Development and Characterization of 32 Microsatellite Loci in Genipa americana (Rubiaceae)

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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF 32 MICROSATELLITE LOCI IN *GENIPA AMERICANA* (RUBIACEAE)[1]

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- *Premise of the study:* Microsatellite primers were developed for the tree species *Genipa americana* (Rubiaceae) for further population genetic studies.
- *Methods and Results:* We identified 144 clones containing 65 repeat motifs from a genomic library enriched for $(CT)_8$ and $(GT)_8$ motifs. Primer pairs were developed for 32 microsatellite loci and validated in 40 individuals of two natural *G. americana* populations. Seventeen loci were polymorphic, revealing from three to seven alleles per locus. The observed and expected heterozygosities ranged from 0.24 to 1.00 and from 0.22 to 0.78, respectively.
- *Conclusions:* The 17 primers identified as polymorphic loci are suitable to study the genetic diversity and structure, mating system, and gene flow in *G. americana*.

Key words: conservation genetics; *Genipa americana*; microsatellite markers; population genetics; Rubiaceae; tree species.

Genipa americana L. (Rubiaceae) is widespread throughout Brazil and tropical America, both in plantations and in the wild (Durigan and Nogueira, 1990). It is a dioecious tree species (Mielke et al., 2003) and is used in forest gallery restoration (Salvador, 1986) and in mixed plantings of swampy and degraded permanent preservation areas, because of its high tolerance to flooding (Lorenzi, 2002). As a result of habitat fragmentation and damage caused by human activities, the remaining natural populations of *G. americana* currently occur in small forest fragments and protected parks. This represents a potentially serious long-term threat to global biodiversity. Thus, it is necessary to understand the effects of forest fragmentation on the genetic diversity and structure, mating system, and gene flow of the species for conservation and sustainable use. Therefore, assessment of genetic studies using molecular markers is urgently needed, particularly for the conservation of genetic resources of the remaining populations. For this purpose, the development of microsatellite markers for the species is useful, due their high level of polymorphism, and will create new opportunities for conservation research that can be used to minimize the negative implications of population fragmentation. In this study, we created a set of 17 polymorphic microsatellite loci for *G. americana* that were confirmed to be reproducible in assessing its genetic diversity, mating system, and gene flow.

METHODS AND RESULTS

Fresh *G. americana* leaves were collected from a single individual to be used in the initial DNA extraction, using the protocol proposed by Doyle and Doyle (1990). A microsatellite-enriched genomic library was constructed following the protocol by Billotte et al. (1999), with slight modifications. Genomic DNA was digested with *Rsa*I enzyme (Invitrogen, Carlsbad, California, USA) for 3 h incubation at 37°C, and the digested fragments were enriched in microsatellite fragments using $(CT)_8$ and $(GT)_8$ motifs. Digested fragments were then ligated to the double-stranded *Rsa*I adapters Rsa21 (5′-CTCTTGCTTACGCGTGGACTA-3′) and Rsa25 (5′-TAGTCCACGCGTAAGCAAGAGCACA-3′) for 2 h incubation at 20°C. Hybridized DNA was captured by streptavidin-coated magnetic probe beads (MagneSphere Magnetic Separation Products, Promega Corporation, Madison, Wisconsin, USA). The enriched fragments were amplified by PCR, the product was cloned into pGEM-T Easy Vector (Promega Corporation), and ligation products were used to transform Epicurian Coli XL1-Blue *Escherichia coli*–competent cells (Stratagene, Agilent Technologies, Santa Clara, California, USA). A total of 192 recombinant colonies were obtained, and 144 were selected and sequenced in an automated ABI 3500xL Genetic Analyzer (Perkin Elmer–Applied Biosystems, Foster City, California, USA) using T7 and SP6 primers and the BigDye Terminator version 3.1 Cycle Sequencing Kit (Perkin Elmer–Applied Biosystems); 65 of these contained microsatellite motifs. Dinucleotide motifs were the most abundant, followed by mono-, tetra-, tri-, and pentanucleotide motifs (approximately 80.49%, 10.97%, 4.88%, 2.44%, and 1.22%, respectively). Oligonucleotides complementary to the genomic sequences flanking the microsatellite region were designed using Primer3Plus (Untergasser et al., 2007) according to the following criteria: size of primers preferably between 18 and 22 bp; melting temperature (T_m) between 45°C and 60°C; amplified product length between 100 and 300 bp; and GC content between 40% and 60%. Developed primer pairs were assessed using 40 samples by PCR amplification on a Mastercyler thermocycler (Eppendorf, Hamburg, Germany) in a final volume of 16 µL using GoTaq Colorless Master Mix (Promega Corporation) containing 6.0 µL

