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PRIMER NOTE

Twelve microsatellite loci in *Manilkara huberi* (Ducke) Standl (Sapotaceae), an Amazonian timber species

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Abstract

***Manilkara huberi* is a timber species, found and intensely exploited in the Amazonian forest. Twelve highly polymorphic microsatellite loci were developed from a genomic library enriched for AG/TC repeats. Levels of polymorphism were evaluated using a total of 12 adult trees from a natural population. An average of 6.43 alleles per locus were detected, and expected heterozygosity ranged from 0.721 to 0.862. These loci represent a powerful tool in investigating the mating system, gene flow, parentage and population dynamics in natural populations of *M. huberi*, all of which are needed to implement sound management.**

Keywords: conservation, genetic diversity, Manilkara huberi, microsatellite, Sapotaceae

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Simple Sequence Repeats (SSR) or microsatellites exhibit high levels of variability because of differences in the number of repeated units. The high allelic diversity and abundance of microsatellites in the eukaryotic genome make these co-dominant molecular markers popular for detailed genetic studies. The Dendrogene Project (*Genetic Conservation within Managed Forests in Amazônia*) is interested in the conservation and management strategies of timber trees from the Amazonian forest (Kanashiro *et al*. 2001), such as *Manilkara huberi* (Ducke) Standl (Sapotaceae), a species intensively harvested by the timber industry. As part of this project, we report the development of 12 microsatellite markers; the first on microsatellite cloning for *M. huberi*.

Total genomic DNA was extracted (Ferreira & Grattapaglia 1998) from expanded leaves of a single individual of *M. huberi*, and digested with *Tsp*509 I. Fragments were separated on 2% agarose gel, and those from 300 to 800 bp were used to construct an enriched genomic library, as described by Rafalski *et al*. (1996). These fragments were ligated into a pGEM-T Easy vector (Promega, Madison, WI) and transformed in *Escherichia coli* XL1-Blue, which were grown overnight on 1× LB agar containing ampicilin, Xgal and IPTG (Sambrook *et al*. 1989). Positive clones were selected by hybridization with a poly AG/TC probe and sequenced on an ABI 377 Applied Biosystem (Perkin Elmer, CA) automatic sequencer. Primers to the flanking regions were designed using the PRIMER 3 Output software (Rozen & Skaletsky 2000). Microsatellite loci were amplified using polymerase chain reaction (PCR) in 13 μL containing: DNA (3 ηg), PCR reaction buffer (1×), forward primer (0.28 μM), and reverse primer (0.28 μM), $MgCl_2$ (1.5 mM), BSA (0.25 mg/mL), dNTP (0.25 mM) and *Taq* polymerase (1.3 U). PCR conditions were: denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at T_a (Table 1) for 1 min, and extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min. Reaction products were separated on polyacrylamide gel and visualized by silver staining. Polymorphism was evaluated using a total of 12 adult trees from a natural population from the National Forest, Tapajós, Pará, Brazil. Alleles were sized relative to a 10 bp ladder. Number of alleles per locus, mean observed and expected heterosygosities were calculated using GENETIC DATA ANALYSIS version 1.0 (GDA) (Lewis & Zaykin 2001). Probabilities of paternity exclusion (Slate *et al*. 2000) were estimated using CERVUS (Marshal, 1998–2001) (Table 1).

Twenty nine clones contained both microsatellite and appropriate flanking regions for primer design. We successfully amplified 23 primer pairs. Of those, nine were monomorphic, and 14 were variable. Levels of variability detected in 12 loci were high, with the number of alleles ranging from four to eight, selected to be used for genetic analysis. Ten of the 12 loci had observed heterozygosity

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Table 1 Information on the 12 microsatellite marker loci of *Manilkara huberi*. Allele size range (bp), annealing temperature (T_a °C), number of individuals (N), total number of alleles per locus (A), expected heterozygosity (H_E), observed heterozygosity (H_O), paternity exclusion probabilities ($Pr(Ex_1)$ and $Pr(Ex_2)$) in 12 individuals

