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TECHNICAL NOTE

Isolation of microsatellite loci from endangered members of *Lotus* (Fabaceae) subgenus Syrmatium

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Abstract *Lotus* subgenus Syrmatium is a group of 11 plant species that exhibit extensive ecological and morphological diversity throughout the California floristic province. Fifteen polymorphic microsatellite loci were isolated from two taxa, *Lotus argophyllus* var. *adsurgens* and *L. dendroideus* var. *traskiae*, and were screened for variability in 15 additional taxa within *Lotus* subgenus Syrmatium. Moderate levels of variability were observed with mean numbers of alleles per locus ranging from 1.3 to 7.3. The mean observed and expected heterozygosities ranged from 0.09 to 0.47 and 0.10 to 0.79, respectively. These new loci will be useful in conservation genetic and evolutionary studies within *Lotus* subgenus Syrmatium.

Keywords California Channel Islands · Conservation genetics · Lotus · Microsatellite

Lotus subgenus Syrmatium is a diverse group of 11 species within the pea family (Fabaceae), occurring predominantly in the California floristic province (Isely 1998; Allan and Porter 2000). Within this subgenus one taxon, *Lotus dendroideus* var. *traskiae*, is recognized by the U.S. Fish and Wildlife Service (USFWS) as endangered (USFWS 1977), and an additional three taxa, *L. argophyllus* var. *adsurgens*, *L. argophyllus* var. *niveus*, and *L. nuttallianus*, are recognized as species of concern (USFWS 1980). These taxa have been impacted by coastal development, military training, and introduced herbivores, the genetic effects of which warrant further study. Here we report the characterization of eight microsatellite loci isolated from *L. argophyllus* var. *adsurgens* and seven microsatellite loci isolated from *L. dendroideus* var. *traskiae*, useful for conservation genetic and evolutionary studies within *Lotus* subgenus Syrmatium.

Genomic DNA was isolated from leaf tissue using the DNeasy Plant Mini Kit (Qiagen). Microsatellite libraries were constructed individually for two taxa, *L. argophyllus* var. *adsurgens* and *L. dendroideus* var. *traskiae*. Isolation of microsatellite loci was performed following the subtractive hybridization method of Hamilton et al. (1999) with some modifications. Digested DNA was enriched for eight oligonucleotide repeats $(AC)_{15}$, $(AG)_{15}$, $(AT)_{15}$, $(CG)_{15}$, $(CCG)_{10}$, $(AAC)_{10}$, $(AGG)_{10}$, and $(CAC)_{10}$. Fragments were cloned using pBluescript II SK-Phagemid vector and the XL1-Blue MRF' bacterial host strain (Stratagene). Color-positive clones were screened for microsatellite regions using a membrane based 'dot blot' method (Glenn and Schable 2002) and the Phototope chemiluminescent detection system (New England Biolabs). A total of 227 positive clones were screened for insert size by PCR using a MJ Research PTC-200. The 25 µl reactions contained 1 µl template DNA, 0.8 µM each of primers T3 and T7 (Integrated DNA Technologies), 1× Thermopol Reaction Buffer (New England Biolabs),

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Table 1 Primer sequences and diversity statistics for eight microsatellite loci isolated from *Lotus argophyllus* var. *adsurgens* (LOAR) and seven microsatellite loci isolated from *L. dendroideus* var. *traskiae* (LODE)

