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Short Communication

Characterization of 12 microsatellite loci for the Pacific lamprey, *Entosphenus tridentatus* (Petromyzontidae), and cross-amplification in five other lamprey species

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ABSTRACT. The Pacific lamprey (*Entosphenus tridentatus*) is an anadromous fish that is of conservation concern in North America and Asia. Data on Pacific lamprey population structure are scarce and conflicting, impeding conservation efforts. We optimized 12 polymorphic microsatellite loci for the Pacific lamprey. Three to 13 alleles per locus were observed in a sample of 51 fish collected from the West Fork Illinois River, Oregon. Observed heterozygosity ranged from 0.235 to 0.902 and expected heterozygosity ranged from 0.214 to 0.750. Cross-species amplification produced 8 to 12 polymorphic

loci in four other *Entosphenus* species and in the western brook lamprey (*Lampetra richardsoni*). Two loci appear to be diagnostic for distinguishing *Entosphenus* from *Lampetra*. These markers will be valuable for evaluating population structure and making conservation decisions for *E. tridentatus* and other lamprey species.

Key words: *Entosphenus*; *Lampetra richardsoni*; Microsatellite; Pacific lamprey; Petromyzontidae; Western brook lamprey

The Pacific lamprey (*Entosphenus tridentatus*, Petromyzontidae) is an anadromous fish that lives along the Pacific coast of North America from Alaska to California and in Asia as far south as Japan (Scott and Crossman, 1973). Although its numbers have decreased dramatically in the United States since the 1960s (Close et al., 2002), the United States Fish and Wildlife Service declined to protect the Pacific lamprey under the Endangered Species Act (United States Fish and Wildlife Service, 2004), due in part to a lack of information on population structure. Studies examining possible Pacific lamprey residency in Japanese rivers have suggested that Asian populations are not well established (e.g., Yamazaki et al., 2005), and the Japanese government has listed the Pacific lamprey as "data deficient" for conservation purposes (Japan Ministry of the Environment, 2003).

Studies on population structure in the Pacific lamprey have been conflicting; Goodman et al. (2008) found no evidence of geographical population structure using mitochondrial DNA, whereas Beamish and Withler (1986) and Lin et al. (2008) found small but significant differences between Pacific lampreys from different locations using allozymes and amplified fragment length polymorphisms, respectively. Microsatellites, the marker of choice for examining population structure within a species, were not available for the Pacific lamprey, and those from other lamprey species failed to amplify or had low polymorphism (Close and Aronsuu, 2003). We, thus, developed and optimized 12 microsatellite loci for use in Pacific lamprey. These loci will be valuable in analyzing Pacific lamprey population structure and determining appropriate conservation strategies.

Microsatellite loci were isolated from a Pacific lamprey genomic DNA library (Genetic Identification Services, Chatsworth, CA, USA). Blunt-end enzymes *Rsa*I, *Hae*III, *Bsr*I, *Pvu*II, *Stu*I, *Sca*I, and *Eco*RV were used to partially digest genomic DNA. Blunt-ended adapters with a *Hin*dIII restriction site were ligated to DNA fragments 300 to 750 bp long. Four libraries were prepared in parallel using biotin-CA_8, biotin-GA_8, biotin-AAC_8, and biotin-CAG_8 as capture molecules (MPG beads, PureBiotech LLC); these were PCR-amplified and digested with *Hin*dIII to remove adapters. The fragments were ligated into the *Hin*dIII cut site of the pUC19 plasmid. Recombinant plasmids were electroporated into *Escherichia coli* (DH5α), and clones were randomly chosen for sequencing. The CA library produced 31 microsatellites, GA = 25, AAC = 17, and CAG = 17. PCR primers were designed for 24 microsatellite loci using Designer PCR v. 1.03 (Research Genetics, Inc.).

The 24 loci were tested in Pacific lamprey larvae from the Fraser River, British Columbia (N = 7). DNA was extracted using the Wizard® Genomic DNA Purification kit (Promega). PCR amplification was performed in 10-µL reaction volumes with approximately 50 ng genomic DNA, 1X KAPA Taq Buffer A, 2.25 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer, and 0.2 U Taq DNA polymerase (KAPA Biosystems). PCR conditions

were as follows: an initial denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 s, primer annealing at 51-62°C (Table 1) for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 5 min. Testing was performed with non-dye-labeled primers, and PCR products were visualized on a 1.5% agarose gel under UV light.

