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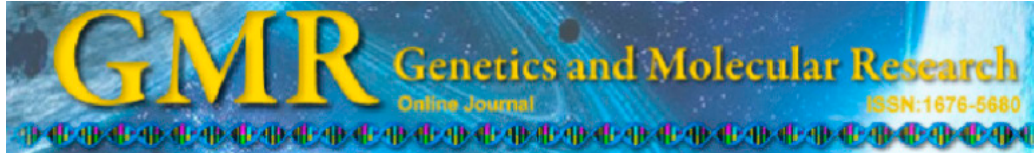
Microsatellite markers for the Cabreúva tree, *Myroxylon peruiferum* (Fabaceae), an endangered medicinal species from the Brazilian Atlantic Forest

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ABSTRACT. The Cabreúva tree, *Myroxylon peruiferum*, is an endangered tropical species from Brazil used in forest restoration projects. It is known for its medicinal properties. Eleven microsatellite markers were developed for this species, from a microsatellite-enriched library. Nine of these markers, characterized in 30 individuals from a semideciduous forest remnant population in southeast Brazil, were

polymorphic, with allele numbers ranging from 2 to 8 per locus; expected and observed heterozygosities ranged from 0.103 to 0.757 and 0.107 to 0.704, respectively. One locus (Mpe-C04) showed significant deviation from Hardy-Weinberg equilibrium, probably due to null alleles. Two other loci (Mpe-E09 and Mpe-H07) were monomorphic in this population. These microsatellite loci should be useful for future population genetic studies of this species.

Key words: Molecular marker; Atlantic Forest; Medicinal plants; Fragmentation; Forest restoration; Conservation genetics

INTRODUCTION

Myroxylon peruiferum L.f. (Fabaceae-Leguminosae: Papilionoideae) is a native Brazilian species, present all over the country, especially at the semideciduous forests of the Atlantic Forest hotspot. It is a deciduous tree, occurring both inside dense primary forest as well as in secondary ones. The dispersion syndrome of its seed is anemochoric and the pollination takes place through anemophily (Yamamoto et al., 2007). This species comes the Peruvian balsam, used in popular medicine as an anesthetic for urinary issues, cough, bronchitis, diabetes, and against the Gram-negative mycobacteria *Helicobacter pylori*. This balsam is made of a mix of benzoic and cinnamic acids, terpenes, alcohols, and phenylpropanoids.

Isoflavones, flavones, isoflavonoids, among others, come from the *M. peruiferum* trunk. Substances like 3',4',7-trimethoxyisoflavone (cabreuvina) and 6-hydroxy-4',7-dimethoxyisoflavone and germacrene D were isolated from its leaves, and showed activities against *Mycobacterium tuberculosis*, *M. avium* and *M. kansasii* (Carvalho et al., 2008). Also, there are records of *M. peruiferum* extract activity against *Streptococcus pyogenes*, *Shigella sonnei* and *Staphylococcus aureus* (Ohsaki et al., 1999, Gonçalves et al., 2005). Besides its medicinal properties, other uses of *M. peruiferum* include cosmetics, perfumery, construction wood and carpentry, besides landscaping and forest restoration of degraded areas. Excessive exploitation of its wood has provoked a reduced number of individuals in this species, causing it to be included in the list of endangered species of the State of São Paulo (Secretaria do Meio Ambiente SP, 2004).

Atlantic Forest is a biome spreading all over the Brazilian coast from the State of Rio Grande do Norte all the way to Rio Grande do Sul, penetrating the land, crossing the States of Minas Gerais and São Paulo further away to Paraguay and the north of Argentina. Today, less than 12% of its original formation remain mostly as small, degraded and isolated forest patches in different levels of anthropization (Ribeiro et al., 2009). Aiming at preserving this ecosystem, the Brazilian government, NGOs and private entrepreneurs have invested in two main actions: 1) conservation and protection of the best preserved remaining areas of the Atlantic Forest, and 2) the forest large-scale restoration of the partially or totally degraded areas (Calmon et al., 2011). As a species currently represented by scarce populations occurring in isolated and degraded forest tracts, it is essential to know the diversity and genetic structure of *M. peruiferum* to better design conservation strategies, sustainable management for medicinal uses and ecological restoration for implementing populations able to self-perpetuate even without a significant contribution of gene flow from surrounding remnants.

