

CHARACTERIZATION OF MICROSATELLITE LOCI IN *HIMANTHUS*  
*DRASTICUS* (APOCYNACEAE), A MEDICINAL PLANT FROM THE  
BRAZILIAN SAVANNA<sup>1</sup>

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- *Premise of the study:* We developed a new set of microsatellite markers for studying the genome of the janaguba tree, *Himantanthus drasticus* (Mart.) Plumel, which is used in folk medicine in northeastern Brazil. These novel markers are being used to evaluate the effect of harvesting on the genetic structure and diversity of natural populations of this species.
- *Methods and Results:* Microsatellite loci were isolated from an enriched *H. drasticus* genomic library. Nine primer pairs successfully amplified polymorphic microsatellite regions, with an average of 8.5 alleles per locus. The average values of observed and expected heterozygosity were 0.456 and 0.601, respectively.
- *Conclusions:* The microsatellite markers described here are valuable tools for population genetics studies of *H. drasticus*. The majority of the primers also amplified sequences in the genome of another species of the same genus. This new set of markers may be useful in designing a genetic conservation strategy and a sustainable management plan for the species.

**Key words:** conservation; harvesting; medicinal plants; molecular markers; sustainable management.

*Himantanthus drasticus* (Mart.) Plumel, commonly known as janaguba, is an important medicinal tree species of the Brazilian savanna that has been used by native American populations of northeastern Brazil since the 1970s in the treatment and healing of a great number of diseases. Recently, the medicinal value of the genus *Himantanthus* Willd. ex Schult. was confirmed through pharmacological studies (Colares et al., 2008; Ferreira et al., 2009). This may increase the level of harvesting of bark and latex and lead to an overexploitation of natural populations. Harvesting can affect not only individual physiology and metabolism, but also genetic diversity and structure of managed populations (Ticktin, 2004).

The characterization of genetic diversity, associated with demographic studies, has been applied to the establishment of exploitation criteria for some plant species (Reis et al., 2000; Mariot et al., 2002). However, our present knowledge of the genetic diversity and structure of harvested species is still incipient, especially regarding savanna species. To evaluate the

impact of bark and latex harvesting on the genetic diversity of *H. drasticus* populations, we developed a set of highly polymorphic microsatellite markers, which can be used to determine the genetic diversity and structure, spatial genetic structure, and gene flow of *H. drasticus* populations and to perform paternity analysis. The genetic information obtained with these novel markers will be applied in a sustainable management plan for the species and may also be employed in conservation and genetic improvement programs.

## METHODS AND RESULTS

Genomic DNA was isolated from dried leaves of an individual *H. drasticus* tree, which were sampled in northeastern Brazil (07°23'51"S, 39°20'02"W) using a DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA). A microsatellite-enriched library was constructed according to the protocol described by Billotte et al. (1999). Genomic DNA samples were digested with the *RsaI* restriction enzyme, and the resulting DNA fragments were linked to *RsaI* adaptors. A library was enriched for dinucleotide sequences using (CT)<sub>8</sub> and (GT)<sub>8</sub> biotin-labeled probes bound to Streptavidin MagneSphere Paramagnetic Particles (Promega, Fitchburg, Wisconsin, USA). Selected DNA fragments were PCR-amplified using primer sequences complementary to the adaptors, and the amplicons were cloned into a pGEM-T Easy (Promega) vector. Plasmids were transformed into *Escherichia coli* XL1-Blue competent cells, and positive clones were selected using the β-galactosidase gene and grown overnight in an HM/FM medium with ampicillin. A total of 96 positive clones were bidirectionally sequenced in an automated ABI 377 sequencer (Applied Biosystems, Foster City, California, USA) using T7 and SP6 primers and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

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TABLE 1. Characteristics of nine microsatellite primers developed for *Himatanthus drasticus*. Depicted for each primer pair are the forward and reverse sequences, repeat motifs, sizes of the original fragments (bp), annealing temperatures when analyzed individually ( $T_a$ ), and GenBank accession numbers.

