Development and Characterization of Microsatellite Primers in the Federally Endangered Astragalus bibullatus (Fabaceae)

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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE PRIMERS IN THE FEDERALLY ENDANGERED *ASTRAGALUS BIBULLATUS* (FABACEAE)[1]

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- *Premise of the study:* Microsatellites were developed for *Astragalus bibullatus* (Fabaceae), a federally endangered narrow endemic, to investigate reproductive ecology and species boundaries among closely related taxa.
- *Methods and Results:* Next-generation sequencing was used to develop 12 nuclear microsatellite loci that amplify in *A. bibullatus*, as well as in *A. crassicarpus* var. *trichocalyx*, *A. gypsodes*, and *A. tennesseensis*. Identified loci were di- and trinucleotide repeats, with 1–15 alleles per locus. Observed and expected heterozygosities ranged from 0.000–0.938 and 0.000–0.860, respectively. Cross-amplification of three loci previously published in *A. michauxii* was also confirmed for the taxa included here.
- *Conclusions:* These data indicate the utility of novel microsatellite loci for conservation genetics and reproductive ecology in closely related *Astragalus* species.

Key words: *Astragalus bibullatus*; *Astragalus* sect. *Sarcocarpi*; cedar glades; conservation genetics; Fabaceae.

Astragalus bibullatus Barneby & E. L. Bridges (Fabaceae; Pyne's ground plum) is a herbaceous perennial endemic to the limestone cedar glades of the Central Basin of Middle Tennessee (Barneby and Bridges, 1987). It is a federally endangered species, known only from eight extant locations in Rutherford County (U.S. Fish and Wildlife Service, 2011). Previous studies using allozymes (Baskauf and Snapp, 1998; Morris et al., 2002) and amplified fragment length polymorphisms (AFLPs) (Baskauf and Burke, 2009) to investigate population structure have found limited genetic diversification among sites. Demographic studies have shown that population sizes fluctuate, in some cases dramatically, from year to year (U.S. Fish and Wildlife Service, 2011). More importantly, seed recruitment and fertility appear to vary by site (Albrecht, 2011). The genetic consequences of these combined factors (long-lived, genetically diverse seed bank, fluctuating population sizes, and differential recruitment success) have yet to be evaluated.

Many recognize nuclear microsatellites as the preferred molecular approach for ecological and conservation genetic studies, due to their highly variable, codominant nature (Selkoe and Toonen, 2006). The greatest disadvantage to microsatellites is likely the need for prerequisite knowledge of the genome of the target species, as amplification primers are designed to match a specific location within the genome, unlike AFLPs, random-amplified polymorphic DNA (RAPDs), and intersimple sequence repeats (ISSRs) (Selkoe and Toonen, 2006). Here we present a suite of novel nuclear microsatellite loci in *A. bibullatus*, developed using next-generation sequencing (Davey et al., 2011; Ekblom and Galindo, 2011; Zalapa et al., 2012) on an Illumina platform. The resulting loci will be useful in long-term conservation studies of the target taxon, as well as in broader taxonomic studies of closely related taxa. In addition, the methodological approach used here provides a relatively straightforward workflow for the user new to microsatellite development and characterization, regardless of study system.

METHODS AND RESULTS

Total genomic DNA of a single individual of *A. bibullatus* collected from a natural population by Carol Baskauf (Austin Peay State University, Clarksville, Tennessee, USA) was extracted using the QIAGEN DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA). The sample was submitted to the Evolutionary Genetics Core Facility (EGCF) at Cornell University (Ithaca, New York, USA) to generate and then sequence an enriched genomic library using an Illumina MiSeq platform (San Diego, California, USA) to target di-, tri-, tetra-, penta-, and hexameric microsatellite repeats (Andrés and Bogdanowicz, 2011). EGCF supplied files containing more than 3000 possible primer pairs, which were identified in MSATCOMMANDER 1.0.3 (Rozen and Skaletsky, 1999; Faircloth, 2008). We selected 46 primer pairs for our initial screen, with an emphasis on tri- and dinucleotide repeat motifs. Repeat counts of targeted loci ranged from five to 15, and targets were chosen with an effort to diversify the motifs selected. Contigs of selected targets were aligned and compared to verify that all loci were unique. All PCR reactions were three-primer reactions following Schuelke (2000). A 17-base tail (5′-GTAAAACGACGGCCAGT-3′) was added to the 5′ end of each forward primer; a 7-base PIG-tail (5′-GTTTCTT-3′) was added to the 5′ end of each reverse primer. A third primer identical