MATERIAL AND METHODS

Samples and DNA extraction

Thirty *M. peruiferum* individuals were randomly sampled from the semideciduous forest at Santa Genebra Wood (Campinas, SP, Brazil), a natural Brazilian Atlantic Forest remnant. Santa Geneva is the largest remaining rain forest in Campinas, and the second largest urban forest of Brazil. It has an area of 251.77 ha, average altitude of 670 m and an average annual temperature of 20.6ºC. It is defined as a semideciduous forest and was declared ARIE (Area of Ecological Interest) by the Federal Government in 1985.

The total genomic DNA was extracted from young leaves of *M. peruiferum* following a protocol adapted from Doyle and Doyle (1990), using an extraction buffer containing 2% CTAB, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, 2% PVP-40 (w/v), 1.42 M NaCl, 3% beta-mercaptoethanol (v/v).

Microsatellite-enriched library construction and sequencing

From the DNA of one single individual of *M. peruiferum*, 5 μg was digested with 50 U *Afa*I restriction enzyme, used for the development of a microsatellite-enriched library according to the methodology described by Billotte et al. (1999). The DNA of the library clones was sequenced using primer SP6 (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') in an ABI PRISM 3100 Genetic Sequencer (Applied Biosystems) at University of Campinas.

In the obtained sequences we looked for repetitions of di- (5 repetitions minimum), tri- (4 repetitions), tetra-, penta-, and hexanucleotides (3 repetitions) using the WebSat program (Martins et al., 2009). The pairs of oligonucleotides chosen to characterize the 17 simple sequence repeat (SSR) loci were designed using the Primer3 program (Rozen and Skaletsk, 1998), then analyzed by the Oligo Explorer program (Javed et al., 2010) in order to exclude those that presented dimer, heterodimer and hairpin formations. The oligonucle-otides located too close to the vector or in regions of low-quality sequence were discharged. We selected the pairs of oligonucleotides with annealing temperature between 55° and 60°C, GC content from 35 to 60%, length between 17 and 25 bases, and product size from 130 and 265 bases. The selected pairs of oligonucleotide were ordered from Eurofins MWG Operon (Huntsville, AL, USA).

Four individuals of *M. peruiferum* were used for quality test of SSR locus amplifica-tion. The total DNA of these and other 26 individuals of a single population collected from the natural remnant of Santa Genebra Wood were extracted as described above. PCRs were conducted in a 15-μL solution containing about 8 ng template DNA, and reagents as follows: 1X reaction buffer containing 20 mM Tris-HCl, pH 8.4, 20 mM KCl and 10 mM $(NH_4)_2SO_4$, 1.2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 U Taq polymerase, 0.2 pmol forward primer, 0.2 pmol reverse primer. PCR was conducted on a Veriti Dx 384-Well thermocycler following two distinct programs of temperature cycles:

A) 95°C for 5 min followed by 8 touchdown cycles of 95°C for 30 s, 66°C for 1 min (minus 2°C per cycle), 72°C for 80 s; then followed by 37 cycles of 95°C for 30 s, 50°C (annealing temperature indicated in Table 1) for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min.

B) 95°C for 5 min followed by 3 touchdown cycles of 95°C for 30 s, 60°C for 1 min (minus 2.5°C per cycle), 72°C for 80 s; a single cycle of 95°C for 30 s, 55°C for 1 min, 72°C for 80 s, then followed by 37 cycles of 94°C for 30 s, 53°C (annealing temperature indicated in Table 1) for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min.

The amplification products were separated by electrophoresis on 7% denaturing polyacrylamide gel subjected to a current of 40 mA for 3 h. The gel was subjected to silver nitrate staining via the consecutive exposure to a fixing solution (10% ethanol, 0.75% glacial acetic acid; 10 min), pre-oxidation solution (1.5% HNO_3; 3 min), staining solution (0.2% $AgNO_3$; 20 min), and revealing solution (3% Na_2CO_3, 0.05% formaldehyde; 10 min). Each locus was genotyped through direct visualization of its bands in a transilluminator. Using all the loci that granted polymorphic bands, we calculated the number of alleles and expected and observed heterozygosities. We performed the exact test for Hardy-Weinberg equilibrium considering the alternative hypothesis (H1) of heterozygotes deficiency using the Markov chain algorithm or, whenever possible, the complete enumeration method (Table 1). For linkage disequilibrium the G-test was used. All of the above mentioned analyses were made with Genepop program (Raymond and Rousset, 1995). The MICRO-CHECKER 2.2.1 software (Van Oosterhout et al., 2004) was used for identifying possible null alleles (1000 randomizations). All tests were corrected for multiple comparisons by Bonferroni's correction (Rice, 1989).

