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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE LOCI FOR THE ENDANGERED SCRUB LUPINE, *LUPINUS ARIDORUM* (FABACEAE)[1]

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- *Premise of the study:* Microsatellite primers were developed in scrub lupine (*Lupinus aridorum*, Fabaceae), an endemic species to Florida that is listed as endangered in the United States, to assess connectivity among populations, identify hybrids, and examine genetic diversity.
- *Methods and Results:* We isolated and characterized 12 microsatellite loci polymorphic in scrub lupine or in closely related species (i.e., sky-blue lupine [*L. diffusus*] and Gulf Coast lupine [*L. westianus*]). Loci showed low to moderate polymorphism, ranging from two to 14 alleles per locus and 0.01 to 0.86 observed heterozygosity.
- *Conclusions:* These loci are the first developed for Florida species of lupine and will be used to determine differentiation among species and to aid in conservation of the endangered scrub lupine.

Key words: Fabaceae; Florida; *Lupinus*; microsatellite; PCR primers.

Scrub lupine (*Lupinus aridorum* McFarlin ex Beckner) is an endangered plant restricted to the Winter Haven and Mount Dora ridge systems in central Florida that has declined primarily due to habitat loss (USFWS, 1987). Polymorphic genetic markers are needed to answer questions about genetic diversity and connectivity among *L. aridorum* populations, genetic relatedness among Florida lupine species including the closely related Gulf Coast lupine (*L. westianus* Small), and hybridization between sympatric populations of *L. aridorum* and sky-blue lupine (*L. diffusus* Nutt.; Bupp, 2013).

METHODS AND RESULTS

Total genomic DNA was extracted from leaf samples of two individuals from *L. aridorum* populations using a QIAamp DNA Mini Kit (QIAGEN, Valencia, California, USA). Using a Covaris S220 (Woburn, Massachusetts, USA), we prepared an Illumina paired-end shotgun library by shearing 1 µg of DNA as described in the Illumina TruSeq DNA Library Kit (Illumina, San Diego, California, USA) and using a multiplex identifier adapter index. Illumina sequencing, with 100-bp paired-end reads, was conducted on a HiSeq 2000 (Illumina). We used the program PAL_FINDER_v0.02.03 (Castoe et al., 2012) to examine five million reads and extracted the reads that contained di-, tri-, tetra-, penta-, and hexanucleotide microsatellites. Positive reads were batched to the program Primer3 (version 2.0.0; Rozen and Skaletsky, 1999) for primer design. We selected loci for primer sequences that only occurred one time in the five million reads to avoid problems with copy number of the sequence in the genome. Ninety loci of the 1740 loci that met this criterion were chosen. One primer from each pair was modified on the 5′ end with an engineered sequence (CAG tag 5′-CAGTCGGGCGTCATCA-3′) to enable use of a third primer in the PCR that was fluorescently labeled with one of three dyes (6-FAM, NED, or VIC; Applied Biosystems, Culver City, California, USA).

Primer pairs were tested for amplification and polymorphism using DNA obtained from four individuals. Amplifications were in 20-µL volumes (250 µg/mL bovine serum albumin [BSA], 2 µL 10× Buffer B, 25 mM $MgCl_2$, 5 µM unlabeled primer, 0.5 µM tag-labeled primer, 5 µM universal dye-labeled primer, 2.5 mM dNTPs, 0.5 units *Taq* DNA polymerase [Fisher Scientific, Pittsburgh, Pennsylvania, USA], and 20 ng DNA template) using a Bio-Rad MyCycler (Hercules, California, USA) thermal cycler. We used touchdown cycling conditions to amplify DNA and to attach the universal dye-labeled primer. Parameters consisted of an initial denaturation step of 2 min 30 s at 95°C; followed by 20 cycles of 95°C for 20 s, 65–50°C annealing temperature for 20 s (decreasing 0.5°C per cycle), and extension step of 72°C for 30 s; followed by 15 cycles of 95°C for 20 s, 55–45°C for 20 s, and 72°C for 30 s. Cycles were followed with a final extension step of 72°C for 10 min. Amplifications were run on an ABI3730XL sequencer (Applied Biosystems).

Twelve of the tested primer pairs amplified high-quality PCR product that exhibited polymorphism in *L. aridorum*, *L. diffusus*, or *L. westianus* (Table 1). We then assessed the variability at these loci using 19–22 individuals of *L. aridorum*, 9–22 *L. diffusus*, and 12–20 *L. westianus* (Table 2). Alleles were scored using GeneMapper software (Applied Biosystems). We evaluated the number of alleles per locus, observed heterozygosity, and expected heterozygosity and tested for Hardy–Weinberg equilibrium (HWE) and linkage equilibrium using Arlequin version 3.5 (Excoffier et al., 2005). Sequences of raw paired-end reads are available in the SRA database of the National Center for Biotechnology Information (bioproject no. PRJNA274660) and as Appendix S1. Vouchers of leaves collected for this study were deposited at Bok Tower Gardens, Lake Wales, Florida (Table 2).

