PERMANENT GENETIC RESOURCES

Isolation and characterization of nine microsatellite loci for the tropical understory tree *Miconia affinis* Wurdack (Melastomataceae)

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Abstract

We isolated nine microsatellite loci from the Neotropical understorey tree *Miconia affinis* (Melastomataceae) and optimized them for research on gene flow and genetic structure. Loci screened in 43 individuals from a 2.26-km² region were shown to be unlinked and polymorphic, with 5 to 14 alleles per locus and observed heterozygosity ranging from 0.394 to 0.810.

The Neotropical understorey tree *Miconia affinis* (Melastomataceae) is broadly distributed in the Neotropics, ranging from southern Mexico to southern Venezuela. *M. affinis* is known in Mexico and Central America as *Cinco Negritos* (five black berries) because of its conspicuous berries, which are borne on magenta stalks during the summer rainy season. Seeds of *M. affinis* are broadly dispersed by Neotropical birds and bats. Although *M. affinis* is most often found in remnant forest, widespread seed dispersal facilitates colonization of *M. affinis* into adjacent agricultural lands. Like many other melastomes, *M. affinis* has deep poricidal anthers which must be vibrated by a pollinator in order for pollen to be released. 'Buzz-pollination' is effected by a select group of bee pollinators (Buchmann 1983). Therefore, the study of *M. affinis* provides an opportunity to evaluate effects of habitat changes on gene flow and reproductive success of a reproductively specialized understorey tree, as well as to study the pattern of seed dispersal between forest remnants and adjacent agricultural habitat. The purpose of the present research was to develop microsatellite markers to examine the breeding structure of *M. affinis* in remnant forest patches and shade coffee farms in Chiapas, Mexico.

DNA was extracted from a single plant using the DNeasy Plant kit (QIAGEN). A library of microsatellite repeats was constructed using a combination of procedures described in Weising *et al.* (2005) and Glen & Schable (2005). First, the

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genomic DNA (6 µg) was digested with restriction enzyme *Rsa*I and ligated with forward and reverse SuperSNX24 adaptors (SuperSNX24 forward 5′-GTTTAAGGCCTAGCT-AGCAGCAGAATC and SuperSNX24 reverse 5′-GATTCT-GCTAGCTAGGCCTTAAACAAAA). The restriction-ligation product was purified (Geneclean kit, QBiogene), and hybridized with biotinylated di- and trinucleotide-specific oligonucleotides [oligo mix 2 = $(AG)_{12}$, $(TG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(ATC)_8$] as described in Glen & Schable 2005). Hybridized fragments were captured twice with streptavidin-coated magnetic beads (Dynal), filtered using a Microcon YM-30 spin filter (Millipore), and then amplified with adaptor-specific primers. The PCR products were ligated into a TOPO TA Cloning Kit (Invitrogen) and selected colonies were amplified and sequenced (ABI Model 3730 Sequencer). Twelve of the 50 clones sequenced (24%) contained microsatellites. Primers were designed for the flanking sequences using Primer 3 (Rozen & Skaletsky 2000).

We initially screened all 12 loci in 43 individuals sampled across a 2.26-km² shade coffee farm in Chiapas, Mexico, which has been colonized by *M. affinis* since the establishment of the farm approximately 100 years ago. Polymerase chain reaction (PCR) was performed in a final volume of 20 µL, containing approximately 2 ng of DNA, 2 µL of 10× PCR buffer, 1.5 mM $MgCl_2$, 300 µM of each dNTP, 1 U of HotStart *Taq* Polymerase (QIAGEN) and 0.25 µM of each primer. The thermal cycle began with a 15-min denaturation step at 95 °C, and was followed by 45 cycles: 30 s at 94 °C, 60 s at the locus-specific annealing temperature (Table 1),

Table 1 Forward (F) and reverse (R) sequences with fluorescent dye label (used for genotyping), repeat motifs, annealing temperatures in °C (T_a), number of alleles (N_A), allelic size range in bp (Size range), observed heterozygosity (H_O), expected heterozygosity (H_E), and GenBank Accession number for nine microsatellite loci for *Miconia affinis*

