Applications in Plant Sciences

Applications in Plant Sciences 2013 1(6): 1200493

Primer Note

Microsatellite markers for *Cariniana legalis* (Lecythidaceae) and their transferability to *C. estrellensis*[1]

Evandro Vagner Tambarussi[2,4], Alexandre Magno Sebbenn[3], Maria Andréia Moreno[2], Elza Martins Ferraz[2], Paulo Yoshio Kageyama[2], and Roland Vencovsky[2]

[2]Escola Superior de Agricultura "Luiz de Queiroz," Universidade de São Paulo, Av. Pádua Dias 11, CP 9, Piracicaba, São Paulo 13418-900, Brazil; and [3]Instituto Florestal de São Paulo, CP 1322, São Paulo, São Paulo 01059-970, Brazil

- *Premise of the study:* Microsatellite primers were developed in the neotropical tree species *Cariniana legalis* (Lecythidaceae) to investigate its genetic diversity, mating system, and gene flow.
- *Methods and Results:* We identified 96 clones containing 82 repeat motifs from a genomic library enriched for $(CT)_8$ and $(GT)_8$ motifs. Primer pairs were developed for 13 microsatellite loci and validated in 51 *C. legalis* specimens and 26 *C. estrellensis* specimens. Eleven loci were polymorphic, revealing a maximum of two to 15 alleles per locus in *C. legalis* and three to 12 in *C. estrellensis*. For *C. legalis*, the observed (H_o) and expected (H_e) heterozygosities ranged from 0 to 0.99 and from 0.07 to 0.90, respectively. For *C. estrellensis*, H_o and H_e ranged from 0 to 0.96 and from 0.14 to 0.91, respectively.
- *Conclusions:* The primers identified polymorphic loci that are suitable to study genetic diversity and structure, mating system, and gene flow in *C. legalis* and the related species *C. estrellensis*.

Key words: Brazilian Atlantic forest; *Cariniana legalis*; conservation genetics; microsatellite markers; population genetics; tropical tree species.

Cariniana Casar. (Lecythidaceae) is a genus of trees native to South America, and many of these tree species are harvested for timber. *Cariniana legalis* (Mart.) Kuntze and *C. estrellensis* (Raddi) Kuntze are two endangered tropical trees with wind-dispersed seeds that are pollinated by bees (Carvalho, 1994). These species are endemic to the Atlantic Forest in Brazil and have a low population density (less than 1 tree/ha). However, the Atlantic Forest has become increasingly fragmented in recent centuries, and today just 12–16% of the original forest remains (Ribeiro et al., 2009). Forest fragmentation isolates and decreases the natural populations of these trees. These changes affect gene flow among populations and can increase the selfing rate, correlated matings, genetic structure, and the relatedness in subsequent generations (Jump and Penuelas, 2006; O'Connell et al., 2006). Recent advances in molecular techniques, such as microsatellite markers, have created new opportunities for conservation research that can be used to minimize the negative implications of population fragmentation. Here, we describe the development of 12 nuclear microsatellite markers for *C. legalis* and the transferability of these markers for studying *C. estrellensis*.

