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TECHNICAL NOTE

Isolation of 13 novel highly polymorphic microsatellite loci for the Amazonian Palm *Mauritia flexuosa* L.f. (Arecaceae)

Sarah Federman · Chaz Hyseni · Wendy Clement · Adalgisa Caccone

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Abstract *Mauritia flexuosa* L.f. (Arecaceae) is a New World tropical palm that generally grows in isolated swamps along meandering rivers and is in danger of fragmentation through unsustainable harvest practices. To explore gene flow among populations of *M. flexuosa* in Amazonia, we developed 13 novel, polymorphic microsatellite loci for *M. flexuosa*. Further studies will employ these loci to investigate the impacts of artisanal gold mining and wild-harvest on gene flow among populations of *M. flexuosa*.

Keywords *Mauritia flexuosa* · Amazonia · Microsatellite · Arecaceae

Mauritia flexuosa L.f. (Arecaceae) is a dioecious, diploid palm (Röser et al. 1997) covering millions of hectares in Amazonia in monodominant stands (Peters et al. 1989). *M. flexuosa* populations are naturally isolated from one another as they generally dominate swamps along meandering rivers that originated as ox-bow lakes (Kalliola et al. 1991). The fruit of *M. flexuosa* has a high wild harvest potential, but harvesters often fell females for easier access to the fruit (Holm et al. 2008). This harvesting strategy, combined with artisanal gold mining that often occurs along meandering rivers in Amazonia in both Peru and Brazil, could restrict gene flow in naturally fragmented populations. To investigate questions regarding habitat fragmentation, impacts of wild-harvest, and natural isolation, we developed 13 novel polymorphic dinucleotide microsatellite loci for *M. flexuosa*.

Microsatellite library development followed a modified version of Glenn and Schable (2005). DNA was extracted from the leaf tissue of one *M. flexuosa* individual from Pará, Brazil, donated by the Montgomery Botanical Center. Dry tissue was homogenized in a Qiagen Tissue Lyser (Qiagen, Inc.) and DNA extracted following the Qiagen DNeasy Plant Kit (Qiagen, Inc.) protocol with modified Buffer AP1 to which we added 40 µL of 10 mg/mL PVPP (polyvinylpolypyrrolidone). DNA was digested with the restriction enzyme *Sfo I* (New England Biolabs) and ligated to double-stranded Super-SNX linkers. Following ligation, the restricted DNA was denatured, hybridized to biotin-labeled oligonucleotides [(GA)12, (CA)12] and captured on magnetic streptavidin coated beads (Invitrogen). Retrieved microsatellite-enriched DNA was amplified via PCR and sent for commercial Rolling Circle Amplification and sequencing to Sequetech (Mountain View, CA). Sequences were searched for the presence of microsatellites using MSATCOMMANDER 0.8.1 (Faircloth 2008) and primers were designed using Primer3 (Rozen and Skaletsky 2000).

We tested 36 primer-pairs on ten individuals of *M. flexuosa* from Estrada do Amapá, Acre, Brazil, collected by collaborators at the University of Acre. Loci were amplified via PCR on an Eppendorf Mastercycler (Eppendorf, Westbury, NY). The M13-tailed primer method (Boutin-Ganache et al. 2001) was used for genotyping individuals. Reactions involved forward primers 5′-tailed with a 15-mer M13 sequence (5′-TCCCAGTCACGACGT-3′), unmodified reverse primers and fluorescently labeled (6-FAM, VIC, NED) 15-mer M13 primers (Applied Biosystems).

S. Federman (✉)
School of Forestry & Environmental Studies,
Yale University, New Haven, CT 06511, USA
e-mail: sarah.federman@yale.edu

C. Hyseni · W. Clement · A. Caccone
Department of Ecology and Evolutionary Biology,
Yale University, New Haven, CT 06511, USA

