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Development of microsatellite markers for *Dimorphandra mollis* (Leguminosae), a widespread tree from the Brazilian cerrado[1]

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- *Premise of the study:* Microsatellite markers were developed for *Dimorphandra mollis* (Leguminosae), a widespread tree in the Brazilian cerrado (a savanna-like vegetation).
- *Methods and Results:* Microsatellite markers were developed from an enriched library. The analyses of polymorphism were based on 56 individuals from three populations. Nine microsatellite loci were polymorphic, with the number of alleles per locus ranging from three to 10 across populations. The observed and expected heterozygosities per locus and population ranged from 0.062 to 0.850 and from 0.062 to 0.832, respectively.
- *Conclusions:* These microsatellites provide an efficient tool for population genetics studies and will be used to assess the genetic diversity and spatial genetic structure of *D. mollis*.

Key words: cerrado; conservation genetics; *Dimorphandra mollis*; genetic structure; Leguminosae; microsatellite.

Dimorphandra mollis Benth. (Leguminosae), popularly known as "faveiro" and "fava d'anta," is a tree that is widely distributed throughout the Brazilian cerrado, a biodiversity hotspot with a savanna-like vegetation that also occurs in parts of Bolivia and Paraguay. *Dimorphandra mollis* is a medicinal plant of high economic value for local populations of central Brazil and has been extensively exploited mainly because of the high concentration of the flavonoid rutin in its fruits. Rutin has antioxidant, antiviral, antitumoral, and anti-inflammatory properties (Féres et al., 2006), and is used in prescriptions for human circulatory diseases. Studies indicate that the species also has a potential use in the food industry because its seeds contain a high amount of galactomannan that can be used as a thickening, stabilizing, and gelling agent (Panegassi et al., 2000).

Unsustainable fruit exploitation may jeopardize the long-term persistence of *D. mollis* populations. Habitat loss due to cerrado fragmentation, as well as an increase in the frequency of fires caused by the expansion of agricultural practices in the last ~60 yr, may also threaten populations of this species. However, despite its high economic value, studies on population genetics of *D. mollis* are scarce; as of today, the genetic structure of only a few populations has been studied (Souza and Lovato, 2010; Gonçalves et al., 2010). Before the current study, microsatellite loci were not available for *D. mollis*. Microsatellites, or simple sequence repeat (SSR) markers, are highly polymorphic codominant markers that have been widely used in studies of genetic diversity and structure of populations. Thus, the development of microsatellite markers for this species will allow more in-depth genetic analyses that are useful for conservation and sustainable management planning, such as mating system and current gene flow. Here, we report the isolation and characterization of nine polymorphic microsatellite loci for *D. mollis*.

METHODS AND RESULTS

Genomic DNA was initially isolated from leaves of one individual of *D. mollis*, from a population of Paranapanema (southeastern Brazil), using the protocol described by Russell et al. (2010). A genomic enriched library was constructed according to the protocol described by Billotte et al. (1999). Genomic DNA was digested with the *Rsa*I restriction enzyme and the fragments were linked to Rsa21 and Rsa25 adapters. The fragments were amplified by PCR and purified using PEG 8000. The library was enriched for dinucleotide and tetranucleotide motifs using the biotinylated probes $(CT)_8$, $(GT)_8$, $(GATA)_4$, and $(GACA)_4$. Target fragments were captured using streptavidin-coated magnetic beads (Promega, Madison, Wisconsin, USA) and amplified by PCR. The DNA fragments were cloned in the plasmid vector pGEM-T Easy vector (Promega) and transformed into competent XL1-blue *Escherichia coli*. Transformed cells were cultivated on agar medium containing X-galactosidase. Single white colonies were transferred to microplates for long-term storage at −80°C. These colonies were amplified using the universal M13 forward and reverse primers before sequencing. A total of 480 positive clones were sequenced using M13 universal primers and the DYEnamic ET-terminator Kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, United Kingdom) on a MegaBACE 1000 automated DNA sequencer (GE Healthcare).

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TABLE 1. Characterization of nine microsatellite loci developed in *Dimorphandra mollis*.