Locus	GenBank accession number	Primer sequence (5′–3′)	5′ Tag	Label dye	Repeat motif	Allele size range	Genetic diversity					
							Species	N_A	H_O	H_E	HWE P value	Null alleles
LOAR_21	HM801119	F-CCTGATAAGATTGTGACGTAAAAG		VIC	$(ACC)_2GCCACCGCC(ACC)_4$	228–243	LODETR	1	0.00	0.00	–	No
		R-AAATGAAAGGACGTGTGGTA	M13R				LOARAD	2	0.04	0.04	1.000	No
							LOARAR	4	0.43	0.55	0.019	No
							Mean	2.3	0.16	0.20		
LOAR_50A	HM801117	F-CAAACCGTCAATAAATGAAACA		PET	$(CA)_8$	265–275	LODETR	1	0.00	0.00	–	No
		R-GCTGAGGAGTGAGGAGTGTTC	M13R				LOARAD	1	0.00	0.00	–	No
							LOARAR	2	0.37	0.30	0.552	No
							Mean	1.3	0.12	0.10		
LOAR_55B	HM801118	F-CCACAACAAGCAAATGGAGA	CAGT	PET	$(CA)_4TAAA(CA)_7$	256–262	LODETR	1	0.00	0.00	–	No
		R-TTCAAAACCTTGTCTTCAAAACC					LOARAD	2	0.03	0.03	1.000	No
							LOARAR	3	0.27	0.54	0.001	Yes
							Mean	2	0.10	0.19		
LOAR_70B	HM801116	F-GGTTTGGCTGTGGTACACG	CAGT	VIC	$(CT)_{13}$	277–335	LODETR	7	0.47	0.75	0.001	Yes
		R-TGTCAAGAAAAATAGTTCAACAACG					LOARAD	3	0.45	0.62	0.003	No
							LOARAR	6	0.47	0.80	0.001	Yes
							Mean	5.3	0.46	0.72		
LOAR_104	HM801115	F-TTGGGAAGTCATCAGAGATCAA	CAGT	VIC	$(GA)_6AA(GA)_5$	246–298	LODETR	9	0.17	0.78	0.001	Yes
		R-GATTTGGCTATGGCTTGTGC					LOARAD	6	0.17	0.82	0.001	Yes
							LOARAR	7	0.33	0.78	0.001	Yes
							Mean	7.3	0.22	0.79		
LOAR_131	HM801120	F-CAATGGAAGAGAGAAGAACA	CAGT	VIC	$(AG)_4GTC(AG)_{14}$	240–260	LODETR	4	0.13	0.16	0.168	No
		R-CAGAAAAGAGATGGAGGAGGAA					LOARAD	3	0.18	0.63	0.001	Yes
							LOARAR	5	0.57	0.72	0.128	No
							Mean	4	0.29	0.50		
LOAR_201	HM801121	F-CTTCCGCTTCGCATCCTTG	CAGT	VIC	$(TTC)_7$	195–213	LODETR	3	0.30	0.53	0.002	No
		R-CTTTGGCGGAAAGGTGCTC					LOARAD	3	0.17	0.47	0.001	Yes
							LOARAR	4	0.20	0.39	0.001	Yes
							Mean	3.3	0.22	0.47		
LOAR_216	HM801122	F-TTTCTCATTCTAACACCAGAGATAC	CAGT	6-FAM	$(TC)_9(AC)_7$	166–190	LODETR	6	0.50	0.71	0.001	No
		R-CAGGTTTATTGGCCCTGTCG					LOARAD	2	0.10	0.38	0.001	Yes
							LOARAR	6	0.60	0.69	0.421	No
							Mean	4.7	0.40	0.59		

Table 1 continued

Locus	GenBank accession number	Primer sequence (5′–3′)	5′ Tag	Label dye	Repeat motif	Allele size range	Genetic diversity					
							Species	N_A	H_O	H_E	HWE *P* value	Null alleles
LODE_16	HM801108	F-CATTACCTAATCAGGATGTGC		6-FAM	$(CA)_{10}$	184–200	LODETR	5	0.23	0.22	1.000	No
		R-TTTCTGTGGCGGAGGA	M13R				LOARAD	1	0.00	0.00	–	No
							LOARAR	3	0.43	0.68	0.040	Yes
							Mean	3	0.22	0.30		
LODE_44	HM801109	F-GAAGAATTGGGGGCAGTGTA		PET	$(TC)_6TT(TC)_5$	362–374	LODETR	1	0.00	0.00	–	No
		R-GATTTTTAATGCACGCTTGG	M13R				LOARAD	3	0.29	0.57	0.001	Yes
							LOARAR	4	0.47	0.54	0.541	No
							Mean	2.7	0.25	0.37		
LODE_48B	HM801110	F-TGTTTGGCAAAATCCAATGA	CAGT	VIC	$(GT)_{13}$	279–295	LODETR	4	0.50	0.53	0.892	No
		R-CCTACCAGTTCCAGTTCATA					LOARAD	3	0.37	0.59	0.004	Yes
							LOARAR	6	0.53	0.72	0.001	Yes
							Mean	4.3	0.47	0.61		
LODE_50	HM801111	F-CCCCACCCCAATTACACTATT	M13R	6-FAM	$(TG)_9$	246–254	LODETR	2	0.30	0.49	0.059	No
		R-GAAGTTCAATGCGTCAAGC					LOARAD	3	0.00	0.13	0.001	Yes
							LOARAR	4	0.30	0.27	1.000	No
							Mean	3	0.20	0.30		
LODE_146	HM801112	F-AAAGGACTGGACCAGGCT		PET	$ATTCTT(GTT)_8$	203–221	LODETR	3	0.34	0.57	0.001	No
		R-GCAGCACCAGGTACAAAG	CAGT				LOARAD	4	0.33	0.57	0.001	Yes
							LOARAR	3	0.32	0.53	0.048	Yes
							Mean	3.3	0.33	0.56		
LODE_246	HM801113	F-TGAGGGAATTGGGTGATTTG	M13R	6-FAM	$(TC)_{10}CC(TC)_6$	256–284	LODETR	6	0.20	0.73	0.001	Yes
		R-CACCAGGAAAAGAAAGAAATCCAG					LOARAD	3	0.37	0.57	0.032	Yes
							LOARAR	2	0.07	0.07	1.000	No
							Mean	3.7	0.21	0.46		
LODE_I	HM801114	F-TGACGCATTAGGTGTTTGGG		PET	$(CTT)_6(CAT)_2CTT$	191–197	LODETR	2	0.10	0.10	1.000	No
		R-GGAACCTACCACAAGCAAGC	CAGT				LOARAD	1	0.00	0.00	–	No
							LOARAR	3	0.17	0.16	1.000	No
							Mean	2	0.09	0.09		