Locus	Primer sequence (5'–3')	Repeat motif	T_a (°C)	N_A	Size range	H_O	H_E	Accession no.
Micaff-5	R: ROX.GGAGGAGAACTCAACACAACCTCAA F: CCACCATGAAAAAGGAGAGACAATG	(TG)$_{11}$	57.0	8	258–276	0.394*	0.618	EU675265
Micaff-7	R: GACAGTCACGGAGCAGTGGAAT F: ROX.TCGATTAATGAACTTTTGAAATCAGG	(CT)$_{11}$	53.3	10	248–298	0.541*	0.804	EU675266
Micaff-8	R: CGTTACAACTGTGTCCTGCTTGTTG F: HEX.AGGACAATGAATTAGCGTTGAACCA	(CT)$_{12}$	56.9	10	278–300	0.800	0.774	EU675267
Micaff-9	R: 6-FAM.CGTTCGTCGTCTTCTTTCATCTTCT F: GGCGAGTCAATTCCCATTTCAAAAA	(AG)$_{19}$	59.8	7	266–290	0.810	0.783	EU675268
Micaff-14	R: 6-FAM.CCCCTTTTCCTTTATCCTGTCTGT F: GAACATGTTATTGGATGGGCTACC	(TG)$_{11}$	54.7	5	214–222	0.400*	0.677	EU675269
Micaff-16	R: HEX.TGGGAAATGGGAAGAAACAAGTAAG F: GCAAAGGTGAGAGCTGTTGAGATTT	(AG)$_{10}$(TG)$_{10}$	56.2	8	242–270	0.682	0.703	EU675270
Micaff-17	R: CGTCCGTCAAGCAACCTATAAAA F: 6-FAM.ATCACGTCCCAATTCCACGTC	(CT)$_9$	56.3	14	272–300	0.775	0.834	EU675271
Micaff-18	R: 6-FAM.ACCATCCGAACAACACAACACAAA F: GCGTCCGTCAAGCAACCTATAAAA	(AG)$_{11}$	57.4	9	206–226	0.464*	0.813	EU675272
Micaff-19	R: HEX.CGTTCTTGACTTTTTCCCTCGACTC F: GAACGTAGGAACGGGAGTTGGAAT	(AG)$_6$G(GA)$_9$	57.9	9	260–276	0.480	0.814	EU675273

*deviation from Hardy–Weinberg equilibrium at $P = 0.001$.

and 30 s at 72 °C, followed by a final extension at 72 °C for 20 min. Nine of the 12 primer pairs generated consistent and scorable amplification products of the expected size across all individuals tested. For these nine primer pairs, one primer from each primer pair was end-labelled with a fluorescent dye, either FAM, HEX or ROX, and genotyped on an ABI 3730 Sequencer. The genotypes were analysed using the ABI Peak Scanner Software version 1.0.

Deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were tested in GenePop version 4.0 (Raymond & Rousset 1995) using the Markov chain method (Guo & Thompson 1992) with 1000 dememorizations, 100 batches and 1000 iterations per batch. A Bonferroni correction for multiple comparisons was applied in both cases to a significance level of $P < 0.05$. The probability of null alleles was calculated using the software Micro-Checker (Van Oosterhout *et al.* 2004).

All of the nine loci were polymorphic (Table 1). Four loci exhibited significant deviations from HWE. This may be due to biparental inbreeding and/or null alleles, although only a single locus, Micaff-19, was indicated as having null alleles in the Micro-Checker calculations. No significant LD was detected between any of the loci. The cumulative exclusion probability estimated from sampled adult individuals reached 0.995 for the first parent and 0.9999 for the second parent. This high level of polymorphism makes these powerful markers for direct and indirect estimates of gene flow and population structure. These are among the first microsatellite DNA markers for the Melastomataceae, which contain more than 3000 tropical woody plant species.

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