New Microsatellite Loci for Prosopis alba and P. chilensis (Fabaceae)

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PRIMER NOTE

NEW MICROSATELLITE LOCI FOR *PROSOPIS ALBA* AND *P. CHILENSIS* (FABACEAE)[1]

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- *Premise of the study:* As only six useful microsatellite loci that exhibit broad cross-amplification are so far available for *Prosopis* species, it is necessary to develop a larger number of codominant markers for population genetic studies. Simple sequence repeat (SSR) markers obtained for *Prosopis* species from a 454 pyrosequencing run were optimized and characterized for studies in *P. alba* and *P. chilensis*.
- *Methods and Results:* Twelve markers that were successfully amplified showed polymorphism in *P. alba* and *P. chilensis*. The number of alleles per locus ranged between two and seven and heterozygosity estimates ranged from 0.2 to 0.8. Most of these loci cross-amplify in *P. ruscifolia*, *P. flexuosa*, *P. kuntzei*, *P. glandulosa*, and *P. pallida*.
- *Conclusions:* These loci will enable genetic diversity studies of *P. alba* and *P. chilensis* and contribute to fine-scale population structure, indirect estimation of relatedness among individuals, and marker-assisted selection.

Key words: Fabaceae; microsatellite; next-generation sequencing data; *Prosopis*.

Prosopis L. (Fabaceae) contains woody species of arid and semiarid areas of the Americas, Africa, and Asia. There is considerable taxonomic confusion about the American sect. *Algarobia* DC., due largely to the phenotypic variation within species and the low genetic differentiation and frequent hybridization among species (Hunziker et al., 1986).

Population genetic, conservation, and evolutionary studies using molecular markers have been generated over the past few years in *Prosopis* (Bessega et al., 2009, 2010). However, the lack of a sufficient number of simple sequence repeat (SSR) markers limits the conclusions that can be inferred. The only available microsatellite markers in *Prosopis* were six loci developed in *P. chilensis* (Molina) Stuntz (Mottura et al., 2005). They revealed cross-species affinity when tested in five species of sect. *Algarobia* and two of sect. *Strombocarpa* Benth. Although these markers proved to be useful loci for population studies in *P. alba* Griseb. (Bessega et al., 2009, 2010), at least 10 microsatellite loci are recommended to accurately estimate relatedness coefficients and diverse population parameters depending on parentage estimates (Wang et al., 2010). In the current study, we developed additional SSRs useful for population genetic analysis including effects of fragmentation on loss of diversity, fine-scale population structure, breeding system and pollen dispersal, and detection of recent bottlenecks in *P. alba* and *P. chilensis*, the most important native forest resources among Argentinean "algarrobos." Also, these SSRs will contribute to more accurate relationships estimates, and these estimates may be used to estimate in situ heritability estimates. Taking advantage of the high genetic similarity among species of sect. *Algarobia* (Bessega et al., 2005) and the observation of cross-amplification in several species of *Prosopis* (Mottura et al., 2005), we searched for possible microsatellite loci from 454 sequence data from a bulk sample of American mesquites introduced to Australia. We then tested their utility in these species and cross-amplification in five other species of sect. *Algarobia*.

METHODS AND RESULTS

We extracted DNA from a bulk collection of leaf material from Queensland, Australia, which originated from American species of sect. *Algarobia*. The sample included *P. pallida* (Humb. & Bonpl. ex Willd.) Kunth, *P. velutina* Wooton, *P. glandulosa* Torr., and putative hybrids involving these species (Appendix 1). Taxonomic identification is difficult due to the high hybridization rate of these invasive populations. Three micrograms of DNA was sent to the Australian Genome Research Facility (www.agrf.org.au/; St. Lucia, Queensland, Australia). The DNA was sheared and one-quarter plate was sequenced on a Roche GS FLX (454 Life Sciences, a Roche Company, Branford, Connecticut, USA) following the manufacturer's protocol. We screened the sequences obtained for perfect di-, tri-, and tetranucleotide repeats using MSATCOMMANDER version 0.8 (Faircloth, 2008). We used the option "Design primer," in which case the software searches for microsatellite repeats and identifies possible primer annealing sites in one step. Primer3 (Rozen and Skaletsky,

