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Primer Note

Microsatellite markers for *Senna spectabilis* var. *excelsa* (Caesalpinioideae, Fabaceae)[1]

M. Cristina López-Roberts[2,4], Ariane R. Barbosa[2], Luciano Paganucci de Queiroz[3], and Cássio van den Berg[3]

[2]Pós-graduação em Botânica, Universidade Estadual de Feira de Santana (UEFS), Av. Transnordestina s/n, 44036-900 Feira de Santana, Bahia, Brazil; and [3]Departamento Ciências Biológicas, Universidade Estadual de Feira de Santana (UEFS), Av. Transnordestina s/n, 44036-900 Feira de Santana, Bahia, Brazil

- *Premise of the study: Senna spectabilis* var. *excelsa* (Fabaceae) is a South and Central American tree of great ecological importance and one of the most common species in several sites of seasonally dry forests. Our goal was to develop microsatellite markers to assess the genetic diversity and structure of this species.
- *Methods and Results:* We designed and assessed 53 loci obtained from a microsatellite-enriched library and an intersimple sequence repeat library. Fourteen loci were polymorphic, and they presented a total of 39 alleles in a sample of 61 individuals from six populations. The mean values of observed and expected heterozygosities were 0.355 and 0.479, respectively. Polymorphism information content was 0.390 and the Shannon index was 0.778.
- *Conclusions:* Polymorphism information content and Shannon index indicate that at least nine of the 14 microsatellite loci developed are moderate to highly informative, and potentially useful for population genetic studies in this species.

Key words: Caatinga; Fabaceae; genetic variation; microsatellite library; polymorphic loci; *Senna spectabilis*.

Senna Mill. is a large, diverse, and widespread genus of Cassiinae (Caesalpinioideae, Fabaceae), with about 350 species. Most species are American, and some species also occur in Africa, Madagascar, Australia, and Asia (Marazzi et al., 2006). *Senna spectabilis* (DC.) H. S. Irwin & Barneby is a deciduous tree or shrub ranging from southern Mexico to the Caribbean and northern Argentina and including two varieties: *S. spectabilis* var. *spectabilis* and *S. spectabilis* var. *excelsa* (Schrad.) H. S. Irwin & Barneby (Irwin and Barneby, 1982). This taxon has been introduced to many countries (including the United States, Malaysia, and Africa) as an ornamental street tree, where it is often regarded as an invasive plant (Richardson and Rejmánek, 2011).

Senna spectabilis var. *excelsa* is widespread in the Caatinga Phytogeographical Domain of northeastern Brazil (Queiroz, 2009). Due to its long flowering period (December to April), it is an important source of pollen for "buzzing bees" (*Xylocopa*, *Bombus*, and *Centris* species), which constitute its main pollinators (Manente-Balestieri and Machado, 1999). This tree has been used by local people as a source of fuel wood and timber, as well as fodder for sheep and goats (Santos et al., 2013). Extracts of this plant also have several applications in folk medicine, and recent studies confirm sedative, anticonvulsive, antimalarial, antimicrobial, cytotoxic, and leishmanicidal activities (de Albuquerque Melo et al., 2014). Despite this, there are no studies that assess the levels of genetic diversity in populations of *S. spectabilis*. The only study in this species assessed the genetic diversity in germplasm collections using RAPD markers (Santos et al., 2013). In the current study, our goal was to develop microsatellite markers to assess the genetic variation and population structure of *S. spectabilis* var. *excelsa*, generating useful tools for future studies and management of this species.