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TABLE 1. Characteristics of 32 microsatellite loci developed in *Genipa americana*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	T_m (°C)	GenBank accession no.
Gam01	F: CATTCCACATTTGCCCTTG R: GCTTTCCTGTTCCCTAAATCC	$(TC)_{16}$	175	60	KF031152
Gam02	F: GCACCAGAGTCTAAAGCCAGA R: TGCACGAGTTCATTGAGATTG	$(CT)_8(TC)_{16}$	186	60	KF031153
Gam03	F: TGAAATTGCCTCTTACACACAC R: AACCACTTCCTTGAACACTGC	$(AC)_9(AT)_5$	245	59	KF031154
Gam06	F: CTCTGCGTGTGTGTGTGTGT R: CAAAGACTGTGCGGCATCT	$(CA)_{10}$	150	60	KF031155
Gam08	F: ACGGCTTTTCTTGTCTGGTC R: GGCATAATCCAAACCTGCTA	$(CCT)_8$	175	59	KF031156
Gam10	F: GGCTCAATGGGTGGCTTA R: TATAAGGGGTCAGAGGAAAACA	$(AC)_8$	150	60	KF611785
Gam11	F: AGCCACTACCACCAGTCCAT R: GGAGACCGAGTGTTACATTTCA	$(TC)_5(AC)_8$	215	59	KF031157
Gam12	F: ACGTGTGTGTGTGTGTGTATTG R: TGCCCTCCTATATTTTTCCTCT	$(GT)_8(GA)_5$	178	58	KF611786
Gam14	F: AAGCGTGTTGACAATGCTG R: CTTCAAGCCAGTCACCTTCC	$(CT)_{19}(CA)_{10}$	198	60	KF611787
Gam16	F: CCGAGTCCTATTTAGTCGCATT R: CCGCTTTGCTTCTATCACAC	$(TG)_6(TG)_6$	158	60	KF031158
Gam20	F: TGTGTGTATGTGTGGGTGTGT R: ACTTGAACCCCTCTCCAAAA	$(TG)_6$	234	59	KF031159
Gam21	F: TGGGTTGAGTAGGAAGAGAGC R: TTGTTCTCCTTTTGCTCGTG	$(AC)_9$	249	59	KF031160
Gam23	F: GGAAAAAGCCGAATGTTAGTC R: TGGGAGCATAAAAGCCTAGAA	$(GCAC)_4(AC)_8(GA)_{29}$	249	59	KF611788
Gam24	F: TGTCCAAAAACCAGAAATC R: CAGCTGGAATAAGAAAATGA	$(GA)_{29}$	189	53	KF031161
Gam25	F: GAAGTTCAGTTAAGACGCATCA R: GAAAACTGTGGAAGCAATGG	$(AC)_8$	238	58	KF611789
Gam26	F: ACTACAAAGGCAGGTGGTCTC R: GTGTCGGGAATGAAAGAAACT	$(TC)_5(CA)_8$	223	58	KF611790
Gam27	F: CCAGCCGTAGTTGAAAAAGA R: ACAAGGCGGTGGAGTTCT	$(AC)_6(AC)_7(AT)_6$	260	58	KF611791
Gam28	F: ACTCAGTCAACCTCCGAA R: TACCCAAAAGATTCAGCC	$(AC)_8$	226	54	KF031162
Gam30	F: TAATGGGGCCTTCAATAAGTT R: CAGGTATGCATTGGAAACACA	$(TG)_6$	241	58	KF611792
Gam31	F: TTTGGCCAATATTTACTGAT R: TTCAATGGTTCATCCTCC	$(AG)_8$	267	54	KF031163
Gam32	F: ATGAGGGAACGCTGCCGAAG R: GGTGGAGGAGGGGGAGGAAG	$(CCT)_8$	174	67	KF031164
Gam33	F: TGTTTCTTTCTCTGCCAT R: GAAAAAGACAACCATACCTT	$(AC)_5$	118	51	KF031165
Gam34	F: GATGATTGCCGATTAGTG R: TCATTAGTATCCATCCCTTAC	$(AG)_9(AG)_5(AAAAC)_3$	213	52	KF611793
Gam35	F: TTCTAACAAATCCTATCTAA R: GCTACTCACAAAAATGAT	$(AC)_5$	214	45	KF611794
Gam36	F: TGACTTGGTGCTGTGAGACGAG R: TCAAAATCCTCCCCGCCTT	$(AC)_5$	214	64	KF031166
Gam37	F: GGACGCTAAAGAGCAAGAGG R: CACAGCAAGGATGGAGCA	$(TG)_8$	209	59	KF611795
Gam38	F: GCATAAATGACCTACACA R: GTTGCTACTTTATCCTCTT	$(GAAA)_3$	140	47	KF031167
Gam39	F: GAACTCGATGGAAATCAG R: CAACTTAATGTGTCTGTAGGA	$(AC)_{10}$	132	51	KF611796
Gam40	F: TGTTCCGTTTGAAACCCTTG R: GAAGCCTAGAACGCATCAGACT	$(TG)_7$	279	61	KF611797
Gam41	F: TCTTGATTACTGGATGACAGAC R: CCTTCACTGCTTCACACAC	$(AC)_{10}$	132	55	KF031168
Gam42	F: ATTTGGCTAGCATTTCTTCTCA R: GCATTTTCTTTAGTGGTCGTGT	$(TC)_7(TG)_7(GA)_8(GA)_5$	185	58	KF611798
Gam43	F: AGCACCTTACCCCTTTCATA R: GCACTTCATTTTCTTTCATTTC	$(AC)_7(AC)_6(AC)_6$	269	56	KF611799