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2000) is implemented in MSATCOMMANDER for primer design according to the following criteria: amplification products within the size range of 100–500 bp, optimal melting temperature (range 57–62°C), optimal GC content of 50%, possession of at least 1-bp GC clamp, low levels of self- or pair-complementary, and maximum end stability (ΔG) of 8.0 (Faircloth, 2008). The 454 sequencing from the Australian sample bulk yielded 66,100 reads, which assembled into 2672 contigs of which 795 were longer than 500 bp. We detected 760 sequences with tandem repeats: 695 di-, 31 tri-, and 10 tetranucleotide microsatellites with at least 10, eight, and eight repeats, respectively. From these, 736 sequences contained suitable primer sites.

To test the amplification success of the microsatellites, DNA was extracted from samples belonging to *P. alba*, *P. chilensis*, *P. ruscifolia* Griseb., *P. flexuosa* DC., *P. kuntzei* Harms, *P. glandulosa*, and *P. pallida* (Appendix 1). Due to limited economic resources, we randomly tested 30 primer pairs (GL1–GL30; Table 1). Untested sequences are planned to be explored for additional primers in the future; in the meantime, the corresponding author can be contacted for information about them. In *P. alba* and *P. chilensis*, we genotyped between eight and 18 individual samples; in *P. ruscifolia*, *P. flexuosa*, *P. glandulosa*, *P. pallida*, and *P. kuntzei*, we only tested microsatellite amplification (between three and five individuals were screened). To confirm that the amplified products corresponded to the microsatellites found bioinformatically in the Australian sequence data set, one allele from each locus of *P. alba* was sequenced (Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina). Table 1 presents the characteristics of the 12 loci studied. The PCR amplifications were carried out in a 50-μL reaction volume containing 10–30 ng DNA, 0.6 μM each primer, 0.2 mM dNTPs, 0.3 U *Taq* DNA polymerase (Invitrogen, Buenos Aires, Argentina), and 1.5 mM $MgCl_2$. A MyCycler Techne thermal cycler (Bio-Rad Laboratories, Hercules, California, USA) was used for amplifications, where the cycling profile was initial denaturation at 94°C for 5 min; followed by 30 cycles at 94°C for 45 s denaturation, primer-specific annealing temperature (58–62°C; see Table 2) for 45 s, and at 72°C for 45 s extension; and a final extension at 72°C for 10 min. PCR products were run in a 6% (w/v) polyacrylamide gel containing 5 M urea in 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8) with a 10-bp DNA Ladder (Invitrogen) size marker. Gels were stained with silver nitrate. For all the polymorphic loci that generated clear patterns, we calculated observed, unbiased, and biased heterozygosity and the effective number of alleles using BIOSYS-2 (Swofford and Selander, 1999). The polymorphism information content (PIC) (Botstein et al., 1980) was estimated.

From the 30 potential microsatellite markers tested, 18 failed to produce a reproducible polymorphic pattern. Twelve primer pairs revealed clear and reproducible variable loci in *P. alba* (Table 2). Eight of these were also polymorphic in *P. chilensis*, GL3 amplified only in these two species, GL6 failed to amplify only in *P. kuntzei*, and GL9 did not amplify in *P. chilensis*. Ten primer pairs amplified DNA fragments in all tested species (Table 2). Across the samples, we found between two and seven alleles, with an average effective number of alleles of 2.75 for *P. alba* and 2.92 for *P. chilensis*. Average heterozygosity estimations in *P. alba* were 0.57, 0.60, and 0.54 (heterozygosity [*H*], unbiased estimated heterozygosity [H_{unb}], and direct count heterozygosity [H_{DC}], respectively). In *P. chilensis*, the estimations were similar, yielding average values of 0.61, 0.65, and 0.59, respectively. Individual H_{DC} did not differ significantly from *H* for any locus. The PIC index describes diversity within populations and characterizes the degree of polymorphism at each locus. Across all loci, the PIC ranged from 0.22 to 0.79 in *P. alba* and from 0.37 to 0.77 in *P. chilensis*. PIC values of less than 0.25 indicate low polymorphism, values between 0.25 and 0.5 indicate average polymorphism, and values higher than 0.5 indicate a highly polymorphic locus (Botstein et al., 1980). Eight out of 12 loci in *P. alba* and five out of eight loci in *P. chilensis* were highly polymorphic.