Primer	Primer sequence (5'–3')	Repeat motif	Size	$T_a$ (°C)	GenBank Accession No.
HD1	F: TTGCTTTTTGTGCTGAGAGG R: CACCAATCCTTGATTCCAGA	(TG) <sub>4</sub> CAG(TG) <sub>4</sub>	155	55	HQ423414
HD3	F: TGGTCTTTCCAGCAGTTGT R: GCGAAAAGTGTAGCACTGGA	(GA) <sub>13</sub>	253	55	HQ423416
HD7	F: CTTGTAGGTAACCGCTTTCACG R: CAAAGTGGCTGACGTTTGG	(CT) <sub>9</sub>	142	57	HQ423419
HD9	F: CTGCTTTGAGTCCATAACCTG R: GGTCCAGCCGATATGATTGT	(GT) <sub>6</sub> (TA) <sub>4</sub>	167	57	HQ423420
HD11	F: CCGCCAATTTCTCTTCCCTCT R: ACCACCACCATCCACTTCAT	(CT) <sub>8</sub> T(TC) <sub>12</sub>	274	53	HQ423421
HD14	F: AGGGAGGGTGGAGAGAGAAG R: TTCGGCACGCTCTATATCCT	(AG) <sub>10</sub>	255	57	HQ423424
HD18	F: CAAGCTTGAATTAACAACCA R: GCATTGCCATTCATCTAGCA	(AAT) <sub>4</sub>	250	57	HQ423426
HD19	F: TGGCAATACACTGTCTCAAGTC R: TCTGGAAAAGCAGCAGAAG	(TG) <sub>9</sub> (GC) <sub>4</sub>	233	60	HQ423427
HD21	F: GCACCTAATTTCCCTACCTC R: CATCTATCTACCGTCAGCA	(AC) <sub>9</sub> AT <sub>4</sub>	285	57	HQ423428

The sequences were assembled and edited using SeqMan software (DNA-STAR, Madison, Wisconsin, USA). MICROSAT software (A. M. Risterucci, CIRAD, personal communication) was employed to eliminate adaptors and restriction sites from the sequences. The repetitive regions were identified using the Simple Sequence Repeat Identification Tool (Temnykh et al., 2001), and primer pairs were designed using Primer3Plus software (Untergasser et al., 2007). After evaluation of the patterns of amplified fragments, nine of the 15 developed markers were selected (Table 1) and the forward primer from each pair was labeled with various fluorochromes (6-FAM, HEX, and TET). The remaining loci were discarded due to amplification failures, nonspecific amplification patterns, or unexpected product sizes.

Microsatellite loci were amplified through PCR performed in 15  $\mu$ L total volume containing 5.0 ng of template DNA, 0.8 mM of each forward and reverse primer, 20 mM Tris–HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl, 0.15 mM of each dNTP, and 1 U of *Taq* DNA polymerase. A PTC-100 thermal cycler (MJ Research, Waltham, Massachusetts, USA) was used for PCR with the following program: 96°C for 1 min followed by 30 cycles of denaturation at 94°C for 1 min, 1 min at a specific annealing temperature ( $T_a$ ), and a final extension of 72°C for 5 min. Amplification products were verified by electrophoresis on 3% agarose gels containing 0.1 mg ethidium bromide per mL in 1 $\times$  TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.0) and were genotyped on 6% denaturing polyacrylamide gels stained with silver nitrate. Amplicons were electrophoretically separated in an ABI 377 automated sequencer (Applied Biosystems) using the GS500 TAMRA marker as the size standard (Applied Biosystems). Fragment size and allele identification were determined using Genescan v. 3.1.2 and Genotyper v. 2.5.2 software (Applied Biosystems). Two populations were sampled for genetic diversity analysis: Population 1 (07°18'40"S/39°27'48"W; n = 51) and Population 2 (7°23'58"S/39°20'00"W; n = 56). Descriptive statistics and tests of Hardy–Weinberg equilibrium were performed using Arlequin 3.0 analysis (Excoffier et al., 2005). Cross-species amplification was evaluated on *H. obovatus* (Müll. Arg.) Woodson and *Plumeria rubra* L.

