American Journal of Botany

American Journal of Botany: e372–e374. 2012.

AJB Primer Notes & Protocols in the Plant Sciences

Development of microsatellite markers for *Anadenanthera colubrina* var. *cebil* (Fabaceae), a native tree from South America[1]

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- *Premise of the study:* Microsatellite primers were developed in the native legume tree *Anadenanthera colubrina* var. *cebil* to study the genetic diversity and genetic structure in natural populations in Argentina.
- *Methods and Results:* Nine microsatellite markers were identified using a genomic library enriched for tandemly repeated motifs, eight of which markers were polymorphic. The polymorphism of these markers was assessed by investigating 20 individuals for fragment polymorphism; three to 13 alleles were observed for each locus. Observed and expected heterozygosities ranged from 0.300 to 1.000 and from 0.463 to 0.900, respectively.
- *Conclusions:* These results confirm that these primers will be useful for investigating the genetic diversity and genetic structure of natural populations of *A. colubrina* var. *cebil* in future studies.

Key words: *Anadenanthera colubrina* var. *cebil*; enrichment protocol; Fabaceae; nuclear microsatellites; polymorphism.

Anadenanthera colubrina (Vell.) Brenan var. *cebil* (Griseb.) Altschul (Fabaceae, Mimosoideae) is a tree species native to subtropical forests in Brazil, Paraguay, Bolivia, Peru, and northern Argentina (Cialdella, 2000). The latter populations are located in the Paranaense and Yungas biogeographic provinces, and are impacted by the advances of cultivated areas and also selective logging.

Anadenanthera colubrina var. *cebil* can reach up to 35 m in height; it has hermaphroditic flowers and long legume fruits (Justiniano and Fredericksen, 1998; Cialdella, 2000). Bees are the main pollinators, and seeds are dispersed by autochory or anemochory (Justiniano and Fredericksen, 1998; Abraham de Noir et al., 2002). Previous studies in this species showed high diversity using chloroplast microsatellites (Barrandeguy et al., 2011). Recently, Feres et al. (2012) have reported 14 polymorphic nuclear simple sequence repeat (SSR) markers for *A. colubrina*. To analyze the nuclear genetic diversity and genetic structure in natural populations of *A. colubrina* var. *cebil*, we developed nuclear microsatellite markers.

[1]Manuscript received 15 February 2012; revision accepted 3 May 2012.

Special acknowledgments are given to Alexandra Dolynska for technical assistance in the laboratory. M.E.B. has been supported by a short-term fellowship provided by the German Academic Exchange Service (DAAD) during this work.

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doi:10.3732/ajb.1200078

METHODS AND RESULTS

Field-collected leaf material from adult trees was preserved in silica gel, and genomic DNA was extracted by the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). Microsatellite markers were developed from two microsatellite-enriched genomic libraries to increase the variability of microsatellite motifs. Both libraries were developed following the protocol described by Fisher and Bachmann (1998) and modified by Prinz et al. (2009). In short, template DNA from one individual was cut with *Rsa*I, and adapters were ligated to the fragments. The restriction–ligation products were heat-denatured and hybridized with biotinylated oligonucleotides, either with an equimolar pool of $(GAA)_8$, $(CAA)_8$, and $(CA)_{10}$ (1 pmol each; hybridization at 74°C) or with $(GA)_{10}$ alone (hybridization at 60°C). Streptavidin-coated magnetic beads (Invitrogen, Carlsbad, California, USA) were used to capture the hybridized fragments. The adapter sequences served as primer templates for the following amplification of the fragments. The enrichment procedure including hybridization, capture, and PCR was repeated once for each library. Final PCR products were ligated into the pCR 2.1-TOPO vector (Invitrogen) and transformed to One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen). Positive bacterial colonies were amplified and sequenced in an ABI Prism 3100 (Applied Biosystems, Foster City, California, USA) automatic sequencer. In total, 204 positive clones from both libraries were sequenced, and 62 out of 97 sequences containing microsatellites showed high quality and long flanking regions to design primers. Primer pairs were created for 30 loci using Primer3 version 2.2.3 (Rozen and Skaletsky, 2000) applying the standard settings (GC: 20–80%, T_a: 57–63°C) and a desired fragment length of 80 to 200 bp depending on the microsatellite motif and total fragment length in the clones. PCR functionality tests were conducted in a final volume of 15 µL using 0.5 ng/µL of genomic DNA, 1× Hot Start Buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U Hot Start DNA Polymerase (5 U/µL HOT FIREPol, Solis BioDyne, Tartu, Estonia), and 0.33 pmol of each primer. PCR was performed in a gradient cycler (Biometra, Göttingen, Germany) using a touchdown program. The temperature regime was: 94°C for 15 min; 10 cycles of 1 min at 94°C, 1 min at 60°C to 50°C with a reduction of 1°C in each cycle, and 1 min at 72°C; followed by 29 similar cycles using 50°C for annealing and a final elongation at 72°C for 20 min. PCR products were separated in 1.5% agarose gels, and allele sizes were scored visually using a 100-bp ladder as a size reference. Sixteen loci showed clear,

TABLE 1. Characterization of nine microsatellite loci developed in *Anadenanthera colubrina* var. *cebil* (n = 20 individuals).