METHODS AND RESULTS

Total genomic DNA was extracted from fresh leaves collected from a single *C. legalis* tree using the protocol proposed by Doyle and Doyle (1987). A microsatellite-enriched genomic library was constructed following the protocol of Billotte et al. (1999). The *Rsa*I enzyme (Invitrogen, Carlsbad, California, USA) was used to digest genomic DNA from one genotype of *C. legalis*, enriched in microsatellite fragments using $(CT)_8$ and $(GT)_8$ motifs. These enriched fragments were cloned into pGEM-T Easy Vector (Promega Corporation, Madison, Wisconsin, USA), and ligation products were used to transform Epicurian Coli XL1-Blue *Escherichia coli*–competent cells (Stratagene, Agilent Technologies, Santa Clara, California, USA). Transformed cells were cultivated on agar plates containing 100 µg/mL ampicillin, 50 µg/mL X-galactosidase, and isopropyl β-D-1-thiogalactopyranoside (IPTG). Single white colonies were selected and stored at −80°C. A total of 96 recombinant colonies were obtained and sequenced using the adapters *Rsa*21 (5′-CTCTTGCTTACGCGTGGACTA-3′) and *Rsa*25 (5′-TAGTCCACGCGTAAGCAAGAGCACA-3′) in a 3730xl DNA Analyzer sequencer (Applied Biosystems, Foster City, California, USA) using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). Ninety-six positive clones from the library were sequenced, and the microsatellites were found in 82 of them. Dinucleotide motifs were the most abundant, followed by mono-, tetra-, tri-, and hexanucleotide motifs (approximately 75.6%, 14.6%, 5%, 2.4%, and 2.4%, respectively); pentanucleotide motifs were not found. Only 13 simple sequence repeat (SSR) markers were selected for primer design because their sequences presented more than five tandem repeats. Vector segments were removed from each of the sequences by VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). Thirteen pairs of primers were designed for SSR flanking regions using Primer3Plus (Untergasser et al., 2007) according to the following criteria: annealing temperature ranging from 52–60°C and GC content between 40% and 60%. Each primer pair was designed to amplify a fragment ranging between 150 and 300 bp. Microsatellite loci were amplified by PCR in a final volume of 15 µL using GoTaq Colorless Master Mix (Promega Corporation) containing 7.5 µL GoTaq Colorless Master Mix

[1]Manuscript received 17 September 2012; revision accepted 18 November 2012.

This research was supported by Fundação de Amparo à Pesquisa do Estado da São Paulo (FAPESP; 2010/10704-7) and by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; 470491/2010-8). The authors would like to thank FAPESP for financial support provided to E.V.T. (scholarship no. 2010/12354-3) and CNPq for financial support to A.M.S. and R.V.

[4]Author for correspondence: evtambarussi@yahoo.com.br

doi:10.3732/apps.1200493

Table 1. Microsatellite primers developed in *Cariniana legalis*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Size range (bp)[b]	Size range (bp)[c]	T_a (°C)	GenBank accession no.
Cle01	F: TTCTCTTCCCCCTCTTCCTC R: TCCTTTCCAAACCAAACCAC	$(AC)_{18}$	154–184	160–181	60	JX466851
Cle02	F: TCTCAAAACTCCCCCTCAAG R: CCGAAGAAATCATCACCTCA	$(AC)_{11}$	150–202	140–202	62	JX466852
Cle03	F: GCCTGTCTACTGATGCCAGA R: GTATTCCTTGGTTTCTTTGCTG	$(AC)_8$	222–241	202–230	62	JX466853
Cle04	F: CAAAGGTTGAGGGTATAAATGC R: GGGAGAACTATCCACATCAAGA	$(TG)_{10}T(TGTA)_5$	266–300	256–296	60	JX466854
Cle05	F: CAAGCCGCACCTTTATCTAT R: GCAGCCAAACAGGATAGCA	$(CA)_8$	188–226	186–232	62	JX466855
Cle06	F: CTCTCCTGCCATTGATTTTG R: ATGACTGACTCTAAATCTTG	$(TG)_8A(GA)_{11}$	198–230	176–202	60	JX466856
Cle07	F: GGGTAGTGACCAACAATCTCG R: ATGATGCTGCCAAGGTAATG	$(CA)_8$	150–160	148–208	56	JX466857
Cle08	F: GCAATCCTCCAAACAGCATT R: CCCTCTCTCCATGACCGTTA	$(AG)_{19}$	152–180	150–180	62	JX466858
Cle09	F: TGGGACAACACATCACAACC R: GAATGAATTGGGAGAAAGTG	$(GT)_{14}$	156–186	158–180	58	JX466859
Cle10	F: AAGTAGAAACCACCTGGCAGA R: CCCTATTTCATCCTCAGCAG	$(TG)_{16}$	156–170	160–182	60	JX466860
Cle11	F: ATGACGCTGATGATGCTGAA R: TGCTCCCTTCTGGCTACTTG	$(GT)_7$	226–230	224–230	58	JX466861
Cle12	F: GCCTTGTTAGATGTTGCCTGT R: TTGGTTAGTCTCCCTGTTAGC	$(AG)_{16}$	202–224	202–244	56	JX466862
Cle13	F: TGCCCAACTCAATTCTGAAAC R: TGACTTCTCCACCTTCAACG	$(TC)_{19}(AC)_8$	NA	NA	40–65	JX466863

Note: NA = not amplified; T_a = annealing temperature when run individually.

