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PRIMER NOTE

Characterization and isolation of DNA microsatellite primers for the tropical tree, *Symphonia globulifera* Linn. f.

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Abstract

Five highly polimorphic microsatellite markers for *Symphonia globulifera* were developed from enriched repeat libraries screened for microsatellite repeats. An average of 5.8 alleles per locus and expected heterozygosity ranging from 0.649 to 0.806 were found in a sample of 12 *S. globulifera* individuals from the Tapajos National Forest, in the state of Pará in the Brazilian Amazon. These loci are being used for analysis of precise paternity testing, estimation of gene flow and parentage coefficients in a managed forest in Pará, Brazil, as part of the Dendrogene Project.

Keywords: Amazon, microsatellites, *Symphonia globulifera*, tropical tree

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Symphonia globulifera Linn.f. (Clusiaceae), known in Brazil as anani, is a widespread species found in southern Mexico, most of Central America, parts of the West Indies, Amazonian and Guyanan South America, and tropical West Africa. This species is of important economic value for timber extraction and for the medicinal properties of the latex used by the local population of the Amazon rainforest (Ribeiro *et al*. 1999).

We are interested in conservation and management strategies of timber trees such as *Symphonia globulifera* from the Amazon rainforest. The Dendrogene proposal is to attempt to predict the effects of forestry practice (selective logging) on the genetic and ecological processes which determine the sustainability of the harvest of the selected species (Kanashiro *et al*. 2002). As part of the Dendrogene Project, we developed a battery of microsatellite markers to allow precise estimation of parentage coefficients among trees in the wild for mating system and gene flow studies.

Protocols of construction of microsatellites markers-enriched genomic libraries described by Rafalski *et al*. (1996) were used. DNA was extracted from expanded leaves of a single individual of *S. globulifera.* The DNA was digested with three different restriction enzymes *Sau*3AI, *Mse*I or *Tsp*509 (Biolabs, New England) in order to select one that would produce a larger amount of fractionated DNA in the range of 300 to 800 pb. Approximately 50 μg of DNA was digested with *Tsp*509I and fragments were separated on a 2% agarose gel, and those from 300 to 800 bp were used to construct an enriched genomic library. The fragments were ligated to adapters *Tsp*509I restriction site. Fragments containing microsatellite sequences were selected by hybridization with biotinylated oligonucleotides complementary to the repetitive poly AG/TC and recovered by magnetic beads linked to streptavidine. Subsequently fragments were ligated into a pGEM-T Easy vector (Promega), transformed by electroporation in *Escherichia coli* strain XL-Blue according to Sambrook *et al*. (1989). Transformants were streaked onto agar plates (120 clones per plate) with duplicate plates on nylon membranes (Hbond N, Amersham-Pharmacia) and were regrown overnight at 37 °C. Positive recombinant clones were identified by hybridization with an end-labelled $(AG/TC)_{13}$ (detection of DIG-labelled nucleic acids with CSPD, Boehringer Mannheim) making a total of 225 positive clones. Transformants were diluted in water and the fragment inserted into the plasmid was amplified using the M13 Universal Primers by polymerase chain reaction (PCR). A total of 186 inserts were sequenced on an Applied Biosystem 377 (Perkin-Elmer) instrument using dye terminator fluorescent chemistry with the M13 forward (–20) primer, according to Amersham's protocol. The positive clones sequenced that contained repetitive sequences GA and CT, which varied in size from 9 to 25,

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Table 1 Information of the five microsatellite marker loci of *Symphonia globulifera*. Annealing temperature (T_a), number of individuals (N), total number of alleles (A), observed heterozygosity (H_O), expected heterozygosity (H_E), and first parent and second parent total exclusionary power [Pr(Ex_1) and Pr(Ex_2)]

Locus	Array	Primer sequences (5′–3′)	Allele size range bp	T_a (°C)	N	A	H_O	H_E	Pr (Ex_1)	Pr (Ex_2)	Accession no.
Sg 01	$(CT)_{13}$	F 5′CTTCCCCTTCCTCATggATT-3′ R 5′TATTggCCAAggTgAAgAgg-3′	240–304	60	12	5	0.677	1.000	0.238	0.402	AY819036
Sg 06	$(CT)_{13}$ $(CTTT)_8$	F 5′ACgACATTgggAggTTTCAG-3′ R 5′CTgAAACTTAAggTgCCAAgAA-3′	211–281	58	12	7	0.822	0.417	0.409	0.587	AY819037
Sg 08	$(CT)_{18}$	F 5′gTTCCAAAgCCCAAgATgAA-3′ R 5′ACTgATTTCCAgCCCCTCTC-3′	171–231	56	10	4	0.721	1.000	0.260	0.424	AY819038
Sg 09	$(CT)_{20}$	F 5′CCCTTTCCATTggTTCACAG-3′ R 5′gAggAgggTAgCTgTTgTgg-3′	130–170	60	12	5	0.710	1.000	0.276	0.456	AY819039
Sg 10	$(CT)_{13}$ $(CTAT)_{14}$	F 5′ACCACAAgTTCACCCTCACC-3′ R 5′gCTACgAAACCAAAgAACTAACAA-3′	149–205	58	12	8	0.840	0.667	0.454	0.630	AY819040
Mean						5.8	0.754	0.816	0.868	0.97	

and the position of the repetitive simple sequence was appropriate for designing the flanking sequences as primers were selected for designing primers pairs using the software PRIMER 3 OUTPUT (Rozen & Skaletsky 2000). Twenty primers were designed and synthesized. Microsatellite loci were amplified using PCR in 13 μL containing: DNA (3 ηg), PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) (1x), primer forward and reverse (0.28 μM each), $MgCl_2$ (1.5 mM), BSA (bovine serum albumin, Biolabs New England) (0.25 mg/mL), dNTP (0.25 mM) and *Taq* polymerase (Invitrogen) 1.3 U. An APTC-100 MJ Research Thermal Cycler was used programmed for initial 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. Eletrophoresis with agarose gel 3.5% to verify the amplification of the primers. Microsatellite amplification occurred in 19 primers (95%).

The polymorphism analysis was carried out using 12 adult trees (Table 1) from the Tapajos National Forest in the eastern Brazilian Amazon. The PCR products were separated on 4% denaturant poliacrilamid gels containing 1× TBE buffer and 7 M Urea at 45 W, 2000 V and 100mA constant power for 30 min according to Bassam *et al*. (1991). The identification of the alleles was based on the disposition of the fragments in relation to the marker 10 pb ladder. Levels of variability were detected in five loci.

Data analysis for allelic frequencies for each locus was obtained directly from genotypes observed for each individual. Number of alleles per locus, mean observed and expected heterozygosities and Hardy–Weinberg and pairwise equilibrium were calculated using GDA version 1.0 (Lewis & Zaykin 2001). Probabilities of paternity exclusion were estimated using CERVUS (Marshal *et al*. 1998) (Table 1).

All microsatellite marker loci were highly polymorphic, with a mean of 5.8 alleles per locus. Mean observed heterozygosity was 0.81 and mean expected heterozygosity was greater than 70%. The locus Sg06 showed departure from Hardy–Weinberg expectations ($P = 3750$, $P < 0.005$), but no pairwise disequilibrium was detected between the five loci. The combined values (over all loci) for first parent and second parent total exclusionary power were 0.868 and 0.971, respectively, with mean PIC value of 0.681.

These markers will have practical application in the estimation of levels of variation between the natural and managed populations of *S. globulifera*, as well as to help understand the complexity of this species in a tropical forest, so conservation strategies can be formulated.

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