RESULTS AND DISCUSSION

Microsatellite sequences

One hundred and ninety regional clones containing microsatellite repetitions (or SSR) were obtained and sequenced. The average length of the inserts was 570 bases. Twenty-three clones were identified as having SSR regions, of which five were discharged for showing SSR regions too close to the restriction sites of the *Afa*I enzyme, or to the insert end, preventing the design of the oligonucleotides. Two clones formed a cluster for which a unique pair of oligonucleotides was designed. Seventeen pairs of oligonucleotides were selected and ordered from Eurofins MWG Operon.

Polymorphism and heterozygosity

Of the 17 loci tested, 9 amplified polymorphic bands among the individuals and 2 others (Mpe-E09 and Mpe-H07) revealed to be monomorphic. The information to all oligonucleotides that produced sharp electropherograms is in Table 1.

We found between 2 and 8 alleles per locus (average = 4.11); the expected heterozygosity varied from 0.103 to 0.757 (average = 0.370) and the observed heterozygosity from 0.107 to 0.704 (average = 0.3006; Table 1). The inbreeding coefficient varied from -0.045 to 0.633 [average = 0.189 (0.043-0.296)]. We did not find any disequilibrium linkage between any pairs of locus (P > 0.05). Only locus Mpe-C04 presented deviation from the expected frequencies in Hardy-Weinberg equilibrium (P < 0.05; Table 1). This same locus (Mpe-C04) showed signs of null alleles in the population studied.

Table 1. Primer sequences and characteristics of 11 *Myroxylon peruiferum* microsatellite markers.

GenBank accession No.	N_A	H_E	H_O	f	P_{HWE}
JX486089	2	0.1197	0.1250	-0.045 (-0.136-0.000)	1.0000
JX486090	8	0.6171	0.5185	0.162 (-0.096-0.398)	0.0839
JX486091	5	0.2883	0.1071	0.633* (-0.025-1.000)	0.0001
JX486092	2	0.4983	0.4815	0.034 (-0.364-0.398)	0.5846
JX486093	8	0.7575	0.7037	0.072 (-0.176-0.296)	0.0094
JX486094	2	0.1032	0.1071	-0.038 (-0.125-0.000)	1.0000
JX486095	2	0.1032	0.1071	-0.038 (-0.125-0.000)	1.0000
JX486096	3	0.6618	0.4444	0.333 (0.040-0.591)	0.0162
JX486097	5	0.1775	0.1111	0.378 (-0.066-1.000)	0.0194
JX486098	1	ICal	ICal	ICal	ICal
JX486099	1	ICal	ICal	ICal	ICal

N_A = number of alleles; H_O and H_E = observed and expected heterozygosities; f = fixation coefficient; P_{HWE} = probability of Hardy-Weinberg equilibrium (significant if lower than 0.0056 after Bonferroni's correction). *Loci showing evidence of null alleles suggested by Micro-Checker; ICal = impossible to calculate in monomorphic loci.

CONCLUSION

Based on our data, we developed 9 polymorphic microsatellite markers for the medicinal tree *M. peruiferum* and 2 other markers that were monomorphic in the single population studied. We demonstrated that these 9 loci are polymorphic in a sample of 30 individuals of the same population of *M. peruiferum*, not presenting any linkage disequilibrium between the pairs of loci. Only one locus (Mpe-C04) showed signs of null alleles presence.

We believe that this set of primers will be useful to perform diversity and genetic structure studies in the natural remnant areas of the Atlantic Forest as well as in the selection of seedlings useful to the implementation of forest restoring programs able not only to recover the floristic diversity, but also to reimplement the maximum possible genetic diversity of the species in forest areas previously subjected to deforestation and anthropization.

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