



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

Authors: Manoel, Ricardo O., Freitas, Miguel L. M., Barreto, Mariana A., Moraes, Mário L. T., Souza, Anete P., et al.

Source: Applications in Plant Sciences, 2(3)

Published By: Botanical Society of America

URL: <https://doi.org/10.3732/apps.1300084>

BioOne Complete (complete.BioOne.org) is a full-text database of 200 subscribed and open-access titles in the biological, ecological, and environmental sciences published by nonprofit societies, associations, museums, institutions, and presses.

Your use of this PDF, the BioOne Complete website, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at www.bioone.org/terms-of-use.

Usage of BioOne Complete content is strictly limited to personal, educational, and non - commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

BioOne sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.

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

RICARDO O. MANOEL^{2,5}, MIGUEL L. M. FREITAS³, MARIANA A. BARRETO⁴,
MÁRIO L. T. MORAES², ANETE P. SOUZA⁴, AND ALEXANDRE M. SEBBENN³

²Faculdade de Engenharia de Ilha Solteira, Universidade Estadual Paulista (UNESP), Av. Brasil Centro 56, CP 31, Ilha Solteira, São Paulo 15385-000, Brazil; ³Instituto Florestal de São Paulo, CP 1322, São Paulo, São Paulo 01059-970, Brazil; and ⁴Centro de Biologia Molecular e Engenharia Genética, Universidade Estadual de Campinas (UNICAMP), CP 6010, Campinas, São Paulo 13083-970, Brazil

- *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 *RsaI* 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 *RsaI* adapters Rsa21 (5'-CTCTT-GCTTACGCGTGGACTA-3') and Rsa25 (5'-TAGTCCACGCGTAAGCAA-GAGACA-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 Mastercycler thermocycler (Eppendorf, Hamburg, Germany) in a final volume of 16 µL using GoTaq Colorless Master Mix (Promega Corporation) containing 6.0 µL

¹Manuscript received 30 October 2013; revision accepted 3 December 2013.

This research was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; 2010/19613-4) and by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; 473677/2010-5). The author would like to thank FAPESP for financial support provided to R.O.M. (scholarship no. 2011/01518-8).

⁵Author for correspondence: rickom.is@gmail.com

doi:10.3732/apps.1300084

Applications in Plant Sciences 2014 2(3): 1300084; <http://www.bioone.org/loi/apps> © 2014 Manoel et al. Published by the Botanical Society of America. This work is licensed under a Creative Commons Attribution License (CC-BY-NC-SA).

