | FAM             | MP2             | 0.30      | KT897320              |
|           | R: AGATGCTTGTGACTTTGACTCTT  |              |                              |                 |                 | 0.30      |                       |
| Singleplex PCR\(^d\)                          |                                                          |                |                              |                 |                 |           |                       |
| Lm4293    | F: TCACCTTGAAGTAGTGTGAGG    | (GTAT)\(_{7}\) | 138–200                      | FAM             | 1               | 0.08      | KT897321              |
|           | R: AGTACTGAAGTGGACGGCC       |              |                              |                 |                 | 0.32      |                       |
| Lm1750    | F: TGCACTTAGATGTTGAGGGAGG   | (TACA)\(_{10}\) | 218–234                      | ATTO565         | 1               | 0.08      | KT897322              |
|           | R: ATGACATTGCTGAGGAGGGC      |              |                              |                 |                 | 0.32      |                       |
| Lm2407    | F: GGGGACCTACCTCAGGGCC       | (ACAT)\(_{10}\) | 84–112                       | FAM             | 1               | 0.06      | KT897323              |
|           | R: TCCTGACCCCTGAGCTAGAC      |              |                              |                 |                 | 0.24      |                       |
| Lm6026    | F: AGAGCCATTTCGCGCAACCC     | (TATG)\(_{4}\) | 147–225                      | YY              | 1               | 0.06      | KT897324              |
|           | R: ATGACATCTATGAGGCCTTCC     |              |                              |                 |                 | 0.24      |                       |
| Lm0144    | F: ACATCTCATGTAGGAGGACTTCC  | (TAGA)\(_{8}\) | 244–280                      | ATTO550         | 1               | 0.06      | KT897325              |
|           | R: CATGTTCCTCAGGTAAACCC     |              |                              |                 |                 | 0.24      |                       |
| Lm2071    | F: CCTACTCAGCGCCATTTTCCC    | (GA)\(_{3}\) | 104–138                      | FAM             | 2               | 0.08      | KT897326              |
|           | R: TACGACATCTACCTCTTCC      |              |                              |                 |                 | 0.32      |                       |
| Lm7170    | F: ACAGATTGAGGAAATCCTCAC    | (ATAC)\(_{6}\) | 216\(^e\)                    | FAM             | 2               | 0.08      | KT962232              |
|           | R: ATGAGGGCGCTGTGCAGAAAT    |              |                              |                 |                 | 0.32      |                       |
| Lm1012    | F: GTGATGAGTGTGCCTAACTTG    | (TACA)\(_{7}\) | 251\(^e\)                    | ATTO565         | 2               | 0.08      | KT962233              |
|           | R: CCTGCTTATCATGAAAGTGCG    |              |                              |                 |                 | 0.32      |                       |
| Lm5950    | F: ACCGAAATGACAAAACTACAC    | (TATC)\(_{7}\) | 180\(^e\)                    | ATTO565         | 2               | 0.08      | KT962234              |
|           | R: CGTTAGAAAATGAGAAAAACG    |              |                              |                 |                 | 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′-TGTAATGACGCGGCAAGT-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′-TGTAATGACGCGGCAAGT-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.
| Locus  | Vallée de Mai (n = 482) | Fond Peper (n = 293) | Fond Ferdinand (n = 265) | Curieuse (n = 212) |
|--------|------------------------|---------------------|-------------------------|-------------------|
|        | A          | H<sub>e</sub> | H<sub>o</sub> | HWE<sup>+</sup> | A          | H<sub>e</sub> | H<sub>o</sub> | HWE<sup>+</sup> | A          | H<sub>e</sub> | H<sub>o</sub> | HWE<sup>+</sup> |
| 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 | 1824.678*** | 17           | 0.543          | 0.909 | 1103.038*** | 18           | 0.598          | 0.895 | 807.148*** | 16          | 0.612          | 0.880 | 384.399*** |
| Lm8553  | 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           | 0.598          | 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*** | 15           | 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 | 224.227*** | 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 | 370.669*** |
| Lm2071  | 15          | 0.838          | 0.852 | 161.679*** | 15           | 0.771          | 0.823 | 110.342 ns | 13           | 0.817          | 0.853 | 94.965 ns | 13          | 0.830          | 0.830 | 77.941 ns |

**Note:** A = number of alleles; H<sub>e</sub> = expected heterozygosity; H<sub>o</sub> = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; n = number of individuals sampled.

- 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′46″S, 55°43′25″E.
- Sixteen individuals were tested from each population using the three monomorphic loci.
- Deviations from HWE using χ² tests: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001; ns = not significant.

**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.).

**LITERATURE CITED**

Bailey, L. H. 1942. Palms of the Seychelles. *Gentes Herbarum* 6: 1–48.

Dakin, E. E., and J. C. Avise. 2004. Microsatellite null alleles in parentage analysis. *Heredity* 93: 504–509.

Edwards, P. J., J. Kollmann, and K. Fließmann. 2002. Life history evolution in *Lodoicea maldivica* (Arecales). *Nordic Journal of Botany* 22: 227–238.

Edwards, P. J., F. Fleischer-Dogley, and C. N. Kaiser-Bunbury. 2015. The nutrient economy of *Lodoicea maldivica*, a monodominant palm producing the world’s largest seed. *New Phytologist* 206: 990–999.

Faure, A. A. 1909. Unpublished documents on the history of the Seychelles Islands anterior to 1810: together with a cartography enumerating 94 ancient maps and plans dating from 1501, and a bibliography of books and mss. concerning these islands. Government Printing Office, Mahé, Seychelles.

Fleischer-Dogley, F. 2006. Towards sustainable management of *Lodoicea maldivica* (Gmelin) Persson. Ph.D. thesis, University of Reading, Reading, United Kingdom.
Appendix 1. Locations and DNA bank information for populations of Lodoicea maldivica used in this study.a

| Population            | Cohort    | UTM coordinatesb | 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                  |

aSilica gel–dried leaf samples deposited at the Tissue Collection of the Royal Botanic Gardens, Kew, Richmond, Surrey, United Kingdom.
bUniversal Transverse Mercator coordinates: WGS 84, UTM Zone 40S.