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TABLE 1. Characterization of novel nuclear microsatellite loci developed for the federally endangered legume *Astragalus bibullatus* (Pyne's ground plum).

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	GenBank accession no.
Abib028	F: ACCAGCGAATAGTGCTTACGTG	$(AAC)_5$	212	KT905411
	R: CTGTAGCCATTGAAGGAACCAC			
Abib051	F: AGTCTGTACATTGCGAACTCAAC	$(AG)_6$	170	KT905412
	R: AGTCTGTACATTGCGAACTCAAC			
Abib059	F: CATTTCTTGTAACGCCAGAAACG	$(AAC)_7$	342	KT905413
	R: GATGAGTTGTGAAGAAGACTGGG			
Abib083	F: AATCTCAGAGGCATAGAGGGTAG	$(AC)_9$	197	KT905414
	R: TGAAATAGGAGATGATTATGGCCC			
Abib093	F: ACTTATCCTCTCACTCCAACCTC	$(AAG)_5$	343	KT905415
	R: CTCAACTCTCTGCCACTTGAATC			
Abib094	F: AGATCCAAAGTTTGCCATCCATC	$(AG)_8$	186	KT905416
	R: TTCCTTCACTTCCGCCATTAATG			
Abib095	F: CAGGCATGCAAATGGGATAATTG	$(AG)_9$	215	KT905417
	R: TAATCACATTCTCTCATGCACGC			
Abib113	F: CTCTTCTCTCGAATCATCATCCC	$(AG)_{11}$	228	KT905418
	R: CTCTATCCTCTACCACCGCTTC			
Abib120	F: TTCTTCATCAGTTGCTAAGCCAC	$(AGG)_6$	267	KT905419
	R: TATACTTCAGAACGGTGAGGAGG			
Abib152	F: TGCTACCTACAATGCCACTATTG	$(AG)_{10}$	235	KT905420
	R: TGCTTTAACTAGTGCTTTGTCAC			
Abib156	F: AAGTGTGTGCGGTGATTAGAAAG	$(AAG)_6$	212	KT905421
	R: AAGTGTGTGCGGTGATTAGAAAG			
Abib170	F: ATTTGTCACCTTTCTCCACATGC	$(AAC)_5$	350	KT905422
	R: ATTTGTCACCTTTCTCCACATGC			

to the tail on the forward primer was fluorescently labeled with one of three standard tags (FAM, VIC, or NED). Loci were initially screened in singleplex using eight individuals from two natural populations, with reactions having the following final concentrations: 1× Platinum *Taq* Buffer, 2 mM $MgCl_2$, 200 nM total dNTPs, 0.15 µM fluorescently labeled primer, 0.05 µM forward primer, 0.2 µM reverse primer, and 1 unit of Platinum *Taq* DNA Polymerase (Invitrogen by Life Technologies, Grand Island, New York, USA). A touchdown cycling profile was used for all amplifications: six cycles of 95°C for 45 s, followed by annealing at 61°C (dropping one degree per cycle) for 45 s, and 72°C for 1 min; the remaining 29 cycles consisted of 95°C for 40 s, 55°C for 45 s, and 72°C for 1 min. All loci were initially run in singleplex on an ABI 3130xl DNA Analyzer at Middle Tennessee State University using a GeneScan 500 LIZ Size Standard (Applied Biosystems by Life Technologies, Grand Island, New York, USA). Traces were analyzed in GeneMarker MTP 2.6.0 (SoftGenetics, State College, Pennsylvania, USA).