CONCLUSIONS

We succeeded in developing highly polymorphic SSRs in *P. alba* and *P. chilensis*. These microsatellite primer pairs are an important addition to the number of genetic markers previously available, increasing from six to 18 loci. They allow accurate estimates of genetic parameters for population structure analysis, including genetic diversity, genetic structure, mating system, pollen dispersal, relatedness among individuals, and heritability of quantitative traits. The cross-amplification observed in *P. kuntzei*, the most differentiated species so far studied from sect. *Algarobia*, suggests that the loci described might also be useful for population studies in several other species of the section.

TABLE 1. Characteristics of the polymorphic microsatellite markers developed for *Prosopis alba* and *P. chilensis* in this study.

Locus	Primer sequences (5′–3′)	T_a (°C)	Repeat motif	Product size (bp)	GenBank accession no.[a]
GL3	F: CACCGATCTCACAAAGCTGC R: AATGGATCTGGTGTTGTCGC	58	$(AAT)_5$	230–280	JX136853
GL6	F: CTGGTTGCTGTGATTGGAGG R: CTCCAGGGATCACAAGACAAAC	62	$(GAT)_7$	180–210	JX136854
GL8	F: CAGGTGGGCATGAAGTTTCC R: CCAAGAACAACCTGCCGAAG	58	$(AT)_{12}$	150–180	JX136855
GL9	F: ACTCTGCGGGTTAGGTAAGC R: ACCTGGAGCTGACATGGATC	58	$(AC)_8$	180–220	JX136856
GL12	F: GAGTGAAGGTCGGGAAGAGG R: CCATTGGACCAAGGCAGAAC	58	$(CT)_5(ATCT)_3(CT)_5$	340–390	JX136857
GL15	F: GTGTTATGGTCCCAACAGCC R: TGAAGAGGGAGGAATCGCAG	58	$(AG)_{12}$	160–220	JX136858
GL16	F: GTTGGATTTCACGGAAGGGC R: TCAGCTAAGTGGCCATACGG	58	$(AT)_{16}$	250–300	JX136859
GL18	F: GAGAATCTGGAGCAGCAACG R: AAGGTAGCGTCCCAGGTATG	58	$(AT)_8$	220–250	JX136860
GL21	F: ATCTCCGTCACAACTTGCAC R: ACCCTCACTCCCGAATGATG	58	$(AG)_5$	190–230	JX136861
GL23	F: GTCTTCTCTCCCGTGGATCC R: TGAGGCAAAGGAAGAGCAAC	58	$(AT)_{10}$	300–330	JX136862
GL24	F: CCTTAATCTCCCTCTCGGCC R: AACCAGGCTCTGCAGAAATG	58	$(AC)_{11}$	260–330	JX136863
GL26	F: CGAATGTGGATCTTCTGCGG R: TTAAGCGGCCCAAGTTTCTC	58	$(GT)_8$	180–220	JX136864

Note: F = forward; R = reverse; T_a = annealing temperature.
[a]The GenBank accession number corresponds to the alleles sequenced in *P. alba*.

TABLE 2. Descriptive statistics for polymorphic microsatellites in *Prosopis alba* and *P. chilensis* and evaluation of amplification success in related species.