Locus	Repeat array	Primer sequence (5′–3′)	Allele size range bp	T_a °C	A	H_E	H_O	$Pr(Ex_1)$	$Pr(Ex_2)$	Accession no.
Mh 03	$(CT)_{17}$	F: CACTTCTGTCTCTCTCCTCGT R: GGGTGATTGCAGAGACGTA	176–204	56	7	0.859	1.000	0.477	0.650	AY514005
Mh 04	$(CT)_{12}$	F: GCACTCTCCATGGTTCCAGT R: AAAGAGTCAATGGCGTGAGC	189–209	52	5	0.768	0.583	0.319	0.492	AY514006
Mh 06	$(GA)_{14}$	F: ACACGCACAAAACAAACCAA R: TTCTTGAAGGAGGGTTGCTC	162–188	56	7	0.754	0.417	0.325	0.502	AY514007
Mh 07	$(CT)_{23}$	F: ATTGCAGCATATCCACACCA R: GCAAAGGGTGATGGGTTAGA	153–187	56	4	0.746	0.750	0.300	0.473	AY514008
Mh 08	$(CT)_{11}$	F: GTAATGGGAGCCGTTTGAGA R: CTGGGTAGCATTTGTTGCAT	172–202	56	7	0.862	0.917	0.485	0.659	AY514009
Mh 12	$(CT)_9(AC)_6$	F: TGCGGAACTGTGGAAAGAGT R: ATCCACAGCAATGACTGACG	187–211	56	7	0.859	0.667	0.481	0.655	AY514010
Mh 17	$(CT)_{13}$	F: CACGATGACCTCTCAGTGGA R: CCTGTGTATGCGTTCGATTG	240–274	56	6	0.721	0.750	0.295	0.480	AY514011
Mh 19	$(CT)_{21}$	F: AATTACAACCAAAGCTCCACTT R: TGAGAGTCTTTTCGCACTTTCA	146–164	56	7	0.862	0.750	0.500	0.672	AY514012
Mh 20	$(GA)_{13}$	F: GAAGTTTTGACCATTTGGGAAT R: GACATAACACTAACCCTTCACGA	134–166	56	8	0.848	0.833	0.458	0.632	AY514013
Mh 22	$(CT)_{15}$	F: CCCATTATAGCCCTCCACCT R: AGAGAGCACATGCAAGCTCA	180–206	56	7	0.841	0.833	0.449	0.627	AY514014
Mh 24	$(CT)_{17}$	F: CCACTTCTGTCTCTCTCCTCGT R: GACATTGTGGGTGATTGCAG	181–209	60	7	0.815	0.167	0.404	0.582	AY514015
Mh 26	$(CT)_{14}$	F: TGCTCAGACTGCTTTCTTTTTG R: TGCAATAAGTGTGATTTGGAGAA	224–250	58	6	0.819	0.583	0.400	0.579	AY514016
Mean					6.43	0.813	0.688	0.408	0.514	

greater than 50%. Two loci (Mh06 and Mh24) showed departure from Hardy–Weinberg expectations ($P < 0.005$), but no pairwise disequilibrium was detected between the 12 loci. The deficits of heterozygous genotypes are consistent with the presence of null alleles in these two loci, at a frequency of 0.298 for Mh06 and 0.652 for Mh24. These two loci with high null allele frequencies should be used only with great caution for paternity exclusion analysis because of the possibility of excluding the true father or nonexcluding a wrong father. The first estimate of paternity exclusion probability $Pr(Ex_1)$, when the offspring is sampled but the mother is not, varied from 0.295 to 0.5 (mean = 0.408) for individual loci and 0.9983 for the combined loci. The second estimate, $Pr(Ex_2)$, when both the mother and the offspring are sampled, varied for individual loci from 0.473 to 0.672 (mean = 0.514) and was 0.99998 for the combined loci (Table 1).

This study shows that these SSR loci allow very precise individual discrimination and paternity testing. The 12 microsatellites developed exhibited a large number of alleles per locus and high heterozygosity. This suggests that these loci will be useful for further population genetic studies of natural populations. As part of the Dendrogene Project, we are currently using these markers to investigate questions of genetic diversity, paternity, spatial genetic structure, and mating system in a natural population of *M. huberi* with the aim of applying scientific knowledge to promote sustainable management in the Brazilian Amazon forest.

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References

Ferreira ME, Grattapaglia D (1998) Introdução ao uso de marcadores moleculares em análise genética. 2nd ed Embrapa Cenargen, Brasília, DF. p. 220.

Kanashiro M, Thompson IS, Yared JAG *et al*. (2001) Improving conservation values of managed forests: the Dendrogene Project in the Brazilian Amazon. *Unasylva* 209.

Lewis PO, Zaykin D (2001) *GENETIC DATA ANALYSIS: Computer: Program for the Analysis of Allelic Data. Version 1.0 (d16c).* http://lewis.eeb.uconn.edu/lewishome/software.html.

Marshal T (1998–2001) CERVUS Version 2.0, University of Edinburgh.

Rafalski JA, Morgante M, Powell W, Vogel JM, Tingey SV (1996) Generating and using DNA markers in plants. In: *Analysis of non Mammalian Genomes — a Practical Guide* (eds Birren B, Lai E), pp. 75–134. Academic Press, New York.

Rozen S, Skaletsky HJ (2000) *PRIMER3* on the frodo.wi.mit.edu/cgi-bin/primer3/primer3_http://www.cgi for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds Krawetz S, Misener S), pp. 365–386. Humana Press, Totowa, NJ.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: Laboratory Manual*, 2nd edn. CSHL, Cold Spring Harbor, NY.

Slate J, Marshall T, Pemberton J (2000) A retrospective assessment of the accuracy of the paternity inference program CERVUS. *Molecular Ecology*, **9**, 801–808.