Following this initial screen, 12 loci were selected for further data collection based on clarity of amplification and ease of scoring from the initial eight samples. Additional samples were selected from three reintroduction sites (Couchville, Stones River National Battlefield [SRNB] Glade 7, and SRNB Glade 54), which originated from multiple natural seed source populations in Rutherford County, Tennessee, and therefore, likely represent the range of diversity expected in this endangered species. All three sites are in Rutherford County, Tennessee. Locality data are not reported here and population vouchers were not collected due to the sensitive nature of ongoing recovery efforts. A total of 109 individuals were genotyped at all loci following the same protocol as above, pooling two to three loci where possible as determined by allele size range. In addition, all loci were tested for cross-amplification in *A. crassicarpus* Nutt. var. *trichocalyx* (Nutt.) Barneby (n = 9), *A. gypsodes* Barneby (n = 2; TEX201642, TEX201643), and *A. tennesseensis* A. Gray ex Chapm. (n = 5) using the same parameters and strategies described above. Additional locality data are not reported here and population vouchers were not collected for *A. crassicarpus* var. *trichocalyx* or *A. tennesseensis*, due to both species being of conservation concern. Sequences on which primers were designed were deposited in GenBank (Table 1). A total of 12 novel loci were identified as polymorphic in the *A. bibullatus* samples included in this study. GenAlEx 6.502 (Peakall and Smouse, 2012) was used to calculate the number of alleles per locus and expected and observed heterozygosity by population (Table 2). The number of alleles per locus per population ranged from one to 15, with observed heterozygosity ranging from 0.000 (Abib120 exhibited a single allele within *A. bibullatus*) to 0.938, and expected heterozygosity ranging from 0.000 to 0.860. All loci

TABLE 2. Genetic properties of the 12 newly developed microsatellites of *Astragalus bibullatus*.[a]

	Couchville (n = 71)			Stones River 54 (n = 16)			Stones River 7 (n = 22)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
Abib028	2	0.892	0.494	2	0.938	0.498	2	0.909	0.496
Abib051	15	0.554	0.779	9	0.500	0.830	12	0.636	0.860
Abib059	6	0.531	0.611	5	0.867	0.711	5	0.682	0.600
Abib083	6	0.597	0.686	4	0.875	0.658	4	0.667	0.672
Abib093	3	0.263	0.325	2	0.200	0.180	2	0.100	0.180
Abib094	6	0.493	0.538	2	0.467	0.464	3	0.474	0.566
Abib095	4	0.443	0.485	3	0.188	0.271	4	0.500	0.439
Abib113	7	0.676	0.654	5	0.750	0.574	6	0.682	0.613
Abib120	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
Abib152	3	0.014	0.042	2	0.063	0.061	2	0.045	0.044
Abib156	3	0.471	0.517	3	0.688	0.471	3	0.789	0.528
Abib170	3	0.500	0.501	3	0.438	0.420	3	0.350	0.401

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

[a]Because this is a federally endangered species, geographic coordinates are restricted. All three populations represent reintroductions and are located in Rutherford County, Tennessee, USA.

successfully cross-amplified and were polymorphic in *A. crassicarpus* var. *trichocalyx*, *A. gypsodes*, and *A. tennesseensis*. Seven recently published loci (AM_15, AM_18, AM_25, AM_29, AM_34, AM_46, and AM_71) developed for *A. michauxii* (Kuntze) F. J. Herm. (Fabaceae; Wall et al., 2014) were also tested for amplification in all species included here. Of these, three successfully amplified (AM_15, AM_46, AM_71) in all species, except AM_71, which did not amplify in *A. tennesseensis*.

CONCLUSIONS

The loci developed here for *A. bibullatus* are now being used to assess population genetic structure in complement to an ongoing demographic study of five natural populations in addition to the three reintroduction populations presented here. These data will be combined with long-term demographic data of monitored individuals to better understand the reproductive ecology of this system. Additionally, preliminary data indicate that at least some of the markers developed here exhibit private alleles among each of the congeners evaluated in this study, suggesting that these markers will be valuable tools in systematic and phylogeographic investigations of closely related *Astragalus* species.

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