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Genetic diversity of *Metrodorea nigra* (Rutaceae) from a small forest remnant in Brazil assessed with microsatellite markers

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ABSTRACT. *Metrodorea nigra* (Rutaceae) is an endemic Brazilian tree of great ecological importance, frequently found in the submontane regions of ombrophilous dense and semideciduous forests. This tree is useful for reforesting degraded areas and the wood can be employed in construction. We developed 12 microsatellite markers from a genomic library enriched for GA/CA repeats, for this species. Polymorphisms were assessed in 40 trees of a highly fragmented population found in Cravinhos, State of São Paulo, in southeastern Brazil. Among the 12 loci, 8 were polymorphic and only one had fixed alleles in this population. The number of alleles per locus and expected heterozygosity ranged from 2 to 11 and from 0.190 to 0.889, respectively. These results revealed mod-

erate levels of genetic variation in *M. nigra* population when compared to other tropical species. Additionally, transferability of the 12 primers was tested in seven other Brazilian Rutaceae tree species (endemics: *M. stipularis*, *Galipea jasminiflora*, *Esenbeckia leiocarpa* and non-endemics: *E. febrifuga*, *E. grandiflora*, *Balfourodendron riedelianum*, *Zanthoxylum riedelianum*). Transferability ranged among species, but at least 8 loci (~67%) amplified in *M. stipularis*, demonstrating a high potential for transferring microsatellite markers between species of the same genus in the Rutaceae family.

Key words: Tropical forest species; Carrapateira; Population genetics; SSR markers; Rutaceae

INTRODUCTION

Metrodorea nigra A. St.-Hil., commonly known as "chupa-ferro", "carrapateira" or "caputuna preta", is a small perennial tree (4-6 m in height) of the Rutaceae family, frequently found in the submontane of Brazilian ombrophilous dense and semideciduous forests from the State of Paraná to Bahia (Souza et al., 2004) and south of Piauí (Pirani and Skorupa, 2002). The anatomical and morphological characteristics (Souza et al., 2004, 2008), phenology, floral biology, breeding systems, and flower visitors of this species (Pombal and Morellato, 2000) have been extensively studied. Small brownish violet or dark purple flowers, with a faint smell of rotting flesh (personal annotation), are observed between August and November depending on the region (Pombal and Morellato, 2000; Souza et al., 2004). Because of the abundance and floral morphology, this species is important for maintaining high pollinator diversity.

Reproductive system experiments have shown that *M. nigra* is a self-incompatible species presenting an allogamous reproductive system associated with dichogamy (Pombal and Morellato, 2000). It is an autochoric species, dispersing its seeds via explosive dehiscence (Schwarcz et al., 2010).

In Brazil, *M. nigra* wood is widely used in interior construction. It is recommended for forest densification in landscaping and restoration of degraded areas and legal reserve (Lorenzi, 2008). Only one study of genetic diversity using isoenzymes has been carried out with populations of this species (Schwarcz et al., 2010); therefore, very little is known about the population genetic structure and gene flow of the *M. nigra* species. This species is commonly found in the most conserved and shaded parts of semideciduous forests and their small size facilitates access to flowers, which raises the possibility of being used as a model species for fine-scale genetic studies, to quantify the effects of habitat fragmentation and to guide conservation strategies.

Microsatellite markers or SSR (simple sequence repeats) have become powerful research tools because they are (mostly) co-dominant, abundant in genomes, highly reproducible, and some have a high ability to amplify orthologous loci in related species (Feres et al., 2009; Nazareno et al., 2009; Guidugli et al., 2010). In this study, we developed microsatellite loci for *M. nigra* in order to facilitate detailed study of its genetic structure, gene flow and mating systems. We also investigated the possibility of using microsatellite markers developed for *M. nigra* in seven Rutaceae-related species.

MATERIAL AND METHODS

Forty *M. nigra* trees were sampled in a disturbed and isolated fragment of 8.0 ha formed by semideciduous forest and located in the private farm Águas Claras in the municipality of Cravinhos (São Paulo State, southeastern Brazil 21°17'47''S; 47°40'29''W). It is one of the few remaining areas in this region and is surrounded by a sugar cane plantation and cattle pasture. The geographic coordinates of all sampled individuals were recorded using GPS (GARMIN eTrex® Vista Cx) (Figure 1). A voucher (collector Groppo #1971) was deposited at the Herbarium of the Department of Biology (Herbarium SPFR) under record SPFR 12706. The leaf tissues were stored at -20°C until DNA extraction. Purity and concentration of the genomic DNA were determined with a spectrophotometer (Nanodrop).

