Development and Characterization of Microsatellite Markers for the Endangered Amazonian Tree Aniba rosaeodora (Lauraceae)\textsuperscript{1}

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The Brazilian rosewood, Aniba rosaeodora Ducke (Lauraceae), is a slow-growing hardwood tree that occurs in nonflooding forests of the Amazon rainforest, Brazil. 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 Sau3AI and the appropriate lengths of 200–900 bp were ligated to double-stranded linkers, Er1Blunt-5′-CGGAATTCAGTGGATCCTGCC-3′ and

- 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.
ErI1HgATCSticky-5'-GGCTTAAATG-GAGCGTCT-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)n. Enriched DNA was ligated into GEM-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). Allele sizes were scored against an internal GeneScan 500 TAMRA size standard (Life Technologies). Individuals were genotyped using 6-FAM, HEX, or TET fluorescent dye for genotyping on an ABI Prism 310 Genetic Analyzer (Life Technologies) with the following cycling conditions: 94°C for 1 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s; a final extension step of 72°C for 1 min; and a final hold at 4°C. The gel was stained with silver nitrate (Crestec, Madison, Wisconsin, USA) and 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 10-bp DNA ladder standard (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).

**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<sub>r</sub> (°C) | GenBank accession no. |
|-------|--------------|--------------------------|-----------------------|----------------|----------------------|
| Ar02  | (GA)<sub>17</sub> | F: GAGCCAGAGAATGGAAATGGC R: GCTCTCTCCTCCCTCCTCCTC | 164–178 62 | JX679089 |
| Ar03  | (GA)<sub>9</sub>(AG)<sub>6</sub> | F: TCTGTCATCCACAGAATTTGCG R: CATCACCACATCTCTGTGGC | 165–211 60 | JX679090 |
| Ar05  | (GA)<sub>10</sub> | F: CCCCACAGCTCACAAGAGAGA R: GCTCTTGTGAGCAAGGTTA | 160–206 58 | JX683390 |
| Ar13  | (TC)<sub>2</sub>(CT)<sub>3</sub> | F: GGAGACTCTCCACAGAATGTGA R: CCCACCTCTCTCCCAAAATCTC | 229–259 62 | JX679091 |
| Ar18  | (CT)<sub>7</sub>(CT)<sub>12</sub> | F: AGCGAAATTTTCAGCGAATGT | 190–220 62 | JX679092 |
| Ar23  | (GA)<sub>3</sub>GAGC(GA)<sub>13</sub> | F: CCGAGGAGAGAGAGAAGAGA R: AAGCCAAAAAATTGCTATCG | 101–141 60 | JX683391 |
| Ar24  | (GA)<sub>9</sub> | F: TTCCCATGCTGGTTTTTCCTC R: CGGTTAGGAGAGAGAAGAGA | 170–220 58 | JX679093 |
| Ar29  | (AG)<sub>17</sub> | F: GAGGGAGAGAGAGAGAGAGA R: CGTTAACCCCTTTATGATCTGTT | 205–275 58 | JX679094 |
| Ar30  | (GA)<sub>10</sub> | F: TGGGCTTTAACAAATTGAGCC R: CGTTAAGGGGGGAGAGAGA | 170–198 60 | JX679095 |
| Ar33  | (GA)<sub>12</sub> | F: GCTATGGGCGAATGGGTATT | 250–280 58 | JX679096 |
| Ar39  | (GA)<sub>8</sub> | F: TGCTGATGCTGCTGCAACCA R: TGCTCCTTTTGGCAGGATGTTCA | 190–204 58 | JX679097 |

Note: T<sub>r</sub> is annealing temperature.
Table 2. Population genetic parameters estimated per SSR locus over two populations of *Aniba rosaeodora* from central Amazonia, Brazil.

| Locus  | *A* | *H*  | *F*  | *H*  | *F*  | *F*  | *F*  | *F*  |
|--------|-----|------|------|------|------|------|------|------|
| 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*  = inbreeding within populations; *F*  = total inbreeding; *G*  = genetic diversity among populations; *H*  = expected heterozygosity; *H* = observed heterozygosity; *N* = number of sampled plants.

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

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