Development and Characterization of Microsatellite Markers for the Endangered Amazonian Tree Aniba rosaeodora (Lauraceae)

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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE MARKERS FOR THE ENDANGERED AMAZONIAN TREE *ANIBA ROSAEODORA* (LAURACEAE)[1]

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- *Premise of the study:* Microsatellite loci were isolated and characterized for Brazilian rosewood (*Aniba rosaeodora*), an endangered neotropical hardwood tree, to investigate population and conservation genetics of this highly valuable nontimber forest resource.
- *Methods and Results:* We used an enriched genomic library method to isolate and characterize 11 nuclear microsatellite loci for *A. rosaeodora*, which exhibited an average of 9.6 and 8.7 alleles per locus in two populations from central Amazonia. Mean observed and expected heterozygosities over the 11 loci were 0.604 and 0.687, and 0.807 and 0.828, respectively, in the two populations.
- *Conclusions:* The polymorphic microsatellite loci developed for *A. rosaeodora* showed highly informative content and can be used as a powerful tool in genetic diversity and population structure, gene flow, and mating system studies for conservation purposes.

Key words: Amazonia; *Aniba rosaeodora*; Brazilian rosewood; neotropical tree; nontimber forest products (NTFP); simple sequence repeat (SSR) loci.

The Brazilian rosewood, *Aniba rosaeodora* Ducke (Lauraceae), is a slow-growing hardwood tree that occurs in nonflooded forests of the Amazon rainforest. The species has its highest population density in central Amazonia, Brazil. It is considered one of the most valuable nontimber forest products (NTFP) in Brazilian Amazonia, and is mainly used as source for essential oil (linalool) in perfumes and aromatherapy (May and Barata, 2004). The oil is extracted almost entirely from the wood, and current extraction methods are destructive to the tree. Due to its high economic value and low-cost unsustainable oil extraction methods, natural stocks of *A. rosaeodora* have been severely depleted. The species is considered endangered and was listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in 2010. Effective strategies for the conservation and management of this resource should be urgently implemented, taking into consideration ecological and genetic data, among other factors. DNA microsatellite markers (simple sequence repeats [SSRs]) have been widely recognized as highly informative, and are frequently used in population and conservation genetics studies of tropical forest tree species. However, despite its high social and economic value, no microsatellite markers are available for *A. rosaeodora* or its congeners. Here, we report on the isolation and characterization of 11 highly variable microsatellite loci for *A. rosaeodora*, aiming to characterize population genetic diversity and structure, gene flow, and mating system of this threatened species. These are important factors in determining how genetic variation is distributed among populations and should be considered in long-term conservation and management strategies for this valuable tropical tree species.

METHODS AND RESULTS

For the construction of an enriched library, total genomic DNA was extracted from silica gel–dried leaves collected from an adult tree (voucher no.: INPA 208904) sampled in a natural population of *A. rosaeodora* located at Ducke Forest Reserve, Manaus, Amazonas (2°53′18″S, 59°58′18″W). For population genetic analysis, total genomic DNA was extracted from 68 adult trees sampled from this population and a second location at Maués, Amazonas (3°22′82″S, 57°43′13″W) (voucher no.: INPA 237807), both in central Amazonia, Brazil. A standard cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987) was used for DNA extraction.

The microsatellite loci were isolated and identified from the library enriched for CA repeats following the protocol described in Farias et al. (2003). Approximately 10 µg of extracted genomic DNA was digested with *Sau*3AI and the appropriate lengths of 200–900 bp were ligated to double-stranded linkers, Er1Bh1*Blunt*-5′-CGGAATTCAGTGGATCCTGCC-3′ and

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Er1Bh1*GATCSticky*-5′-GCCTTAAGTCACCTAGGACGGCTAG-3′. Magnetic beads linked to streptavidin (Dynal, Life Technologies, Grand Island, New York, USA) were used to select fragments containing microsatellites hybridized to the 5′ biotinylated probe $(GA)_{10}$. Enriched DNA was ligated into pGEM-T Easy Vector (Promega Corporation, Madison, Wisconsin, USA) and transformed into *Escherichia coli* strain DH5α. Transformed cells were grown and screened according to the manufacturer's recommendation, with PCR insert screening based on the T7 and Sp6 primers. PCR products were purified and sequenced with T7 and Sp6 primers using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Life Technologies). The amplified products were electrophoresed on an ABI Prism 377 sequencer (Life Technologies) following the manufacturer's instructions.