Primer[a]	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	T_a (°C)	GenBank accession no.
Ac34.3*	F: CCATTCTACAACACGCAAGTG	$(CT)_{21}(GT)_3$	171–197	TD 60–50°C (multiplex PCR)	JQ086537
	R: CCTCCAATTCCTCCAACTCC				
Ac48.1*	F: GAGACCCAACCACACGAGTT	$(GA)_{30}$	124–171	TD 60–50°C (multiplex PCR)	JQ086538
	R: GGTGTAATTCCATAACTCTCTTCTCTG				
Ac11.2*	F: CAAGCGTTTCCTGATATTTATTG	$(GT)_{11}$	110–114	TD 60–50°C (multiplex PCR)	JQ086539
	R: TTGCCATTTCCTTATTTAGTATGA				
Ac28.3*	F: GAGCAGCCATGTTTGGAGTA	$(GAA)_4N_{35}(GA)_{25}$	207–237	65°C	JQ086540
	R: CCCACTTCTGCCTTGCTATT				
Ac157.1*	F: CCACCCTCCATTTTTATTTATCT	$(CT)_{15}(CA)_3(CT)_6(CA)_{10}$	106–189	65°C	JQ086541
	R: CAGAAAGAAACCACGGCAAC				
Ac41.1**	F: ACGAGCTCCACATTCATGC	$(TC)_{10}(AC)_7$	125–144	TD 60–50°C (multiplex PCR)	JQ086542
	R: GAGCCAGTTTCGTTTGAAGG				
Ac172.1*	F: TCTAAATTACGTGGAGAAAACGAA	$(A)_5G(A)_4(CT)_{19}$	87–122	TD 60–50°C (multiplex PCR)	JQ086543
	R: TGACAGGACCCTCACCATGT				
Ac162.1*	F: TGTATGTGTGAATATGGAAGTTGC	$(GA)_{24}$	114–162	TD 60–50°C	JQ086544
	R: GCAGTGCATGTGACCACCTT				
Ac29.2**	F: GCCAGTGTGATGGATATCTGC	$(GT)_{15}(GA)_{28}$	327	TD 60–50°C	JQ806379
	R: TCAAGTAGTGGCTTTCAACTTCC				

Note: T_a = annealing temperature; TD = touchdown.

* 5′-FAM labeled primer.

** 5′-HEX labeled primer.

[a]For population analysis, two groups of primers (Ac34.3, Ac48.1, and Ac11.2; and Ac41.1 and Ac172.1) were each multiplexed in one reaction.

well-defined single bands at expected sizes; the remaining 14 loci showed unspecific or unclear amplification patterns.

PCR products for all 16 loci were cloned, resequenced, and aligned to the original fragment obtained from the enrichment procedure. Sequences obtained from 15 loci matched with the original sequences while the remaining sequence did not. The corresponding primers were resynthesized incorporating a fluorescent tag (FAM = blue or HEX = green) on the 5′ end. PCR amplifications were performed following the conditions of the functionality tests. Two loci (Ac28.3 and Ac157.1) were amplified without the touchdown program and the annealing temperature was 65°C (Table 1). Electrophoresis was carried out in an ABI Prism 3100 (Applied Biosystems), and fragment sizes were scored with GENESCAN analysis software using the internal size standard GS 500 ROX (Applied Biosystems). Six out of 15 loci showed patterns with three or four peaks. As a result of this, nine loci were included in the reported analysis. Polymorphism assay was performed in 20 individuals of *A. colubrina* var. *cebil* from two Argentinean populations (Appendix 1). For population analysis, two (Ac41.1 and Ac172.1) or three primers (Ac34.3, Ac48.1, and Ac11.2) were multiplexed in one reaction. For locus Ac48.1, the allele 171 was observed once; in this case some samples were amplified without multiplexing to verify its accuracy. Eight primers revealed polymorphism and one was monomorphic in both populations.

The total number of alleles as well as observed and expected heterozygosities were determined by GenAlEx version 6.41 (Peakall and Smouse, 2006). A test of Hardy–Weinberg equilibrium was performed by GenAlEx version 6.41 (Peakall and Smouse, 2006). Genotypic linkage disequilibrium was tested for each pair of loci across population by creation of contingency tables for all pairs of loci in each population, and then a probability test for each table was computed using the Markov chain algorithm. Finally, a global test (Fisher's method) was performed across population using GENEPOP version 4.0.10 (Raymond and Rousset, 1995). Results of the initial primer screening in two populations of *A. colubrina* var. *cebil* are shown in Table 2. The number of alleles ranged from three to 13. Observed and expected heterozygosities ranged from 0.300 to 1.000 and from 0.463 to 0.900, respectively. All loci did not significantly differ from Hardy–Weinberg equilibrium, and all of them did not show linkage disequilibrium ($P > 0.05$).

CONCLUSIONS

We developed nine microsatellite loci specific for *A. colubrina* var. *cebil*, eight of which were polymorphic in the studied populations. These markers will be applied to investigate the genetic diversity and structure in natural populations of *A. colubrina* var. *cebil* in future studies.

TABLE 2. Results of the initial primer screening in two populations of *Anadenanthera colubrina* var. *cebil*.

	Candelaria (n = 10)			Libertador General San Martín (n = 10)		
Locus	A	H_o	H_e	A	H_o	H_e
Ac34.3	7	0.800	0.740	8	0.556	0.840
Ac48.1	8	0.800	0.795	8	0.625	0.836
Ac11.2	5	0.600	0.480	3	0.300	0.635
Ac28.3	8	0.500	0.850	6	0.800	0.800
Ac157.1	8	1.000	0.805	8	0.667	0.784
Ac41.1	6	0.556	0.463	4	0.800	0.575
Ac172.1	4	0.600	0.610	13	0.900	0.900
Ac162.1	7	0.700	0.605	12	1.000	0.900

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity.

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APPENDIX 1. Population and geographic coordinates of individuals of *Anadenanthera colubrina* var. *cebil* used in this study.

Population	Geographic coordinates
Candelaria, Misiones Province, Argentina (Paranaense biogeographic province)	27°26′58.200″S, 55°44′20.184″W
Libertador General San Martín, Jujuy Province, Argentina (Yungas biogeographic province)	23°45′15.012″S, 64°51′12.996″W

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