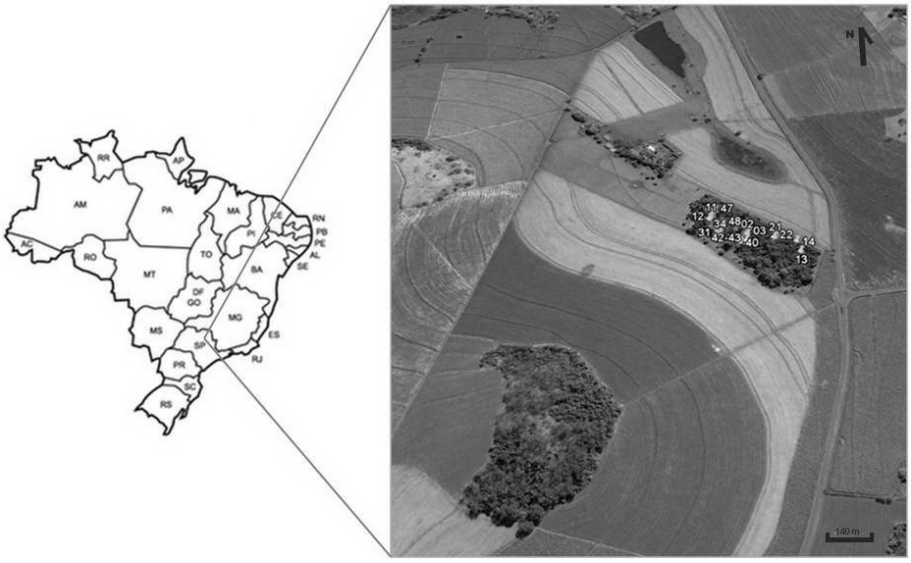

Figure 1. Target *Metrodorea nigra* individuals assessed by SSR loci in isolated forest remnant surrounded by sugar cane (Cravinhos, SP, Brazil).

Protocols described by Billotte et al. (1999) were used for construction of a $(GA)_n$ and $(CA)_n$ microsatellite-enriched library. Total genomic DNA was extracted from an individual of *M. nigra* according to Alzate-Marin et al. (2009) and was digested with *Rsa*I restriction enzyme. The fragments were then linked to adapters (Rsa21 5'-CTCTTGCTTACGCGTGGACTA-3' and Rsa25 5'-TAGTCCACGCGTAAGCAAGAGCACA-3'), amplified by polymerase chain reaction (PCR) and purified by Quiaquick PCR purification kit (Qiagen). Fragments containing microsatellite sequences were selected by hybridization with biotinylated oligonucleotides $(CT)_8$ and $(GT)_8$ and recovered by streptavidin-coated paramagnetic beads. After this step, a second PCR was carried out in order to increase the number of enriched fragments and then an aliquot of the PCR product was linked to the pGEM-T vector (Promega) and transformed into *Escherichia coli* XL-1 Blue strains. A total of 96 clones were sequenced using an ABI 377

and a Big Dye Terminator Kit (Applied Biosystems). Only 19 of these clones presented microsatellite repeats suitable for primer design. Primers complementary to flanking regions of the repeated sequences were designed with the PRIMER3 software (Rozen and Skaletsky, 2000).

Nine individuals of *M. nigra* were used for primer screening. Microsatellite loci were amplified individually in 10-µL reactions containing 2.5 ng template DNA, 0.3 µM of each primer, 0.25 mM of each dNTP, 1X PCR buffer [75 mM Tris-HCl, pH 9.0, 50 mM KCl, and 20 mM $(NH_4)_2SO_4$], 1.5 mM $MgCl_2$ and 1 U *Taq* DNA polymerase (Biotools). Amplifications were performed with an Eppendorf MasterCycler using the following protocol: 96°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing at the primer-specific temperature (Table 1) for 1 min, 72°C for 1 min, and a final elongation step at 72°C for 7 min. PCR products were denatured and separated on 10% denaturing polyacrylamide gels stained with silver nitrate. Allele sizes were estimated by comparison to a 10-bp DNA ladder standard (Invitrogen) and the original DNA used for library development.