Microsatellite repeats were found in 72 of 196 sequenced clones using TROLL software (Castelo et al., 2002). Primer pairs complementary to regions flanking microsatellite repeats were successfully designed for 40 clones using Primer3Plus version 1.1.4 (Untergasser et al., 2007). The remaining 32 sequences containing microsatellites did not allow optimal primer design. Optimization of PCR conditions was performed for each locus using total genomic DNA extracted from four adult trees of *A. rosaeodora*. These individuals were sampled from the same population selected for SSR loci development. PCRs contained 10 ng of DNA template, 0.9 μM of each primer, 1 U *Taq* DNA polymerase, 200 μM of each dNTP, 0.25 μg of bovine serum albumin (BSA), and 1× reaction buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$) in a total volume of 13 μL. PCRs were performed on a Veriti Thermal Cycler (Life Technologies) with the following cycling conditions: 96°C for 2 min; followed by 30 cycles of 94°C for 1 min, 44°C to 58°C (according to the annealing temperature of the primer) for 1 min (Table 1), 72°C for 1 min; and a final elongation step of 72°C for 10 min. The PCR products were visualized in 3% agarose gel containing 0.1 μg/mL of ethidium bromide in TBE buffer (89 mM Tris-borate, 2 mM EDTA [pH 8.3]) and sized with a 1-Kb DNA ladder (Life Technologies).

For a preliminary polymorphism analysis, loci with clear and robust amplified products in the agarose gel were later resolved on 4% denaturing polyacrylamide gel electrophoresis (PAGE). The gel was stained with silver nitrate (Creste et al., 2001) and sized by comparison to a 10-bp DNA ladder standard (Life Technologies). Eleven SSR loci produced clearly interpretable and polymorphic bands in PAGE. The forward primer of each pair was fluorescently labeled with 6-FAM, HEX, or TET fluorescent dye for genotyping on an ABI Prism 377 sequencer (Life Technologies). Allele sizes were scored against an internal GeneScan 500 TAMRA size standard (Life Technologies). Individuals were genotyped using GeneScan Analysis version 3.1 and Genotyper version 2.5 softwares (Life Technologies).

Eleven polymorphic SSR loci were characterized (Table 1) by genotyping 68 adult individuals of *A. rosaeodora* sampled from the two populations. GenAlEx version 6.1 (Peakall and Smouse, 2006) was used to estimate total number of alleles (A), observed (H_o) and expected (H_e) heterozygosities, and Wright's F-statistics (i.e., inbreeding within populations [F_{IS}], total inbreeding [F_{IT}], and genetic diversity among populations [F_{ST}]) for each locus and overall means. We also tested the statistical power and informative content of the 11 SSR loci by inferring genetic identity (I) (Taberlet and Luikart, 1999) and paternity exclusion (Q) (Weir, 1996). The paternity analysis considers exclusion probabilities to exclude a random individual from paternity, which are based on the allele frequencies of a locus. Considering n independent loci, Q_i is the exclusion probability for one locus (i), and the combined probability of exclusion (Q) equals $1 - [\prod (1 - Q_i)]$ for the set of loci (Weir, 1996). The linkage disequilibrium between loci was estimated using GENEPOP version 4.0.7 (Raymond and Rousset, 1995).

The number of alleles observed per locus over two populations of *A. rosaeodora* ranged from six to 14. H_o varied from 0.400 to 0.811 and H_e ranged from 0.611 to 0.884 (Table 2). All pairwise tests for linkage disequilibrium among the 11 SSR loci were nonsignificant. Three (Ar18, Ar30, and Ar33) and six (Ar05, Ar18, Ar23, Ar29, Ar33, and Ar39) loci deviated from expectations for Hardy–Weinberg equilibrium in the Manaus and Maués populations, respectively, after Bonferroni correction (adjusted P value = 0.0045). The probabilities of genetic identity per locus ranged from 0.012 to 0.080, and the combined probability of genetic identity (I) was 6.38×10^{-17}. The paternity exclusion probabilities ranged from 0.594 to 0.843, and the combined probability (Q) was 0.9999995, clearly indicating that these loci allow very precise individual discrimination.

CONCLUSIONS

The high allelic variability and individual discrimination power found for the 11 microsatellite loci allow application of these markers as powerful tools for future studies on population genetics of *A. rosaeodora*. We aim to use these markers to estimate gene flow and to characterize population genetic diversity and structure and mating system of the species. These data will contribute to effective strategies for the conservation and management of this threatened Amazonian forest resource.

Table 1. Characteristics of 11 microsatellite loci developed for *Aniba rosaeodora*.