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Allele size (bp)	No. of alleles	GenBank accession no.
Dmo5	F: *TTGAATTGTCCCTTCAACAT	$(AG)_{18}$	57	200–250	8	JN571428
	R: CGTGCACTCTTCCATCTATC					
Dmo7	F: *CCATTCCTCCTGATCTTCTC	$(CT)_{19}$	62	164–208	10	JN571429
	R: TCTTGAAGAGGCTCACACAC					
Dmo8	F: *TAATCACATCACCCAATGAA	$(CA)_{13}$	59	102–132	8	JN571430
	R: AGAAGCAAATTTGGTGTTTTA					
Dmo11	F: *GCTGTAGAACGCCATCAATA	$(GT)_{13}$	60	268–274	4	JN571431
	R: AAAGACATGCACACAGAACA					
Dmo13	F: *AAACCTCTGCAACATGATTT	$(TG)_{18}$	62	204–246	10	JN571432
	R: AGATTGGCATATCGACACAC					
Dmo14	F: *CCTTCTCACTTTGGGGTGAA	$(TG)_{18}$	63	192–220	6	JN571433
	R: TAACCTCTTTGCCCACCTTG					
Dmo20	F: *AAAAATAAGCTGCCATCACA	$(TG)_{16}$	62	206–220	5	JN571434
	R: CACATTAGCAGGAAAACCAC					
Dmo21	F: *CCATGTCTCCATGATAGGAA	$(TG)_{17}$	60	234–238	3	JN571435
	R: GAGAAAGTGGTTGCTGAAAA					
Dmo25	F: *ATAGCTCCCTCAGCAGAACT	$(TC)_{18}$	59	190–214	6	JN571436
	R: CGTGTCTTAACACCTGTTTGT					

Note: T_a = annealing temperature.
* M13 tag (TTTTCCCAGTCACGAC) added to the 5′ end of the forward primer for amplification with fluorescently labeled M13.

Sequences were analyzed for microsatellites using the identification tool for simple repetitive sequences of the Gramene Project Site (www.gramene.org/db/markers/ssrtool). One hundred of these clones contained microsatellite sequences with more than five repeats. Primers were designed with the software Primer3 (Rozen and Skaletsky, 2000) for 21 fragments containing microsatellite inserts with more than 10 repeat motifs and sufficiently long flanking sequences for primer design. Forward primers were appended with an M13 tag (5′-TTTTCCCAGTCACGAC-3′) to allow labeling with a tailed fluorescent dye M13 primer during genotyping.

Microsatellite polymorphism was analyzed using 56 individuals from three populations of *D. mollis*. Voucher specimens for the sampled populations have been deposited in the Herbarium of the Departamento de Botânica da Universidade Federal de Minas Gerais (BHCB; see Appendix 1 for voucher information). Amplifications were performed in a total volume of 13 μL, using the following conditions: 10–20 ng genomic DNA, PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.4], 0.1% Triton X-100 [Phoneutria, Belo Horizonte, Brazil]), 0.86 mM of $MgCl_2$, 0.25 mM of each dNTP, 0.05 μM forward primer, 0.10 μM reverse primer, 0.10 μM M13 fluorescent-labeled primer, and 1 U *Taq* polymerase (Phoneutria). PCRs were performed on a Mastercycler thermocycler (Eppendorf, Hamburg, Germany) using an initial denaturation at 94°C for 5 min, followed by 34 cycles of denaturation at 94°C for 1 min; annealing at 57–63°C depending on the primer (Table 1) for 1 min; extension at 72°C for 1 min followed by eight cycles at 94°C for 30 s, 53°C for 45 s, 72°C for 45 s; and a final extension at 72°C for 10 min. Loci were genotyped on a MegaBACE 1000 automated sequencer, using 0.1% Tween 20 and ROX-500 Size Standard (GE Healthcare). Alleles were identified by their size using the MegaBACE Fragment Profiler version 1.2 software (GE Healthcare). The number of alleles, observed and expected heterozygosities, and deviations from Hardy–Weinberg equilibrium were estimated for each locus and population using the software Arlequin version 3.1 (Excoffier et al., 2005). Linkage disequilibrium for all pairs of loci was tested using a randomization-based test with Bonferroni correction using FSTAT version 2.9.3.2 (Goudet, 2002). The presence of null alleles at each locus was tested using MICRO-CHECKER version 2.2.3 (van Oosterhout et al., 2004).