Descriptive statistics, such as the number of alleles per locus (N_A), observed (H_O) and expected (H_E) heterozygosities and the intrapopulation fixation index (F_{IS}) were estimated using the GDA software (Lewis and Zaykin, 2002). Deviations from Hardy-Weinberg equilibrium were also computed in GDA using the Fischer exact test. FSTAT (Goudet, 2002) was used to test all loci for linkage disequilibrium, applying the Bonferroni correction for multiple comparisons. Probabilities of paternity exclusion were estimated using CERVUS version 3.0 (Kalinowski et al., 2007).

RESULTS AND DISCUSSION

From the 19 primer pairs tested in a minimum of 9 *M. nigra* individuals, 12 (~63%) yielded clear PCR products. The characterization of these loci was carried out with 40 individuals of *M. nigra*. For this group, we identified a total of 8 polymorphic loci (Table 1). The SSR markers, Mtn3, Mtn13, Mtn19, Mtn21, detected 6 to 11 alleles and another 4 markers (Mtn1, Mtn4, Mtn15, Mtn16) showed moderate levels of variation, exhibiting 2 to 4 alleles. Particularly for the Mtn15 locus, two alleles were found in all 40 individuals sampled, suggesting possible fixation of alleles in this small population. Therefore, statistical analyses were conducted for those seven primer pairs that did not show fixed alleles (Table 1).

The H_O and H_E revealed average polymorphism rates of 0.353 and 0.588, respectively (Table 1). The mean value for H_E in *M. nigra* is comparable to *Psidium guajava* (H_E = 0.62; Risterucci et al., 2005), but lower than other tropical trees: *Cariniana estrellensis* (H_E = 0.73; Guidugli et al., 2009) and *Eugenia uniflora* (H_E = 0.83; Ferreira-Ramos et al., 2008). No evidence of linkage disequilibrium was found between any locus pairs, indicating physical independence of the polymorphic loci. The genotypic frequencies did not fit Hardy-Weinberg expectations with significant deficits of heterozygous genotypes, except for Mtn1 and Mtn19. The average fixation index over all loci was 0.403 (95%CI = 0.190 to 0.621), suggesting biparental inbreeding. The paternity exclusion probability for the second parent over all loci was very high (Table 1), demonstrating clearly that these markers are capable of readily discriminating individuals of *M. nigra*, and will allow detailed parentage studies in natural populations.

Cross-amplification was tested for all 12 *M. nigra* loci in 7 other Brazilian Rutaceae tree species (endemics: *M. stipularis*, *Galipea jasminiflora*, *Esenbeckia leiocarpa* and non-endemics: *E. febrifuga*, *E. grandiflora*, *Balfourodendron riedelianum*, *Zanthoxylum riedelianum*;

Table 1. Microsatellite loci isolated from *Metrodorea nigra*.