Table 1 13 polymorphic microsatellite loci for *M. flexuosa*

Locus	Primer sequence (5′–3′)	Genbank acc. no.	T_A (°C)[a]	Repeat motif	Size range (bp)	n[b]	NA[c]	H_E[d]	H_O[e]	P_{HWE}[f]	$N_{0\,(M)}$[g]	$N_{0\,(F)}$[h]
Mf04	F: CCACGGGGCTGTCATATTTC R: AGGAAAACGGCAAGAAGTGC	JN803910	54	(AC)11(AG)23	224–260	24	15	0.918	0.958	0.449	−0.037	0.000
Mf13	F: TTACAAGCGACCCCTCGTC R: CGTCGAATAGGGTTTCAGTGG	JN803911	57	(CT)14	230–264	25	10	0.780	0.800	0.772	−0.026	0.000
Mf14	F: TAGGTCCTGCTTCTGTGCC R: TGGATCCGGTCCGTTGATAG	JN803912	54	(TC)22	233–275	25	11	0.871	0.920	0.714	−0.046	0.000
Mf17	F: GACAGCTTGTCATCCTCGC R: TTCCATCCCAGTTCTCCCC	JN803913	54	(GA)18	210–232	24	10	0.819	0.833	0.929	−0.016	0.000
Mf19	F: AGCCACGTGACACTCTACC R: CTATAGGACCGGCCACCTG	JN803914	57	(CT)10	239–261	25	7	0.813	0.800	0.128	−0.008	0.003
Mf22	F: GCATGGTGTAGCTCGTATCTG R: CGCACCATACTTGGCTTGC	JN803915	57	(CT)15(GT)17	226–276	25	14	0.884	0.840	0.328	0.014	0.007
Mf25	F: CCCCATTTTCCAATTTGATGCG R: TGGATGTTCAGTTTGGATGCC	JN803917	54	(CT)17	199–225	25	12	0.878	0.840	0.442	0.015	0.000
Mf34	F: GGACAGTTGCCTGTCTTGC R: CAAAGCTAGCACAACCTGGG	JN803922	57	(TC)14	180–220	25	14	0.872	0.840	0.389	0.000	0.009
Mf24	F: TCACATTAGTAGTCAAGGGTAGC R: GGGTGTTAAGCATTCGGGC	JN803916	54	(TC)20	189–215	25	12	0.808	0.600	0.012*	0.130**	0.084**
Mf28	F: TCCCACACTCTCTTGCCAC R: TGAGGGCTGCGTTATGGTC	JN803918	57	(GA)9(GG)(GA)11	184–200	25	8	0.777	0.560	0.077	0.134**	0.109**
Mf30	F: GAGGGGAGCTTCCTTGCTG R: ATTGGCGAAGGTCCAGGG	JN803919	57	(CT)14	231–245	25	6	0.724	0.480	0.015*	0.161**	0.125**
Mf31	F: GCGCTAGAAGCATGATCACC R: TCTCAGCCATCATATTCAGTTATCTTC	JN803920	54	(CT)18(CA)6	225–259	25	11	0.833	0.640	0.007*	0.111**	0.091**
Mf33	F: TGCCGCATTTAGGCTTTGG R: GGCCGGCGATTTATAACGG	JN803921	57	(CT)10	215–229	24	6	0.582	0.500	0.044*	0.024	0.095**

[a] Annealing temperature, [b] sample size, [c] number of alleles, [d,e] observed and expected heterozygosity, [f] Hardy–Weinberg equilibrium *P* value, [g,h] frequency of null alleles estimated using: MICROCHECKER ($N_{0(M)}$) & FREENA ($N_{0(F)}$)

* Deviation from HWE prior to sequential Bonferonni correction ($P > 0.004$); ** null allele frequency greater than 0.05

Reactions were performed in 10 μL volumes using 1× Qiagen Type-It master mix (Qiagen, Inc.), 0.05 μM Forward Primer, 0.5 μM Reverse Primer, 0.5 μM M13 primer and ~20 ng template DNA. The thermal cycling conditions used for PCR amplification included an initial denaturation step at 94°C for 8 m followed by 35 cycles of 30 s at 94°C, 30 s at 54 or 57°C (Table 1), 30 s at 72°C, and a final extension step at 72°C for 5 m. PCR products were run through an Applied Biosystems 3730xl DNA Analyzer at the DNA Analysis Facility on Science Hill at Yale University (http://www.dna-analysis.research.yale.edu) and the data were analyzed in GENEMARKER v1.91 (SoftGenetics, State College, PA).

Observed and expected heterozygosity were calculated using GENALEX 6.41 (Peakall and Smouse 2006). We used GENEPOP 4.0.10 (Rousset 2008) to test for departures from Hardy–Weinberg equilibrium (HWE) and to determine possible linkage disequilibrium (LD) between loci using a burn-in of 100,000 and 1,000 batches with 10,000 iterations per batch. We performed 10,000 permutations to estimate null allele frequencies via two different methods: Dempster et al. (1977), as implemented in FREENA (Chapuis and Estoup 2007), and Oosterhout et al. (2006), as implemented in MICROCHECKER (Oosterhout et al. 2004).

Table 1 shows the results of the 13 loci that were polymorphic and easily scored out of the original 36 tested. Those 13 loci were tested on 25 individuals from a single population in Estrada do Amapá, Acre, Brazil. Loci ranged in size from 180 to 291 bp. Number of alleles ranged from 6 to 15 and expected heterozygosity varied from 0.582 to 0.918. All loci were in HWE ($P > 0.004$) after sequential Bonferroni correction (Holm 1979). Additionally, no loci were in LD after sequential Bonferroni correction. Nine loci were in HWE ($P > 0.05$) before Bonferroni correction, eight of which had null allele frequencies below 5%. The higher null allele frequencies and possible deviation from HWE in these loci are likely to be fixed by lowering the annealing temperature and should still be useful for future genetic studies.

These polymorphic loci provide an opportunity for the study of gene flow in a largely unstudied system with high potential for conservation initiatives and sustainable wild harvest management programs (Peters et al. 1989; Holm et al. 2008). In future studies, we will apply these microsatellite loci to multiple populations of *M. flexuosa* in Brazil that encompass a variety of land-use types to inform conservation and management initiatives.

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