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TECHNICAL NOTE

Development and characterization of sixteen microsatellite markers for the federally endangered species: *Leptodea leptodon* (Bivalvia: Unionidae) using paired-end Illumina shotgun sequencing

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Abstract We isolated and characterized a total of 16 microsatellite loci in *Leptodea leptodon*. Loci were screened in 24 individuals from across the species current range in Missouri. The number of alleles per locus ranged from 3 to 17, observed heterozygosity ranged from 0.208 to 1.000, and the probability of identity values ranged from 0.014 to 0.67. These new loci will be used for conducting investigations into the genetic structure and diversity of extant populations of this federally endangered species.

Keywords *Leptodea* · Microsatellite · PAL_FINDER · PCR primers · SSR

The scaleshell *Leptodea leptodon* (Rafinesque, 1820) is a federally endangered species (USFWS 2001). Whereas this species once occurred in 56 rivers in 13 states within the Mississippi River Basin, it is now restricted to 13 streams in Missouri, Arkansas and Oklahoma, with the largest known populations occurring in the Meramec, Bourbeuse and Gasconade rivers in Missouri (USFWS 2010). Degradation of water quality, sedimentation, channel destabilization, and habitat destruction are all considered to be contributing factors in the decline of the scaleshell throughout its range. Very little is known about the life history, reproductive biology and the extant population size of *L. leptodon* (USFWS 2010), and the development of species specific genetic markers are consistent with the goals outlined in the USFWS recovery plan, including improving our understanding of the biology and ecology of the scaleshell mussel.

Total DNA was extracted from one individual of *Leptodea leptodon*, following the protocol of Puregene Buccal Cell DNA Kit (Gentra Systems, Inc. Minneapolis, MN) for use in isolation of microsatellite loci. An Illumina paired-end shotgun library was prepared by shearing 1 μg of DNA using a Covaris S220 and following the standard protocol of the Illumina TruSeq DNA Library Kit and using a multiplex identifier adaptor index. This library was pooled with those from other species and Illumina sequencing was conducted on the HiSeq with 100 bp paired-end reads. Five million of the resulting reads were analyzed with the program *PAL_FINDER_v0.02.03* (Castoe et al. 2012) to extract those reads that contained di-, tri-, tetra-, penta-, and hexanucleotide microsatellites. Once positive reads were identified in *PAL_FINDER_v0.02.03* they were batched to a local installation of the program Primer3 (version 2.0.0) for primer design. To avoid issues with copy number of the primer sequence in the genome, loci for which the primer sequences only occurred one or two times in the 5 million reads were selected. Forty-eight loci of the 3905 that met this criterion were chosen. One primer from each pair was modified on the 5′ end with an engineered sequence (CAG tag 5′-CAGTCGGGCGTCAT CA-3′) to enable use of a third primer in the PCR (identical to the CAG tag) that was fluorescently labeled. The

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sequence GTTT was added to primers without the universal CAG tag addition.

Forty-eight primer pairs were tested for amplification and polymorphism using DNA obtained from eight individuals. PCR amplifications were performed in a 12.5 µl volume (10 mM Tris pH 8.4, 50 mM KCl, 25.0 µg/ml BSA, 0.4 µM unlabeled primer, 0.04 µM tag labeled primer, 0.36 µM universal dye-labeled primer, 3.0 mM $MgCl_2$, 0.8 mM dNTPs, 0.5 units AmpliTaq Gold® Polymerase (Applied Biosystems), and 20 ng DNA template) using an Applied Biosystems GeneAmp 9700. Either touchdown thermal cycling programs (Don et al. 1991) encompassing a 10 °C span of annealing temperatures ranging between 65–55 °C (TD65) or standard thermal cycling parameters with an annealing temperature of 65 °C were used for all loci (Table 1). Touchdown cycling parameters consisted of an initial denaturation step of 5 min at 95 °C followed by 20 cycles of 95 °C for 30 s, highest annealing temperature (decreased 0.5 °C per cycle) for 30 s, and 72 °C for 30 s; and 20 cycles of 95 °C for 30 s, lowest annealing temperature for 30 s, and 72 °C for 30 s. Standard cycling parameters consisted of an initial denaturation step of 5 min

Table 1 Details for 16 polymorphic microsatellite loci developed for *Leptodea leptodon*