All of the loci were polymorphic and presented a varied degree of diversity (Table 2). The average number of alleles was 8.5, ranging from two to 14 alleles per locus. Observed and expected heterozygosities ranged from 0.018 to 0.774 (0.456 on average) and from 0.018 to 0.868 (0.601 on average), respectively. The fixation index presented values between –0.020 and 0.491 (0.215 on average). Six loci in Population 1 and eight in Population 2 significantly departed from the Hardy–Weinberg equilibrium ( $P < 0.05$ ). The excess of homozygotes detected in this finding may be related to a partial self-incompatibility and mating among relatives.

Five of the nine loci were positively amplified in *H. obovatus* (55.5%). Some of the primer pairs that demonstrated specificity to *H. drasticus* (HD1, HD18, HD19, and HD21) may also be useful in differentiating these two species, which can coexist in some regions. For *P. rubra*, cross-amplification failed for

the majority of loci, and locus HD1 alone was amplified in this species. These results were expected, as the species belongs to another genus of the Apocynaceae family.

## CONCLUSIONS

The microsatellite loci isolated and characterized in this work are potentially useful tools for genetic studies of *H. drasticus* populations. These microsatellites can be used to analyze genetic diversity, mating systems, and gene flow; to fine-scale spatial genetic structure; and to perform paternity analysis. All

TABLE 2. Results of initial primer screening in populations of *Himatanthus drasticus*. Shown for each primer pair are the number of alleles ( $A$ ), number of effective alleles ( $A_e$ ), values of observed ( $H_o$ ) and expected ( $H_e$ ) heterozygosity, fixation index ( $F$ ), and  $P$  values for Hardy–Weinberg equilibrium tests (HW) using the Markov chain method with 100,000 dememorization steps. The sample size for each population is exhibited in parentheses.

Population	Primer	$A$	$A_e$	$H_o$	$H_e$	$F$	HW
Pop 1 (n = 51)	HD1	2	1.040	0.039	0.038	–0.020	1.000
	HD3	12	5.255	0.412	0.810	0.491	0.000
	HD7	4	2.088	0.333	0.521	0.360	0.000
	HD9	2	1.963	0.510	0.491	–0.039	1.000
	HD11	12	6.208	0.529	0.839	0.369	0.000
	HD14	13	5.513	0.468	0.819	0.427	0.005
	HD18	5	2.021	0.510	0.505	–0.009	0.053
	HD19	8	3.217	0.686	0.689	0.004	0.024
	HD21	5	3.102	0.451	0.678	0.334	0.000
	Pop 2 (n = 56)	HD1	12	1.018	0.018	0.018	–0.009
HD3		5	6.892	0.679	0.855	0.206	0.018
HD7		8	1.585	0.214	0.369	0.420	0.000
HD9		5	2.116	0.393	0.527	0.255	0.001
HD11		13	7.593	0.571	0.868	0.342	0.000
HD14		14	5.381	0.774	0.814	0.005	0.005
HD18		9	3.640	0.679	0.725	0.064	0.001
HD19		13	3.325	0.407	0.699	0.417	0.000
HD21	11	3.502	0.536	0.714	0.250	0.000	

of this information can be applied to a sustainable management plan for this species. Because cross-tests were positive at certain sequences, the primers might be broadly applicable for genetic studies in *H. obovatus*. These results also indicate a high level of genetic diversity in the studied populations, suggesting that conservation in situ may be an important strategy for the species.

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APPENDIX 1. Taxa used in this study. Specimens were deposited in the Universidade de Brasília herbarium (UB) and the Universidade Estadual de Campinas herbarium (UEC). Information presented: taxon, specimen voucher, collection locale.

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*Himatanthus drasticus*—UB 222, Crato

*Himatanthus obovatus*—UEC 5013, Chapada dos Guimarães

*Plumeria rubra*—UEC 496, Campinas

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