METHODS AND RESULTS

A leaf sample from a single individual of *S. spectabilis* var. *excelsa* cultivated on the campus of Universidade Estadual de Feira de Santana was collected and deposited in the herbarium of Universidade Estadual de Feira de Santana (HUEFS; *H. Huaylla & M. C. L. Roberts 3737*, see Appendix 1). Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) 2× protocol (Doyle and Doyle, 1990) adapted for Eppendorf microtubes (Axygen Scientific, Union City, California, USA). Two types of microsatellite libraries were constructed: a microsatellite-enriched library, following the protocol of Billotte et al. (1999), and an intersimple sequence repeat (ISSR) library (Provan and Wilson, 2007). Fragments obtained by both strategies were linked into the pGEM-T Easy Vector System (Promega Corporation, São Paulo, Brazil) and subsequently transformed and cloned into TOP10 competent cells (Invitrogen, Life Technologies, São Paulo, Brazil). The positive clones were amplified by PCR using the T7 (5′-TAATACGACTCACTATAGGG-3′) and SP6 (5′-ATTTAGGTGACACTATAGAA-3′) primers with the following program: premelt of 94°C for 5 min, 35× (denaturation at 94°C for 15 s, annealing at

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[4]Author for correspondence: macrissroberts@gmail.com

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55°C for 35 s, and extension at 72°C for 90 s), followed by a final extension at 72°C for 7 min. After quantification performed in agarose gel electrophoresis with a 100-bp ladder (Ludwig Biotec, Alvorada, Rio Grande do Sul, Brazil), fragments larger than 500 bp were sequenced using the Big Dye 3.1 Terminator Cycle Sequencing Kit and an ABI 3130XL automated sequencer (Applied Biosystems, São Paulo, Brazil). Identification of simple sequence repeats was performed with the software SSRIT (Simple Sequence Repeat Identification Tool; http://archive.gramene.org/db/markers/ssrtool) and Imperfect Microsatellite Extractor (Mudunuri and Nagarajaram, 2007). Primer design was done using the software Primer3Plus (Untergasser et al., 2007) with the following criteria: product size range 100–300 bp, primer melting temperature (T_m) 50–60°C (with a maximum difference of 2°C), and GC content 40–60%.

Genotyping reactions were performed in 10-μL reactions with Top Taq Master Mix (QIAGEN, São Paulo, Brazil), adding 0.15 μM of the forward primer (tailed at the 5′ with the M13 universal sequence: 5′-CACGACGTTGTAAAACGAC-3′), 0.30 μM of the reverse primer, 0.060 μM of the M13-labeled tail (with 6-FAM, VIC, NED, or PET fluorescent dyes; Applied Biosystems) and approximately 5 ng of individual DNA. Optimal annealing temperature for each primer pair was determined using touchdown (60–54°C, 58–48°C) and gradient (from 62–45°C) PCR programs. Amplicons were visualized by agarose gel electrophoresis (1.5%) with a 100-bp ladder. Microsatellite profiles were analyzed with GeneMapper 4.0 (Applied Biosystems). For polymorphism assessment, we sampled 61 individuals from six populations of *S. spectabilis* var. *excelsa* (8–21 individuals per population; Appendix 1). Cross-amplification was tested in *S. spectabilis* var. *spectabilis* using a foliar sample from one individual deposited at HUEFS (*Queiroz L. P. de 13481*, see Appendix 1). The estimated number of alleles, observed heterozygosity (H_o), expected heterozygosity (H_e), Shannon index, and Hardy–Weinberg equilibrium (HWE) deviations were calculated using GenAlEx 6.5 (Peakall and Smouse, 2006). We also performed a linkage disequilibrium test using GENEPOP (Raymond and Rousset, 1995) to check if genotypes at one locus are independent from genotypes at the other loci. The polymorphism information content (PIC) was calculated with the online tools of the Centre for Genomic Research at the University of Liverpool (http://www.genomics.liv.ac.uk/animal/Protocol1.html).

We found 146 perfect and imperfect microsatellite loci in 79 fragments of the enriched library, with motifs of four or more repeats. In the ISSR library, we found 54 microsatellites in only 13 sequences. The proportions of perfect and imperfect-motif microsatellites obtained are shown in Appendix S1. From both sets of loci, we designed a total of 53 primer pairs, of which 34 were designed from the enriched library.