Locus	Repeat motif	Primer sequences (5′–3′)	Allele size range (bp)	T_a (°C)	GenBank accession no.
Ar02	$(GA)_{17}$	F: GAGCCAGAGAATGGAAATGG R: GGCTCTCTCTCCTGCCTCTC	164–178	62	JX679089
Ar03	$(GA)_9(AG)_6$	F: TCTGTCATCCACGAATTTGC R: CATCCACACATCCTTGATGC	165–211	60	JX679090
Ar05	$(GA)_{10}$	F: CCCACACGCATACAAAGAGA R: GCCTTTGTGGACGAAGGTTA	160–206	58	JX683390
Ar13	$(TC)_9C(CT)_9$	F: GGGACGTCTCCCAAGTATGA R: GAATGCAGATTTTAAAAGATTGAGA	229–259	62	JX679091
Ar18	$(CT)_7(CT)_{12}$	F: CCACTCTCTTCTCCCAATTACTC R: AGCGAAATTTTCACGCATGT	190–220	62	JX679092
Ar23	$(GA)_8GAGC(GA)_{13}$	F: CCGAGCGAGAGAGAGAGAGA R: AAGCAAAACCCTAGCCTATCG	101–141	60	JX683391
Ar24	$(GA)_{19}$	F: TTTCCATTCGCTTTTCCTTC R: CACGCTACATTGACAACAAGG	170–220	58	JX679093
Ar29	$(AG)_{17}$	F: CCCCACTGGAAATGAATCAC R: CGTAACCCCTTTTTATGTATCTGTT	205–275	58	JX679094
Ar30	$(GA)_{10}$	F: TGGCCTTAATCAATTGTACGC R: CGTTAATGAGGGGGAGAGAG	170–198	60	JX679095
Ar33	$(GA)_{12}$	F: GTCATGGAGCCAATTGCTTT R: TCCAATGGTGTTATGCAAGC	250–280	58	JX679096
Ar39	$(GA)_8$	F: TGCTCAATTGTGCTGAAACA R: TGTTCCTTTCGCAGGTATTCA	190–204	58	JX679097

Note: T_a = annealing temperature.

Table 2. Population genetic parameters estimated per SSR locus over two populations of *Aniba rosaeodora* from central Amazonia, Brazil.

	Manaus population (N = 38)			Maués population (N = 30)					
Locus	A	H_o	H_e	A	H_o	H_e	F_{IS}	F_{IT}	F_{ST}
Ar02	6	0.607	0.729	8	0.739	0.800	0.119	0.179	0.068
Ar03	17	0.811	0.890	14	0.643	0.884	0.181	0.210	0.036
Ar05	11	0.714	0.865	7	0.667	0.819*	0.180	0.196	0.019
Ar13	11	0.649	0.813	7	0.567	0.809	0.251	0.271	0.027
Ar18	6	0.633	0.779*	8	0.400	0.769*	0.332	0.397	0.096
Ar23	11	0.786	0.890	9	0.545	0.835*	0.228	0.235	0.009
Ar24	11	0.767	0.873	10	0.583	0.810	0.198	0.252	0.068
Ar29	10	0.632	0.786	9	0.667	0.788*	0.175	0.203	0.033
Ar30	8	0.615	0.843*	9	0.722	0.840	0.205	0.274	0.086
Ar33	8	0.763	0.773*	7	0.464	0.705*	0.170	0.196	0.031
Ar39	7	0.579	0.611	8	0.654	0.794*	0.123	0.242	0.136
Mean	9.6	0.687	0.807	8.7	0.604	0.828	0.197	0.241	0.055

Note: A = number of alleles; F_{IS} = inbreeding within populations, F_{IT} = total inbreeding; F_{ST} = genetic diversity among populations; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of sampled plants.

* Significant deviation from Hardy–Weinberg equilibrium after Bonferroni correction.

LITERATURE CITED

Castelo, A. T., W. Martins, and G. R. Gao. 2002. TROLL—Tandem repeat occurrence locator. *Bioinformatics (Oxford, England)* 18: 634–636.

Creste, S., A. Tulman-Neto, and A. Figueira. 2001. Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. *Plant Molecular Biology Reporter* 19: 299–306.

Doyle, J. J., and J. L. Doyle. 1987. A rapid DNA isolation procedure from small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

Farias, I. P., T. Hrbek, H. Brinkmann, I. Sampaio, and A. Meyer. 2003. Characterization and isolation of DNA microsatellite primers for *Arapaima gigas*, an economically important but severely overexploited fish species of the Amazon basin. *Molecular Ecology Notes* 3: 128–130.

May, P. H., and L. E. S. Barata. 2004. Rosewood exploitation in the Brazilian Amazon: Options for sustainable production. *Economic Botany* 58: 257–265.

Peakall, R., and P. E. Smouse. 2006. GenAlEx 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6: 288–295.

Raymond, M., and F. Rousset. 1995. GENEPOP (version 1.2): Population genetics software for exact test and ecumenism. *Journal of Heredity* 86: 248–249.

Taberlet, P., and G. Luikart. 1999. Non-invasive genetic sampling and individual identification. *Biological Journal of the Linnean Society* 68: 41–55.

Untergasser, A., H. Nijveen, X. Rao, T. Bisseling, R. Geurts, and J. A. M. Leunissen. 2007. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research* 35: W71–W74.

Weir, B. S. 1996. Genetic Data Analysis II. Sinauer Associates, Sunderland, Massachusetts, USA.