Note: T_m = melting temperature.

[a]All values are based on two populations located in Selvíria, Mato Grosso do Sul (22°22′02″S, 51°25′08″W), and Mogi-Guaçu, São Paulo (22°17′25″S, 47°10′55″W), Brazil.

TABLE 2. Results of initial polymorphic microsatellite marker screening in two populations of *Genipa americana*.[a]

	Selvíria (N = 19)				Mogi Guaçu (N = 21)			
Locus	A	H_o	H_e	F	A	H_o	H_e	F
Gam01	3	0.866	0.590	–0.468*	6	1.000	0.783	–0.277*
Gam02	5	0.789	0.624	–0.265*	5	0.889	0.662	–0.343*
Gam03	4	0.579	0.652	0.112*	5	0.471	0.561	0.161*
Gam06	8	0.750	0.750	0	6	0.714	0.728	0.019*
Gam08	4	0.579	0.623	0.070*	4	0.400	0.459	0.129*
Gam11	4	0.789	0.564	–0.399*	7	0.951	0.678	–0.402*
Gam16	5	0.895	0.642	–0.394*	3	0.842	0.548	–0.536*
Gam20	5	0.883	0.688	–0.283*	4	0.867	0.676	–0.282*
Gam21	4	0.555	0.668	0.169*	3	0.368	0.468	0.213*
Gam24	8	0.631	0.700	0.098*	5	0.476	0.670	0.290*
Gam28	4	0.477	0.560	0.149*	4	0.300	0.612	0.510*
Gam31	3	0.850	0.595	–0.429*	5	0.857	0.644	–0.331*
Gam32	3	0.524	0.629	0.167*	5	0.571	0.468	–0.221*
Gam33	5	0.500	0.650	0.231*	6	0.619	0.483	–0.281*
Gam36	3	0.429	0.581	0.262*	2	0.571	0.481	–0.188*
Gam38	4	0.579	0.640	0.096*	4	0.239	0.223	–0.070*
Gam41	6	0.619	0.671	0.078*	4	0.572	0.670	0.147*
Mean	4.58	0.664	0.637	–0.043	4.58	0.630	0.577	–0.091