A total of 35 microsatellite loci were successfully amplified, from which 14 were polymorphic. Variation was observed in both perfect and imperfect motifs, particularly in di- and trinucleotide repeats (Table 1). The linkage disequilibrium test detected significant values for the following pairwise comparisons: SS_5/SS_51 (P = 0.0131) and SS_51/SS_52 (P = 0.0097), which should be used carefully. The allele number per locus ranged from two to five, with a total of 39 alleles identified (Table 2). H_o and H_e values varied from 0.037 to 0.727 and 0.036 to 0.743, respectively. Significant deviations from expected HWE proportions (P < 0.01) were detected in seven loci. Values of PIC ranged from 0.037 to 0.697 with a mean of 0.390. The Shannon index varied from 0.092 to 1.423 (mean 0.778). Nine loci presented values of PIC > 0.3 and Shannon index > 0.6, indicating that these are moderately to highly informative microsatellites. Successful cross-amplification was observed in the single tested individual of *S. spectabilis* var. *spectabilis* (Table 2).

CONCLUSIONS

We successfully developed and amplified a set of polymorphic microsatellite markers in *S. spectabilis* var. *excelsa*, which could prove very useful in population genetic studies. Due to the successful cross-amplification of these microsatellite loci in *S. spectabilis* var. *spectabilis*, we expect these markers could also be used to assess genetic variation of germplasm collections and to support plant breeding programs in this species. *Senna spectabilis* var. *excelsa* is one of the few tree species from the caatinga for which microsatellites have been developed, and their use should foster the understanding of patterns of diversity in this fragile ecosystem with high levels of endemic biodiversity.

Table 1. Characterization of 14 polymorphic microsatellite markers developed for *Senna spectabilis* var. *excelsa*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)[a]	M13 5′ PIG-tail	T_a (°C)	GenBank accession no.
SS_1	F: TTCTGAAGCAGACGGAAGGT R: CTCACCTTCCAAATTCAAAGC	$(AGAGA)_4$	132–142	PET	58	KJ398419
SS_5	F: GTCCTTTTCACCGATTGA R: CTCCTGTCAAAACTCACAAA	$(AG)_9$	136–148	6-FAM	59	KJ398420
SS_10	F: CACAGCCAAGAGGGAGAAAG R: CCTTCTCAATGGCCTTCTCTT	$(AGA)_5$	187–196	VIC	58	KJ398421
SS_14	F: GAGTATGGGGAATACGAATC R: TGGGAAGTCAGATACCAT	$(TC)_3C(GT)_3$	305–307	NED	56	KJ398422
SS_15	F: GGTATCTGACTTCCCAAA R: AGATGTCCGGATTGCTAC	$(ATTT)_4$	244–248	PET	56	KJ398422
SS_18	F: GCGATGCTCAAGTTTCTCTT R: CAAACTAAACCAAAGGACGA	$(ATC)_3(TTC)_3(TC)_6$	147–149	NED	56	KJ398423
SS_24	F: GCCTGTGATCTGAAGGTGGA R: GGAGCAGTTTAAGCCAGCTTTATT	$(AG)_9$	115–125	NED	56	KJ398424
SS_26	F: GCGCCTCACTCATACATTGA R: GGTCCACCTCCGGGTAGTAT	$(CT)_4$	124–132	VIC	56	KJ398425
SS_27	F: GAACTGGGAAGGCAGAAAAA R: GATTCTGGGCAGGCTCCTAT	$(TC)_4$	314–316	PET	TD 60–54	KJ398426
SS_34	F: GAACTGGGAAGGCAGAAAAA R: ACTTTTCCCAACCTCCGTCT	$(AT)_4$	153–157	VIC	TD 60–54	KJ398427
SS_41	F: CCAGAACACGACACAAATGG R: CGTGGACTAGCCTTCCAATC	$(AGAA)_4$	200–216	6-FAM	56	KJ398428
SS_48	F: TTGTCCTTGGTGATCTCTTCTC R: GCACGTCAGTCAATATGCAA	$(GTT)_3$	215–248	6-FAM	60	KJ398429
SS_51	F: TGGCAGTACCACCGTACAGA R: TCCCAATGGCTCACAGATG	$(ATA)T(ATA)_3n_{90}(TCA)_3$	308–327	VIC	60	KJ398430
SS_52	F: ACTTCTTGATTTGGTGGCTGA R: CCTGCCAACCCAGATAGAAA	$(AGT)_3$	118–127	6-FAM	57	KJ398431

Note: T_a = annealing temperature; TD = touchdown program.
[a] Expected product size in base pairs (bp) including 5′ M13 universal sequence.