Shown are loci names, the GenBank accession numbers, the forward (*F*) and reverse (*R*) primer sequence, the 5′ tag used for incorporation of the fluorescent tag M13R (AGGAAACAGCTATGACCAT) or CAGT (ACAGTCGGGCGTCATCA), labeling dye used, repeat motif of the sequenced clone, allele size range in base pairs, the number of alleles (N_A), observed heterozygosity (H_O), and expected heterozygosity (H_E) determined as the mean value from 30 individuals each of *L. argophyllus* var. *adsurgens* (LOARAD), *L. argophyllus* var. *argenteus* (LOARAR), and *L. dendroideus* var. *traskiae* (LODETR), *P* value associated with departure from Hardy–Weinberg Equlibrium (HWE), and the inferred presence of null alleles

Table 2 Cross amplification of 15 microsatellite loci in 15 members of *Lotus* subgenus Syrmatium

	N	LOAR_21	LOAR_50A	LOAR_55B	LOAR_70B	LOAR_104	LOAR_131	LOAR_201	LOAR_216	LODE_16	LODE_44	LODE_48B	LODE_50	LODE_146	LODE_246	LODE_I
L. argophyllus var. *argophyllus*	13	230–234	267–271	254–262	281	258–274	250–260	201–210	176–192	186–196	370–374	285–305	250–268	203–212	254–282	194
L. argophyllus var. *fremontii*	5	–	271–277	260–262	283–285	256–266	246	204	180–182	198	372	295–307	256	218–221	260–268	194
L. argophyllus var. *niveus*	8	234–243	267	258	281–283	252–256	254–256	195–207	178	196–198	370	281–291	250–252	215–230	256	194
L. benthamii	5	230	267–271	262–266	283–285	260–274	250–256	207	178–188	200–202	354	287–295	254	218–230	268–270	197–200
L. dendriodeus var. *dendroideus*	31	228–243	267–271	256–264	283–303	250–272	242–266	192–204	168–186	192–204	368–380	279–291	250	212–224	256–298	197–203
L. dendriodeus var. *veatchii*	8	228–234	267–271	258	285–291	260–266	240–256	201–204	180–182	192–202	374–376	285–295	250	203–218	268	197–200
L. haydonii	5	242–252	265–271	256–270	285–287	254–274	252–260	198–204	182–188	198–202	362–374	291–301	250–254	212–224	258–264	191–206
L. heermannii var. *heermannii*	13	244–246	267	258–270	281–287	256–262	248–260	204–207	178–182	192–206	370–374	281–291	250–252	212–224	262–268	194–197
L. heermannii var. *orbicularis*	5	230	271	264	293	260	242–244	219	182–184	200	348–356	295–297	252	215	286	191–194
L. junceus var. *junceus*	5	242–248	267–271	262–270	279–281	248–272	252–262	198–207	172–180	200–204	370–372	283–287	250–254	218–221	258	197–206
L. nevadensis var. *davidsonii*	5	230	267–269	262	287–289	250	250–252	204–216	180–186	196–200	370–374	281–291	250–254	218–224	272–300	191–194
L. nevadensis var. *nevadensis*	5	222	271–277	258	–	258–264	244	204–213	196–204	200–202	356–372	295–297	254–256	197–203	250–258	194–203
L. nuttallianus	8	226–240	267–269	246–262	271–287	244–262	250–254	195–207	168–176	196–200	362–368	289–295	248–250	215–221	242–254	194–209
L. procumbens var. *procumbens*	5	230–236	281–289	248–252	277–281	244–262	246–252	201–210	184–202	188–196	370–380	277–283	252–258	215–221	254–270	194–203
L. scoparius var. *scoparius*	13	234–244	267	254–266	279–287	256–278	250–260	195–210	168–184	196–200	368–372	281–295	250–258	212–224	256–264	191–209