[a]All values are based on a variable number of samples representing two populations located in Ibicatu and Mogi-Guaçu, São Paulo, Brazil.

[b]Allele size found for *C. legalis*.

[c]Allele size found for *C. estrellensis*.

(2×), 10 µM of each primer (F and R), 3.0 µL Nuclease-Free Water, and 7.5 ng template DNA. The amplification program for all primers consisted of an initial denaturing step at 94°C for 1 min, followed by 35 cycles of amplification (94°C for 1 min, followed by 1 min at the specific annealing temperature of each primer pair [Table 1], 72°C for 1 min), and a final elongation step at 72°C for 10 min. Amplifications were performed with a Mastercycler (Eppendorf, Hamburg, Germany). Thirteen pairs of primers were designed, but only 12 yielded successfully amplified fragments. The Cle13 locus did not amplify despite using a considerable range of temperatures (40–65°C). The amplification products (2 µL of the total reaction volume) were separated on a Fragment Analyzer Automated CE System (Advanced Analytical Technologies [AATI], Ames, Iowa, USA) using the Quant-iT PicoGreen dsDNA Reagent Kit, 35–500 bp (Invitrogen). Raw data were analyzed using PROSize version 2.0 software (AATI).

We sampled a total of 51 adult *C. legalis* trees from two populations (25 from Floresta Estadual de Ibicatu and 26 from Floresta Estadual de Mogi-Guaçu) and 26 adult *C. estrellensis* trees from Floresta Estadual de Ibicatu in São Paulo State, Brazil (Appendix 1). Genetic diversity, fixation index (*F*), and linkage

Table 2. Results of initial primer screening in two populations of *Cariniana legalis* (Ibicatu and Mogi-Guaçu) and one population of *C. estrellensis* (Ibicatu) from São Paulo, Brazil.

	C. legalis[a] (*N* = 25)				*C. legalis*[b] (*N* = 26)				*C. estrellensis*[a] (*N* = 26)			
Locus	*A*	H_o	H_e	*F*	*A*	H_o	H_e	*F*	*A*	H_o	H_e	*F*
Cle01	10	0.60	0.81	0.262	15	0.58	0.88	0.347*	11	0.65	0.87	0.247*
Cle02	8	0.96	0.81	–0.18	11	0.92	0.87	–0.056	12	0.88	0.86	–0.029
Cle03	6	0.75	0.76	0.014	5	0.69	0.74	0.066	10	0.30	0.86	0.642*
Cle04	13	0.80	0.90	0.114	14	0.88	0.88	0.001	8	0.54	0.69	0.209
Cle05	7	0.99	0.69	–0.465*	10	0.96	0.86	–0.114	8	0.96	0.73	–0.316
Cle06	6	0.40	0.72	0.448*	7	0.65	0.81	0.196	9	0.27	0.84	0.678*
Cle07	4	0.76	0.56	–0.357	6	0.69	0.73	0.054	6	0.16	0.65	0.754*
Cle08	12	0.76	0.88	0.140	15	0.81	0.90	0.109	11	0.33	0.91	0.635*
Cle09	14	0.68	0.82	0.18	7	0.60	0.76	0.021*	8	0.38	0.72	0.462*
Cle10	8	0.76	0.78	0.024	7	0.96	0.80	–0.203	8	0.70	0.78	0.109
Cle11	2	0.00	0.22	1.000*	2	0.00	0.07	1.000*	3	0.00	0.14	1.00*
Cle12	10	0.46	0.82	0.448*	13	0.96	0.87	–0.104	9	0.51	0.76	0.322*
Mean	8.3	0.66	0.73	0.099*	9.3	0.72	0.77	0.029	8.5	0.47	0.73	0.352*

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]Ibicatu: 22°46′32″S, 47°49′03″W.