Table 2. Population genetic parameters for 14 polymorphic microsatellite loci in *Senna spectabilis* var. *excelsa* (n = 61).

Locus	A	H_o	H_e	HWE[a]	I_S[b]	PIC[c]	Cross-amplification in *S. spectabilis* var. *spectabilis*[d]
SS_1	4	0.385	0.385	0.994[ns]	0.672	0.308	Yes
SS_5	5	0.393	0.696	0.000***	1.349	0.649	Yes
SS_10	2	0.200	0.455	0.012*	0.647	0.352	No
SS_14	3	0.111	0.290	0.000***	0.557	0.269	Yes
SS_15	2	0.455	0.351	0.168[ns]	0.536	0.289	Yes
SS_18	2	0.200	0.295	0.107[ns]	0.471	0.252	Yes
SS_24	5	0.727	0.743	0.805[ns]	1.423	0.697	Yes
SS_26	4	0.545	0.479	0.000***	0.879	0.425	Yes
SS_27	2	0.037	0.036	0.922[ns]	0.092	0.037	Yes
SS_34	3	0.273	0.549	0.003**	0.934	0.490	Yes
SS_41	4	0.500	0.696	0.198[ns]	1.291	0.649	Yes
SS_48	3	0.048	0.534	0.000***	0.841	0.435	Yes
SS_51	3	0.409	0.429	0.916[ns]	0.687	0.355	Yes
SS_52	3	0.333	0.285	0.840[ns]	0.519	0.256	Yes
Mean	3.214	0.355	0.479		0.778	0.390	

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = probability of deviation from Hardy–Weinberg equilibrium; I_S = Shannon index; n = sample size; PIC = polymorphism information content.

[a] Significant deviation from Hardy–Weinberg equilibrium: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; ns = not significant.

[b] $I_S > 0.6$ indicates a moderately to highly informative value.

[c] PIC > 0.3 indicates a moderately to highly informative value.

[d] Cross-amplification was tested in a single individual of *Senna spectabilis* var. *spectabilis* (Appendix 1).

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Appendix 1. Voucher and location information for populations of *Senna spectabilis* used in this study for microsatellite development, polymorphism testing, and cross-amplification. Voucher specimens are deposited at the herbarium of the Universidade Estadual de Feira de Santana (HUEFS), Feira de Santana, Bahia, Brazil.

Species	Population no. (n)	Collection locality	Geographic coordinates	Voucher no.
Senna spectabilis var. *excelsa* (Schrad.) H. S. Irwin & Barneby	Cultivated individual	Feira de Santana, Bahia, Brazil	12°11′58″S, 38°58′08″W	*H. Huaylla & M. C. L. Roberts 3737*
	Population 1 (21)	Morro de Chapeu, Bahia, Brazil	11°19′23.7″S, 41°00′51.4″W	*C. C. Santos 828*
	Population 2 (8)	Xique Xique, Bahia, Brazil	10°51′31.9″S, 42°37′09.6″W	*C. C. Santos 833*
	Population 3 (8)	Dirceu Arcoverde, Piauí, Brazil	09°19′53.6″S, 42°26′47.8″W	*C. C. Santos 860*
	Population 4 (8)	Milagres, Ceará, Brazil	7°15′56.7″S, 38°50′45.7″W	*C. C. Santos 917*
	Population 5 (8)	Pena Forte, Pernambuco, Brazil	7°49′08.8″S, 39°05′08.4″W	*C. C. Santos 920*
	Population 6 (8)	Jeremoabo, Bahia, Brazil	9°50′55″S, 38°15′51.2″W	*C. C. Santos 934*
Senna spectabilis (DC.) H. S. Irwin & Barneby var. *spectabilis*	Individual	Salvador de Jujuy, Mendieta, Jujuy, Argentina	24°19′00″S, 64°58′00″W	*Queiroz, L. P. de 13481*

Note: n = sample size.