Shown are the taxa screened, the number of individuals sampled from each taxon (*N*), and the observed allele size range if the amplification was successful

200 μM of each dNTP, and 0.2 units of Vent (exo-) DNA polymerase (New England Biolabs). Clones that exhibited a single amplified band of 400–1000 bp were cleaned using a PEG precipitation procedure and sequenced using the T3 primer and BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) in 1/8 volume reactions. Sequences were electrophoresed on an Avant 3100 Genetic Analyzer (Applied Biosystems). For inserts containing a di- or tri-nucleotide microsatellite motif, the T7 primer was used to generate a complementary reverse sequence. All sequences were aligned using SEQUENCHER 4.1 (GeneCodes).

Of the 227 sequenced inserts, 87 contained a region of at least six repeat units, but only 54 proved suitable for primer design. Primers were designed using the program PRIMER 3 (Rozen and Skaletsky 2000). One primer of each pair was designed with a common tag at the 5′ end following the procedure of Boutin-Ganache et al. (2001; Table 1). Two common tags were used: M13R (AGGAAACAGCTATGACCAT) and CAGT (ACAGTCGGGCGTCATCA). We chose eight primers from *Lotus argophyllus* var. *adsurgens* and seven primers from *L. dendroideus* var. *traskiae* that yielded consistent amplification products. Loci were amplified with a common tag containing one of three fluorescent dyes, 6-FAM, PET, or VIC (Applied Biosystems).

One sample population each of *Lotus argophyllus* var. *adsurgens* and *L. dendroideus* var. *traskiae*, each containing 30 individuals, were used to evaluate variability in the isolated microsatellite loci. An additional population of *L. argophyllus* var. *argenteus*, a common taxon that co-occurs with the rare taxa, was also screened to evaluate variability in a species not of conservation concern. Microsatellite loci were amplified in 10 μl reactions using the Type-it Microsatellite PCR Kit (Qiagen). When possible multiplex PCR with 2–3 loci was used. Manufacturer protocols and thermal cycler programs were used for all amplifications. PCR products were diluted with water and mixed with Hi-Di formamide and LIZ 500 size standard (Applied Biosystems) before electrophoresis on an Avant 3100 Genetic Analyzer. Fragments were sized using the GENEMAPPER software (Applied Biosystems). We calculated observed (H_O) and expected (H_E) heterozygosity, and tested for deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium using GENEPOP version 3.4 (Raymond and Rousset 1995). MICRO-CHECKER version 2.2.3 was used to infer the presence of null alleles with 1,000 bootstrap replicates (Van Oosterhout et al. 2004).

All loci were found to be polymorphic in at least one population. The mean number of alleles per locus ranged from 1.3 to 7.3, with an average of 3.5 (Table 1). The observed and expected mean heterozygosity ranged from 0.09 to 0.47 and 0.10 to 0.79, respectively, with the common taxon, *L. argophyllus* var. *argenteus*, exhibiting the highest levels of diversity. Three loci, LOAR_70B, LOAR_104, and LOAR_201, showed significant ($P < 0.01$) deviations from HWE in all three taxa. One taxon, *L. argophyllus* var. *adsurgens* showed significant ($P < 0.01$) deviations from HWE at every locus. Of the 105 interlocus comparisons, ten exhibited significant ($P < 0.01$) linkage disequilibrium. The observed linkage disequilibrium is likely driven by population differentiation. Tests for null alleles ($P < 0.01$) identified one locus (LOAR_104) that exhibited potential null alleles for each population sampled. Cross amplification was evaluated in 15 additional *Lotus* subgenus Syrmatium taxa, including the two other taxa that are USFWS species of concern (Table 2). The markers described in this paper will be used to investigate the population structure, levels of genetic variability, and patterns of evolutionary history within *Lotus* subgenus Syrmatium.

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