[b]Mogi-Guaçu: 22°17′25″S, 47°10′55″W.

disequilibrium were estimated for each species using the FSTAT program (Goudet, 2002). To test if *F* was significantly different from zero and to test linkage disequilibrium, we used 1000 Monte Carlo permutations (alleles among individuals) and a Bonferroni correction (95%, $\alpha = 0.05$). As noted above, of the 13 tested primers in both species, one (Cle13) showed no amplification (Table 1). The other 12 loci worked well for both species (Table 2). For *C. legalis*, 100 alleles were found (ranging among loci from two to 14, with an average of 8.3) from Ibicatu, and 112 alleles were found from Mogi-Guaçu (ranging among loci from two to 15, with an average of 9.3). For *C. estrellensis*, we identified 103 alleles (ranging from three to 12, with an average of 8.6) (Table 2). For *C. legalis*, the observed (H_o) and expected (H_e) heterozygosities ranged from 0 to 0.99 and from 0.22 to 0.90, respectively, for Ibicatu and from 0 to 0.96 and from 0.07 to 0.90, respectively, for Mogi-Guaçu. For *C. estrellensis*, H_o and H_e ranged from 0 to 0.96 and from 0.14 to 0.91, respectively. Following Bonferroni correction, *F* was significantly different from zero for three loci of *C. legalis* from Mogi-Guaçu and four loci and means over loci from Ibicatu. In *C. estrellensis*, significant *F* values were observed in eight loci and means over loci (Table 2). After Bonferroni correction, no linkage disequilibrium was detected in the studied populations. Of the 13 loci developed, only Cle11 and Cle13 were not useful due to low polymorphism and lack of amplification, respectively. The remaining 11 loci are suitable for studying genetic diversity and structure, mating system, gene flow, and parentage analysis in both species.

CONCLUSIONS

The microsatellite loci showed high levels of polymorphism in both *C. legalis* and *C. estrellensis*. Our data suggest that the microsatellite markers developed in this study are suitable for population genetic studies in both *Cariniana* species. We are currently using these markers for inferring genetic diversity, spatial genetic structure, and gene flow in *C. legalis*. These studies will produce valuable information for managing fragmented populations, including information for breeding, conservation, and reforestation plans.

Appendix 1. List of vouchers of *Cariniana legalis* and *C. estrellensis* used in this paper. Vouchers are deposited in the Herbarium of the Universidade de São Paulo, São Paulo, Brazil (ESA).

Code	Species	Country	Locality (State)	Voucher no.
IBI	*C. estrellensis*	Brazil	Ibicatu (SP)	120064
IBI	*C. legalis*	Brazil	Ibicatu (SP)	120065
MoG	*C. legalis*	Brazil	Mogi-Guaçu (SP)	120489

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.

Carvalho, P. E. R. 1994. Espécies Florestais Brasileiras: Recomendações silviculturais, potencialidades e uso da madeira. EMBRAPA-CNPF, Brasília, Brazil.

Doyle, J. J., and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemistry* 19: 11–15.

Goudet, J. 2002. FSTAT version 2.9.3.2, a program to estimate and test gene diversities and fixation indices. Institute of Ecology, Lausanne, Switzerland. Website http://www2.unil.ch/popgen/softwares/fstat.htm [accessed 20 August 2012].

Jump, A. S., and J. Penuelas. 2006. Genetic effects of chronic habitat fragmentation in a wind-pollinated tree. *Proceedings of the National Academy of Sciences, USA* 103: 8096–8100.

O'Connell, K. M., A. Mosseler, and O. P. Rajora. 2006. Impacts of forest fragmentation on the mating system and genetic diversity of white spruce (*Picea glauca*) at the landscape level. *Heredity* 97: 418–426.

Ribeiro, M. C., J. P. Metzger, A. C. Martensen, F. J. Ponzoni, and M. M. Hirota. 2009. The Brazilian Atlantic Forest: How much is left, and how is the remaining forest distributed? Implications for conservation. *Biological Conservation* 142: 1141–1153.

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.