	P. alba						*P. chilensis*						Cross-amplification[a]				
Locus	*n*	*A*	*H*	H_{unb}	H_{DC}	PIC	A_e	*n*	*A*	*H*	H_{unb}	H_{DC}	PIC	A_e	*P. fle*	*P. rus*	*P. kun*	*P. gla*	*P. pal*
GL3	10	2	0.5	0.53	0.2	0.37	2.00	10	1	0	0	0	0	1	NA	NA	NA	NA	NA
GL6	8	4	0.60	0.64	0.75	0.55	2.51	8	1	0	0	0	0	1	+	+	NA	+	+
GL8	18	7	0.80	0.82	0.89	0.79	5.02	8	6	0.80	0.86	0.87	0.78	5.13	+	+	+	+	+
GL9	10	2	0.25	0.27	0.3	0.22	1.34	10	NA	—	—	—	—	—	+	+	+	+	+
GL12	15	4	0.63	0.65	0.6	0.56	2.72	8	5	0.74	0.79	1	0.70	3.88	+	+	+	+	+
GL15	8	6	0.78	0.83	1	0.75	4.57	8	4	0.69	0.74	0.50	0.64	3.28	+	+	+	+	+
GL16	12	5	0.69	0.72	0.42	0.64	3.24	8	4	0.55	0.579	0.25	0.51	2.25	+	+	+	+	+
GL18	18	3	0.36	0.37	0.28	0.31	1.55	8	3	0.41	0.43	0.50	0.37	1.68	+	+	+	+	+
GL21	18	3	0.6	0.62	0.56	0.52	2.50	8	2	0.50	0.53	0.25	0.37	2.00	+	+	+	+	+
GL23	17	4	0.62	0.64	0.47	0.58	2.64	8	3	0.48	0.51	0.62	0.43	1.91	+	+	+	+	+
GL24	18	4	0.72	0.74	0.67	0.67	3.56	8	5	0.69	0.74	0.75	0.64	3.28	+	+	+	+	+
GL26	8	2	0.30	0.32	0.37	0.26	1.44	8	1	0	0	0	0	1	+	+	+	+	+

Note: + = correct amplification; *A* = number of alleles; A_e = effective number of alleles; *H* = heterozygosity; H_{DC} = direct count heterozygosity; H_{unb} = unbiased estimated heterozygosity (Nei, 1978); *n* = sample size; NA = no amplification; PIC = polymorphism information content.

[a] *P. fle* = *P. flexuosa*; *P. gla* = *P. glandulosa*; *P. kun* = *P. kuntzei*; *P. pal* = *P. pallida*; *P. rus* = *P. ruscifolia*.

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Appendix 1. Information on voucher specimens of *Prosopis* used in this study. Information presented: taxon, collection site, GPS coordinates, collector, voucher specimen, depository.

Prosopis alba Griseb.: Fernandez, Santiago del Estero; 27°55′23.11″S, 63°53′36.76″W; M. Ewens, ME001–ME175, BAFC. ***Prosopis chilensis*** (Molina) Stuntz: Cuesta de Belén, Catamarca; 27°45′45.55″S, 66°44′56.23″W; C. Bessega, CB001–CB010, BAFC. ***Prosopis ruscifolia*** Griseb.: Herrera, Santiago del Estero; 28°23′48.16″S, 62°22′58.78″W; B. O. Saidman, J. C. Vilardi, BOS703–BOS710, BAFC. ***Prosopis flexuosa*** DC.: Quilmes, Tucumán; 26°29′6.47″S, 65°59′53.65″W; B. O. Saidman, J. C. Vilardi, BOS300–BOS309, BAFC. ***Prosopis kuntzei*** Harms: Loreto, Santiago del Estero; 28°17′59.46″S, 64°12′0.61″W; M. Ewens, MEK001–MEK011, BAFC. ***P. glandulosa*** Torr.: Weslaco, Texas; 26°9′37.26″N, 97°59′28.53″W; J. Evans, JE001–JE005, BAFC. ***Prosopis pallida*** (Humb. & Bonpl. ex Willd.) Kunth: Jumana, Perú; 14°42′8.35″S, 75°16′28.01″W; DANIDA Forest Seed Centre, 01622/86, www.dfsc.dk, Humlebaek, Denmark. ***Prosopis glandulosa*** Torr., ***P. velutina*** Wooton, ***P. pallida***, **and putative hybrids**: Queensland, Australia; 20°52′11.57″S, 142°42′9.81″W; R. Watts, RW137, RW138, RW146, RW147, RW158, RW162, RW166, RW170, RW172, RW173, ANH.