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 “algarobos.” 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, 2000).
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-complementarity, and maximum end stability (AG) 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 Tag DNA polymerase (Invitrogen, Buenos Aires, Argentina), and 1.5 mM MgCl₂. AmCycler Thermo 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 across all loci, 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.

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

| Locus | Primer sequences (5’–3’) | Tₘ (°C) | Repeat motif | Product size (bp) | GenBank accession no.a |
|-------|--------------------------|---------|--------------|-------------------|-----------------------|
| GL3   | F: CACCGATCTCAACAACTGCG | 58      | (AT)₃      | 230–280          | JX136853              |
|       | R: AATGGATCTGCTGTTGCG    |         |             |                   |                       |
| GL6   | F: CTGTTGCTGTGTAGTTGAGG | 62      | (GAT)₇     | 180–210          | JX136854              |
|       | R: CTGGTGGGAGTTCAACACAAC | 58      | (AT)₁₂     | 150–180          | JX136855              |
| GL8   | F: CAGTTGGGCTGTAGGTTCCC | 58      | (AC)₈      | 180–220          | JX136856              |
|       | R: CCAGAACCAACCTCGCCAGG |         | (CT)₃,(ATCT)₃,(CT)₃ | 340–390 | JX136857 |
| GL12  | F: GGCGAGACTGCGGGAGAGGG | 58      | (AT)₁₆     | 250–300          | JX136859              |
|       | R: CCATGGCACGACGGAGAAC  |         | (AT)₈      | 220–250          | JX136860              |
| GL15  | F: GTTATGCTGCCAAACCGGC | 58      | (AT)₁₂     | 160–220          | JX136858              |
|       | R: TGAGGAGGAGAGGACAGCG  |         | (AT)₁₆     |                   |                       |
| GL16  | F: GGTATGCTCCAGGAGGGGC | 58      | (AT)₁₆     | 250–300          | JX136859              |
|       | R: TCAGCTAGTGGGCATACCGG |         | (AT)₁₂     | 220–250          | JX136860              |
| GL18  | F: GAGATGCTGGAGCAACAGCG | 58      | (GT)₉      | 190–230          | JX136861              |
|       | R: AGAATGCTGCCAGGATG    |         | (AT)₁₂     | 300–330          | JX136862              |
| GL24  | F: CTTAATCCCTCCCTCCGCC | 58      | (AC)₁₁     | 260–330          | JX136863              |
|       | R: AACCGGCGCTGCCAGAAATG |         | (GT)₉      |                   |                       |

Note: F = forward; R = reverse; Tₘ = annealing temperature.

aThe GenBank accession number corresponds to the alleles sequenced in P. alba.

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

| Locus | P. alba | n | H | A | H DC | H unb | PIC | P. flexuosa | P. glauca | P. kunzei | P. palida | P. ruscifolia |
|-------|---------|---|---|---|------|-------|----|-------------|-----------|----------|----------|-------------|
| GL3   | 10      | 2 | 0.5 | 0.37 | 0.2   | 0.37  | 0.38  | 0.72  | 0.74  | 0.72  | 0.72  | 0.72  |
| GL4   | 8       | 4 | 0.60 | 0.55 | 0.55  | 0.55  | 0.55  | 0.55  | 0.55  | 0.55  | 0.55  | 0.55  |
| GL5   | 8       | 2 | 0.80 | 0.74 | 0.74  | 0.74  | 0.74  | 0.74  | 0.74  | 0.74  | 0.74  | 0.74  |
| GL6   | 8       | 4 | 0.80 | 0.77 | 0.77  | 0.77  | 0.77  | 0.77  | 0.77  | 0.77  | 0.77  | 0.77  |
| GL8   | 18      | 7 | 0.80 | 0.81 | 0.81  | 0.81  | 0.81  | 0.81  | 0.81  | 0.81  | 0.81  | 0.81  |
| GL26  | 8       | 2 | 0.30 | 0.26 | 0.26  | 0.26  | 0.26  | 0.26  | 0.26  | 0.26  | 0.26  | 0.26  |

Note: + = correct amplification; A = no amplification; PIC = polymorphism information content; H DC = direct count heterozygosity; H unb = unbiased estimated heterozygosity (Nei, 1978).
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.