Note: A = number of alleles per locus; F = fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity; N = sample size for each population.

* Significant departures from Hardy–Weinberg equilibrium at $P < 0.05$.

[a] All values are based on two populations located in Selvíria, Mato Grosso do Sul (22°22′02″S, 51°25′08″W), and Mogi-Guaçu, São Paulo (22°17′25″S, 47°10′55″W), Brazil.

GoTaq Colorless Master Mix, 10 µM of each primer (F and R), 2.7 µL of nuclease-free water, and 7.5 ng of template DNA. The PCR program for all primers consisted of 5 min of initial denaturation at 95°C followed by 30 cycles of denaturation at 95°C for 45 s, a primer-specific annealing temperature (Table 1) for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The amplification products were checked with 3% agarose gels, separated in 6% denaturing polyacrylamide gels, and visualized by silver-staining. We used a 10-bp DNA ladder (Invitrogen) as a size standard for allele scoring. The number of alleles per locus (A), the observed (H_o) and expected heterozygosities (H_e), fixation index (F), and genotypic disequilibrium were estimated for each population using the FSTAT program (Goudet, 2002). To test whether the F values and the genotypic disequilibrium were significantly different from zero, we used 1000 Monte Carlo permutations (alleles among individuals) and a Bonferroni correction (95%, $\alpha = 0.05$).

For the polymorphism evaluation, we sampled a total of 40 adult *G. americana* trees from two populations 580 km apart—21 individuals from a small forest fragment in the Ecological Station of Mogi-Guaçu (São Paulo State, Brazil) and 19 individuals from a natural population in Selvíria (Mato Grosso do Sul State, Brazil). Of the 32 designed primer pairs, 17 were polymorphic and 15 were monomorphic in the analyzed populations. In the Selvíria population, among the polymorphic microsatellites, the number of alleles ranged from three (Gam01 and Gam31) to eight (Gam06 and Gam24), with an average of 4.58 alleles per locus (Table 2). In the Mogi-Guaçu population, the number of alleles ranged from two (Gam36) to seven (Gam11), with an average of 4.58 alleles per locus (Table 2). For the Selvíria population, H_o and H_e ranged from 0.43 to 0.89 and from 0.56 to 0.75, respectively, and in Mogi Guaçu these ranged from 0.24 to 1.00 and from 0.22 to 0.78, respectively. The Hardy–Weinberg equilibrium was tested in both populations. All loci were in disequilibrium (except the locus Gam06 in the Selvíria population and the average populations), most likely due to genetic drift caused by forest fragmentation.

CONCLUSIONS

The molecular markers described here are the first microsatellite loci isolated for *G. americana*. The microsatellite loci showed median levels of polymorphism in the two populations analyzed. Based on this, our data suggest that the developed microsatellite markers may constitute new tools for population genetic studies, such as genetic diversity, spatial genetic structure, mating system, and gene flow of *G. americana*. These studies will produce valuable information for managing fragmented populations, including information for breeding, conservation, and reforestation plans.

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