Locus	Primer Sequence 5′ → 3′	Repeat motif	Allele Size (bp)	N	*K*	H_o	H_e	PI	TA
Lele3[a]	F: GAACAGTTATAATAGACCCTTAGTGTTACC[b]	AC	390–410	23	9	0.435	0.817	0.058	TD65
	R: GGAAATCTCTCGACCTTTACTGC								
Lele6	F: GCTGGCTAACGTATCTGTAATGG[b]	ATAC	141–177	24	10	0.708	0.869	0.032	TD65
	R: TTTGCAGTAAATAACTGAACCCG								
Lele7	F: GACCCGACACATACGGCG[b]	ATCT	373–425	24	13	0.833	0.886	0.023	TD65
	R: GCAACATTGACTTACTTGTTCCACC								
Lele8	F: CATTTGACACATATTTCGTTTGGG[b]	TTC	262–334	21	17	0.714	0.914	0.014	65
	R: CATTGACGGTGAAATGTCGG								
Lele9	F: GGTAACAATGGCAATATGTTTGG[b]	AATG	153–193	21	9	0.810	0.794	0.067	TD65
	R: CGAATTTAGTTTCACTTCTTGTGG								
Lele13	F: TGTATGAACGGATTGAACGG[b]	ATCT	121–217	22	15	0.864	0.899	0.019	TD65
	R: TCTTATCCATCATTCATCTGCC								
Lele16	F: CCATCCAACTATAGAATGTCCCG[b]	ATAC	179–243	24	15	0.917	0.898	0.018	65
	R: CGATGCTTTCCCACACTATCC								
Lele18	F: GCTACATATGTTGATATACAAATTCGG[b]	AAC	310–325	24	3	0.208	0.190	0.67	65
	R: TCATTTACAATGATGGCCAGG								
Lele19	F: CGCTAGCTTGATTAGGCTTCAGG[b]	TGCG	194–254	23	13	0.609	0.874	0.027	TD65
	R: TGTGTGAGTGCGTGCTTATGG								
Lele20	F: AGCATTAGACTGATAAATTGTTTGC[b]	AC	137–171	24	15	0.792	0.878	0.025	TD65
	R: ATACATTGTAACTCATGGCTTCG								
Lele24	F: CCATCAAAGACGTCGGAGC[b]	AAAC	198–218	24	6	0.583	0.822	0.057	TD65
	R: CTCGGGTGCAATGTAGAGAGC								
Lele30	F: GCAACGAAGATGGTAATGTTGG[b]	ATC	144–189	24	11	0.833	0.858	0.036	TD65
	R: CACTTGTCGTCAAGATCTCCG								
Lele33	F: GGGTTAAAGTAGAACGGCGG[b]	AAAG	158–210	22	11	0.864	0.877	0.027	TD65
	R: AACAGACCCTTAAATGGTGTCC								
Lele45	F: GCTAAGCTAAAGCTACAAATCACGC[b]	ATCT	216–304	23	14	1.000	0.900	0.019	65
	R: AGTACACCTGAAATGACAATATGGC								
Lele47	F: TTCTTTGAGTGTCATTAGACGTGG[b]	AATG	196–252	24	11	0.875	0.866	0.032	65
	R: TGCCAGGCTGGATTTATAGG								
Lele48	F: TCACAGATGATGTCTACAGACAATAGTAGG[b]	ATAC	216–288	23	14	0.696	0.888	0.022	TD65
	R: CCTTGCGAAATGGTCATTGG								

Allele size includes the length of the CAG tag; *N* number of individuals; *k* number of alleles observed; H_o and H_e are observed and expected heterozygosity, respectively; *PI* the probability of identity for each locus, and *TA* annealing temperature with TD indicating a touchdown protocol was used (see text)

[a] Indicates significant deviations from Hardy-Weinberg expectations after Bonferroni corrections

[b] Indicates CAG tag (5′-CAGTCGGGCGTCATCA-3′) label

at 95 °C followed by 40 cycles of 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s. PCR products were run on an ABI-3130xl sequencer and sized with Naurox size standard prepared as described in DeWoody et al. (2004), except that unlabeled primers started with GTTT. Results were analyzed using GeneMapper version 3.7 (Applied Biosystems). Seventeen of the tested primer pairs amplified high quality PCR product that exhibited polymorphism.

We assessed the variability of the 16 polymorphic loci in 24 specimens collected, from three putative populations in Missouri. Conditions and characteristics of the loci are provided in Table 1. We estimated the number of alleles per locus (*k*), observed and expected heterozygosity (H_o and H_e), and probability of identity (PI) using GenAlEx v6.4 (Peakall & Smouse 2006). Tests for deviations from Hardy-Weinberg equilibrium (HWE) and for linkage disequilibrium were conducted using GENEPOP v4.0 (Rousset 2008). After Bonferroni correction for multiple comparisons one locus showed significant deviations from expectations under HWE and no linkage disequilibrium was detected for any of 120 paired loci comparisons. It is hoped that the development of these microsatellite markers will provide the necessary data to allow us to assess the genetic differentiation within and among extant *L. leptodon* populations, and estimate dispersal and therefore connectivity between populations. These data will provide conservation managers with valuable information and allow them to develop a suitable conservation strategy for this species.

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