We found that the number of alleles per locus ranged from one to seven for *L. aridorum*, one to nine for *L. westianus*, and one to 14 for *L. diffusus*. For

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TABLE 1. Characteristics of 12 microsatellite loci in *Lupinus aridorum*, *L. westianus*, and *L. diffusus*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)[a]	T_a (°C)
Luar3	F: *AGAATATAAAGGTTTACAAGGGC	$(AAAG)_9$	212–226	61/51
	R: AAGCAGTTGTTGACTACACAGATACG			
Luar11	F: *GGATTTAGATTTGCTACTATTGGCCC	$(AACT)_8$	142–193	54/44
	R: CAGTCCGACCAGATAGTTTAACCG			
Luar12	F: *TGGCAGGGAAGGGAAGTAGG	$(ATC)_{20}$	242–248	54/44
	R: CAGTCGCATGAGAGCGGG			
Luar15	F: *GCACATGGCTTAACCAACTTCC	$(ATAGG)_{11}$	151–161	65/55
	R: CTAAGCGTGCGCATGTGG			
Luar31	F: *TTTCCATACCCTGCGTTTCC	$(ATAGG)_{12}$	259–269	54/44
	R: GTGCTGGAACAGGTAGTGGC			
Luar48	F: *GCGTCGTTTGACATTGACG	$(AAAG)_9$	140–166	54/44
	R: GGGAATTGAGAATAAAGAGGG			
Luar69	F: *TCACCATTCATCCCACATTCACC	$(AAC)_{15}$	236–242	65/55
	R: GTGGCCCATTCCAGTTCC			
Luar71	F: *CCGTATCCATATCCACTTTCCC	$(TTC)_{12}$	318–324	55/45
	R: GTTTAGTTCATTGTGCAACCCGC			
Luar73	F: *TGACAGCTAGAGGTTTCAAGGC	$(TTC)_{11}$	147–159	55/45
	R: GTTTAGCTTCTCTTCCACGCAAGC			
Luar74	F: *CTTCTCACCTCATTTCCAATTCC	$(TTC)_{13}$	238–250	55/45
	R: GTTTGGGAACCCATTATTCCGAGG			
Luar84	F: *TTCCTATCTTATCCTATCTTATTGTGTCC	$(ATAGG)_9$	118–138	60/50
	R: GTTTCAGGATATGTTCGGACGGG			
Luar89	F: *ATGTATGAACAACACACGGG	$(AACCCT)_{10}$	408–418	60/50
	R: GTTTAGTTGATCAAATGGCGGAGG			

Note: T_a = annealing temperature for touchdown protocol.
[a]Range of alleles in base pairs including CAG tag (location indicated with asterisk on primer sequence).

polymorphic loci, observed heterozygosity for *L. aridorum* ranged from 0.01 to 0.68, 0.15 to 0.86 for *L. westianus*, and 0.01 to 0.78 for *L. diffusus*. Expected heterozygosity for *L. aridorum* was between 0.08 and 0.73, 0.19 and 0.83 for *L. westianus*, and 0.09 and 0.90 for *L. diffusus*. All loci were in linkage equilibrium but several loci were out of HWE after correction for multiple tests (Table 2). Deviations from HWE are expected because samples were collected from several areas and individuals might be able to self-fertilize (Bupp, 2013; Bupp, personal communication).

TABLE 2. Genetic properties of 12 microsatellites developed for *Lupinus aridorum* with cross-species amplification in *L. westianus* and *L. diffusus*.[a]

	L. aridorum (n = 19–22)			*L. westianus* (n = 10–20)			*L. diffusus* (n = 9–22)		
Locus	A	H_o	H_e[b]	A	H_o	H_e[b]	A	H_o	H_e[b]
Luar3	2	0.08	0.22	2	0.16	0.33	5	0.14	0.25
Luar11	1	—	—	5	0.15	0.24	1	—	—
Luar12	2	0.38	0.31	3	0.20	0.19	2	0.04	0.12
Luar15	5	0.68	0.51	1	—	—	1	—	—
Luar31	2	0.01	0.08	1	—	—	2	0.01	0.09
Luar48	4	0.08	0.26*	6	0.52	0.68	14	0.70	0.90
Luar69	5	0.55	0.73	7	0.41	0.83*	8	0.62	0.87
Luar71	4	0.40	0.55	5	0.68	0.69	8	0.38	0.83*
Luar73	7	0.60	0.66	9	0.86	0.82	8	0.68	0.89
Luar74	6	0.52	0.70	5	0.68	0.67	6	0.78	0.85
Luar84	3	0.40	0.63	5	0.76	0.68	7	0.50	0.72
Luar89	1	—	—	1	—	—	3	0.20	0.65*

Note: A = number of alleles sampled; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled.
[a]Geographic locations for samples are: *Lupinus aridorum* (Orange County, Pines of Wekiva); *L. westianus* (Walton County, Point Washington State Forest); and *L. diffusus* (Brevard County: 28.0176°N, 80.6000°W; 28.7711°N, 80.7829°W; 28.5054°N, 80.6600°W; Hillsborough County: 27.5953°N, 82.2366°W; Polk County: 27.9385°N, 81.5738°W). All samples are from Florida, and vouchers are deposited at Bok Tower Gardens, Lake Wales, Florida (accession no. La61069s).
[b]Loci that are out of Hardy–Weinberg equilibrium are indicated with an asterisk after Bonferroni correction for multiple tests ($P < 0.004$).

CONCLUSIONS

These loci are the first to be developed for Florida lupine species and are polymorphic in at least three of the five species of North American unifoliolate lupines (Dunn, 1971), *L. aridorum*, *L. diffusus*, and *L. westianus*. Although microsatellite primers have been developed for other, distantly related species of lupines (e.g., Gonzalez et al., 2010), cross-species amplification of *L. aridorum* with these primers was unlikely because North American unifoliolate lupines represent a genetically divergent lineage that is distinctly different from other lupines (Mahé et al., 2011).

Lupinus aridorum is a critically endangered species, and a clear understanding of the effects of habitat fragmentation on the genetic diversity of populations and connectivity among populations is needed to inform conservation efforts. In addition, many of these loci are polymorphic in *L. westianus*, a threatened species in the state of Florida (Wunderlin and Hansen, 2008). Conservation genetic studies will aid in management of these rare lupines.

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