From the 21 primer pairs, six did not amplify successfully and another six did not show clear bands in the gel. Nine primer pairs were polymorphic in the three analyzed populations of *D. mollis* (Table 1). The number of alleles per locus ranged from three to 10 across the three populations (Table 1) and ranged from two to nine in each population (Table 2). The observed and expected heterozygosities ranged from 0.062 to 0.850 and from 0.062 to 0.832, respectively (Table 2). Among the nine analyzed loci, three loci (Dmo13, Dmo20, and Dmo25) showed significant deviation from the Hardy–Weinberg equilibrium ($P < 0.05$) in the population RPA (Table 2) and three loci (Dmo8, Dmo14, and Dmo25) in the population PAR (Table 2). The departure from Hardy–Weinberg equilibrium detected in both populations is likely to be due to inbreeding or other factors violating the criteria of an ideal population. The null allele test

TABLE 2. Results of initial primer screening in three populations of *Dimorphandra mollis*.

	RPA population[a] ($N = 20$)			PAR population[b] ($N = 20$)			MGU population[c] ($N = 16$)		
Locus	N_a	H_o	H_e	N_a	H_o	H_e	N_a	H_o	H_e
Dmo5	6	0.700	0.726	6	0.300	0.431	3	0.428	0.373
Dmo7	9	0.750	0.832	7	0.650	0.785	6	0.643	0.746
Dmo8	4	0.850	0.640	6	0.700	0.718*	6	0.692	0.778
Dmo11	4	0.650	0.629	2	0.562	0.498	2	0.187	0.272
Dmo13	9	0.550	0.681*	7	0.500	0.560	2	0.062	0.062
Dmo14	5	0.650	0.656	3	0.400	0.593*	4	0.312	0.490
Dmo20	4	0.300	0.388*	3	0.389	0.341	4	0.687	0.720
Dmo21	3	0.631	0.622	2	0.600	0.508	3	0.600	0.641
Dmo25	5	0.500	0.702*	3	0.250	0.579*	5	0.667	0.764
Mean	5.4	0.620	0.653	4.3	0.483	0.557	3.9	0.476	0.539

Note: H_e = expected heterozygosity; H_o = observed heterozygosity; N = sample size for each population; N_a = number of alleles per locus.
[a] Ribas do Rio Pardo, 20°23′54″S/53°53′54″W.
[b] Paranapanema, 23°23′20″S/48°43′22″W.
[c] Mogi Guaçu, 22°54′06″S/47°48′48″W.
* Significant departures from Hardy–Weinberg equilibrium at $P < 0.05$.

conducted in the MICRO-CHECKER detected the presence of null alleles only in the locus Dmo25 in the population PAR. Linkage disequilibrium was not observed in any loci in the three populations.

CONCLUSIONS

The nine microsatellite markers developed for *D. mollis* exhibited a large number of alleles per locus and high heterozygosity. Our data suggest that these microsatellite markers, which were all polymorphic in the three populations analyzed, are suitable for several population genetics studies. We are currently using these markers for inferring genetic diversity and spatial genetic structure in *D. mollis*. These studies will contribute toward understanding the evolutionary history and population dynamics of this species and will provide information to develop measures for its conservation and sustainable exploitation.

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APPENDIX 1. List of vouchers of *Dimorphandra mollis* used in this paper. Vouchers are deposited in the Herbarium of the Departamento de Botânica da Universidade Federal de Minas Gerais (BHCB).

Code	Country	Locality (State)	Voucher number
RPA	Brazil	Ribas do Rio Pardo (MS)	*127192*
PAR	Brazil	Paranapanema (SP)	*147204*
MGU	Brazil	Mogi Guaçu (SP)	*116522*