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

Locus	Primer sequences (5'–3')	Repeat motif	Allele size (bp)	<i>T_m</i> (°C)	GenBank accession no.
Gam01	F: CATTCCACATTGCCCCCTTG R: GCTTTCCTGTTCCCTAAATCC	(TC) ₁₆	175	60	KF031152
Gam02	F: GCACCAGAGTCTAAAGCCAGA R: TGCACGAGTTCATTGAGATTG	(CT) ₈ (TC) ₁₆	186	60	KF031153
Gam03	F: TGAAATTGCCTCTTACACACAC R: AACCACCTTCCTTGAACACTGC	(AC) ₉ (AT) ₅	245	59	KF031154
Gam06	F: CTCTGCGTGTGTGTGTGTGT R: CAAAGACTGTGCGGCATCT	(CA) ₁₀	150	60	KF031155
Gam08	F: ACGGCTTTTCTGTCTGGTC R: GGCATAATCCAACCTGCTA	(CCT) ₈	175	59	KF031156
Gam10	F: GGCTCAATGGGTGGCTTA R: TATAAGGGGTCAGAGGAAAACA	(AC) ₈	150	60	KF611785
Gam11	F: AGCCACTACCACAGTCCAT R: GGAGACCGAGTGTTACATTCA	(TC) ₅ (AC) ₈	215	59	KF031157
Gam12	F: ACGTGTGTGTGTGTGTATATG R: TGCCCTCCTATATTTTCTCTCT	(GT) ₈ (GA) ₅	178	58	KF611786
Gam14	F: AAGCGTGTGACAATGCTG R: CTTCAAGCCAGTCACCTTCC	(CT) ₁₉ (CA) ₁₀	198	60	KF611787
Gam16	F: CCGAGTCCTATTATGTCGCATT R: CCGCTTTGCTTCTATCACAC	(TG) ₆ (TG) ₆	158	60	KF031158
Gam20	F: TGTGTGTATGTGTGGGTGTGT R: ACTTGAACCCCTCTCCAAAA	(TG) ₆	234	59	KF031159
Gam21	F: TGGGTGAGTAGGAAGAGAGC R: TTGTCTCCTTTTGCTCGTG	(AC) ₉	249	59	KF031160
Gam23	F: GGAAAAAGCCGAATGTTAGTC R: TGGGAGCATAAAAGCCTAGAA	(GCAC) ₄ (AC) ₈ (GA) ₂₉	249	59	KF611788
Gam24	F: TGTCAAAAAACCAGAAATC R: CAGCTGGAATAAGAAAATGA	(GA) ₂₉	189	53	KF031161
Gam25	F: GAAGTTCAGTTAAGACGCATCA R: GAAAACCTGTGGAAGCAATGG	(AC) ₈	238	58	KF611789
Gam26	F: ACTACAAAGGCAGGTGGTCTC R: GTGTGCGGAATGAAAGAACT	(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: TACCCAAAAGATTGAGCC	(AC) ₈	226	54	KF031162
Gam30	F: TAATGGGGCCTTCAATAAGTT R: CAGGTATGCATTGGAACACA	(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: TGTTCCGTTTGAAACCTTG R: GAAGCCTAGAACGCATCAGACT	(TG) ₇	279	61	KF611797
Gam41	F: TCTTGATTACTGGATGACAGAC R: CCTTCACTGCTTCACACAC	(AC) ₁₀	132	55	KF031168
Gam42	F: ATTTGGCTAGCATTCTTCTCA R: GCATTTTCTTTAGTGGTCGTGT	(TC) ₇ (TG) ₇ (GA) ₈ (GA) ₅	185	58	KF611798
Gam43	F: AGCACCTTACCCTTTCATA R: GCACTTCATTTCTTTCATTTCT	(AC) ₇ (AC) ₆ (AC) ₆	269	56	KF611799

Note: *T_m* = melting temperature.

^aAll 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

Locus	Selvária (N = 19)				Mogi Guaçu (N = 21)			
	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 Selvária, 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 Selvária (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 Selvária 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 Selvária 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 Selvária 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.

LITERATURE CITED

- BILLOTTE, N., P. J. L. LAGODA, A. M. RISTERUCCI, AND F. C. BAURENS. 1999. Microsatellite-enriched libraries: Applied methodology for the development of SSR markers in tropical crops. *Fruits* 54: 277–288.
- DOYLE, J. J., AND J. L. DOYLE. 1990. Isolation of plant DNA from fresh tissue. *Focus (San Francisco, Calif.)* 12: 13–15.
- DURIGAN, G., AND J. C. B. NOGUEIRA. 1990. Recomposição de Matas Ciliares. *Série Registros Instituto Florestal de São Paulo* 4: 1–14.
- GOUDET, J. 2002. FSTAT version 2.9.3.2, a program to estimate and test gene diversities and fixation index. Institute of Ecology, Lausanne, Switzerland. Website <http://www2.unil.ch/popgen/softwares/fstat.htm> [accessed 10 July 2013].
- LORENZI, H. 2002. Árvores Brasileiras: Manual de identificação e cultivos de plantas arbóreas do Brasil, 2nd ed. Instituto Plantarum, Nova Odessa, São Paulo, Brazil.
- MIELKE, M. S., A. F. ALMEIDA, F. P. GOMES, M. A. G. AGUILAR, AND P. A. O. MANGABEIRA. 2003. Leaf gas exchange, chlorophyll fluorescence and growth responses of *Genipa americana* seedlings to soil flooding. *Environmental and Experimental Botany* 50: 221–231.
- SALVADOR, J. L. G. 1986. Comportamento de espécies florestais nativas em áreas de depleção de reservatórios. *IPEF* 33: 73–78.
- UNTERGASSER, A., H. NIJVEEN, X. RAO, T. BISSELING, R. GEURTS, AND J. A. M. LEUNISSEN. 2007. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research* 35(Supplement 2): W71–W74.