Primer name	Repeat motif	Primer sequence (5'-3')	Expected size and observed range (bp)	Ta (ºC)	N_A	H_O/H_E	Pr(Ex2)	Accession No.
Mtn1	$(TG)_8$	GCATGAGCATGGAAATGAGA	144	56	2	0.342/0.380	0.848	HQ377229
		TCATGCGGAAGCCATACG	144-146					
Mtn2	$(GT)_{10}$	CGACAGGCTATTACAACGG	197	50	1	-	-	HQ377230
		CGAGGATCCTTTTGCTGTC	197					
Mtn3	$(CA)_{10}N_8(CG)_5(CA)_6$	GGATCAAGCGACCTATATGC	275	50	6	0.605/0.790*	0.427	HQ377231
		GCAGGGTTATATTAGAGATCACC	273-283					
Mtn4	$(CA)_5CG(CA)_4N_{10}(CA)_{10}$ $CG(CA)_9N_4(CA)_9$	CCTCATATCCACTTCTCATCCC	184	58	4	0.217/0.402*	0.788	HQ377232
		GCATTCATGCATACCTAGCCC	184-190					
Mtn11	$(CA)_{10}$	TAAGCGGGAAGTGGTATTGC	183	54	1	-	-	HQ377233
		CCCGGAAACCATGACACC	183					
Mtn13	$(GT)_{14}$	TGTATTCCTACGCGATCTTGG	213	56	11	0.413/0.889*	0.251	HQ377234
		GAAGGCACAGGTGAAGAAGC	203-229					
Mtn15	$(AT)_4$	TATGTTGATGAGGTCTTGAGC	192	52	2	-	-	HQ377235
		TCAATAATGCCATAAAGCG	180-192					
Mtn16	$(TG)_3CA(TG)_{10}$	ATTGATTGTTTGAGCACATCG	203	50	2	0.000/0.190*	0.899	HQ377236
		CGTCATATCATCTCAGTCTCCC	203-205					
Mtn18	$(CT)_4$	AATGGGATGGAATGACATG	166	50	1	-	-	HQ377237
		TGAGGAGTATACCAAGAACCA	166					
Mtn19	$(TA)_3(TG)_6(TA)_3$ $(TG)_{10}TT(TG)_3$	GGTTGAGGGATGTATATGGAAAG	193	56	8	0.692/0.698	0.509	HQ377238
		CCAACAAACTTATACCATGATCC	163-201					
Mtn20	$(AC)_3(AT)_5$	CCTCAAAATCAAAATATGATGC	120	50	1	-	-	HQ377239
		CCAATATCTTGATTTCTCTGGC	120					
Mtn21	$(TA)_4(TG)_6C(TG)_4$	GTTCTGTGTATGTGCATCACG	220	52	9	0.200/0.768*	0.438	HQ377240
		CCTAGAGTATCATGTTCAAGCC	204-250					
H_O/H_E average[1]/Cumulative probability of exclusion[2]						0.353/0.588[1]	0.9856[2]	

Ta = annealing temperature; N_A = total number of alleles per locus; H_O = observed heterozygosity; H_E = expected heterozygosity. *Indicates significant deviation from Hardy-Weinberg equilibrium ($P < 0.05$); Pr(Ex2) = paternity exclusion probability.

according to Pirani and Groppo, 2010) using the same PCR conditions as previously described. The species *M. stipularis*, *G. jasminiflora*, *E. febrifuga*, *E. grandiflora*, *E. leiocarpa*, *B. riedelianum* belong to a predominantly American monophyletic group (Groppo et al., 2008) and *Z. riedelianum* is phylogenetically far from this group.

Transferability ranged among species, but at least eight loci (~67%) amplified in *M. stipularis*. Mtn2 was amplified across all species and only three SSR loci (Mtn4, Mtn11 and Mtn13) were not amplified (Table 2). These results were expected considering the evolutionary distance between species and indicated a high potential for transferring microsatellite markers between species of the same genus in the Rutaceae family.

Table 2. Tests of transferability of the microsatellite loci for seven Rutaceae species.

Loci	Ta (°C)	Cross-species microsatellite amplification						
		Metrodorea stipularis Allele (bp)	*Galipea jasminiflora* Allele (bp)	*Esenbeckia febrifuga* Allele (bp)	*Esenbeckia grandiflora* Allele (bp)	*Esenbeckia leiocarpa* Allele (bp)	*Balfourodendron riedelianum* Allele (bp)	*Zanthoxylum riedelianum* Allele (bp)
Mtn1	56	144	-	-	-	-	-	-
Mtn2	50	197	197	197	197	197	197	197
Mtn3	50	279	-	-	-	-	-	-
Mtn4	58	-	-	-	-	-	-	-
Mtn11	54	-	-	-	-	-	-	-
Mtn13	56	-	-	-	-	-	-	-
Mtn15	52	180/192	180/192	-	-	-	-	-
Mtn16	50	203	-	-	-	-	-	-
Mtn18	50	166	166	166	-	166	166	-
Mtn19	56	163	-	-	-	-	-	-
Mtn20	50	120	-	120	120	-	120	120
Mtn21	52	-	-	216	208/220	-	-	-

Allele size range (bp) based on two individuals of each species (except *M. stipulares* - one individual).

Our study indicates the strong potential of the newly developed microsatellite set for investigating both population genetics and conservation biology of the *M. nigra* species.

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