Nine of the 24 primer pairs from the Pacific lamprey consistently produced fragments of the expected size. In addition, seven primer pairs developed for the western brook lamprey (*Lampetra richardsoni*) (Luzier et al., 2010) and two primer pairs developed for *Lethenteron* sp N brook lamprey (Takeshima et al., 2005) amplified in Pacific lamprey. Thus, we evaluated a total of 18 primer pairs in Pacific lamprey.

Forward primers were 5'-end labeled with 6-Fam or Hex (Sigma Life Science) or Ned or Pet (Applied Biosystems) fluorescent dyes. Fragment size analysis was performed on an ABI 3130 Genetic Analyzer, and allele sizes were determined using Genemapper v. 4.0 (Applied Biosystems). Six of the nine Pacific lamprey primer pairs, five from *L. richardsoni* and one from *Lethenteron* sp N, were well-resolved and polymorphic in Pacific lamprey. These 12 loci were amplified in 51 Pacific lampreys from the West Fork Illinois River, Oregon. Genetic variation was analyzed using Genepop v. 4.0.10 (Raymond and Rousset, 1995; Rousset, 2008).

Mean expected heterozygosity was 0.587 (0.214-0.750); mean observed heterozygosity was 0.558 (0.235-0.902; Table 1). The number of alleles per locus ranged from 3 to 13, with a mean of 6.3. Four loci deviated from Hardy-Weinberg equilibrium in the sampled population; Etr-2 displayed heterozygote deficit, while Etr-3, Etr-4, and Etr-5 displayed heterozygote excess. Linkage disequilibrium was significant for six of 66 possible locus pairings (Etr-3 × Etr-5, Etr-5 × Lri-2, Etr-6 × Lri-9, Lri-2 × Lri-3, Lri-2 × Lri-7, and Lri-3 × Lri-9). This could mean that these loci are physically linked (i.e., in close proximity on the same chromosome); however, Luzier et al. (2010) found no significant linkage disequilibrium between any of the Lri loci, suggesting that the linkage disequilibrium between Lri loci in the 51 individuals tested here is not consistent across all populations. Furthermore, with the exception of Lri-2, all loci in linkage disequilibrium in the West Fork Illinois River population were tested in more than 900 Pacific lampreys from 20 other populations. Etr-6 and Lri-9 were not in linkage disequilibrium in any of the populations, whereas Etr-3 and Etr-5 were in linkage disequilibrium in only two of the 20 populations. Therefore, although Lri-2 should be used with caution until tested on more individuals, there is little reason to be concerned about pseudo-replication errors using the other loci.

Cross-species amplification of these loci was tested using the PCR conditions described above for the following species: Pit-Klamath brook lamprey (*E. lethophagus*) from Fall River, California; Cowichan lamprey (*E. macrostomus*) from Cowichan Lake, British Columbia; Miller Lake lamprey (*E. minimus*) from Miller Lake, Oregon; Klamath lamprey (*E. similis*) from Lewiston Reservoir, California, and *L. richardsoni* from Lockwood Creek, Washington (N = 10 for each species). Eight loci were polymorphic in *E. minimus* and *L. richardsoni*, 11 were polymorphic in *E. lethophagus* and *E. macrostomus*, and 12 were polymorphic in *E. similis* (Table 2). Three loci failed to amplify in *L. richardsoni*. Etr-1 and Etr-6 produced fragments of sufficiently different size in *Entosphenus* spp versus *L. richardsoni* to be distinguished on an agarose gel. These markers will be useful in delineating population structure in the Pacific lamprey and other lamprey species.

Table 1. Characteristics of 12 microsatellite loci developed for Pacific lamprey (*Entosphenus tridentatus*) and tested in 51 individuals from the West Fork Illinois River, Oregon.

Locus	Accession No.	Primer sequence 5'-3' (fluorescent label)	Repeat motif	T_a (°C)	N_A	Size range (bp)	H_E	H_O
Etr-1	HM594248	F: CCA-TCC-ACA-CAC-AGT-CTC-TC (P) R: GTT-AAG-GTG-TCT-CCC-AGT-GTC	Interrupted $[CA]_N$	52	3	225-229	0.214	0.235
Etr-2	HM594249	F: GCC-ACT-GCT-TAT-CCT-CTC-A (H) R: GTA-GGG-TGC-GAA-AGA-AGT-TC	Interrupted $[CT]_N$	62	4	243-249	0.672	0.529*
Etr-3	HM594250	F: ATT-GGC-GTC-AGA-ACG-AAC (F) R: GGT-GCT-ACA-CGC-TAA-CAG-C	Interrupted $[CA]_N$, $[CAA]_N$	58	7	141-173	0.700	0.902*
Etr-4	HM594251	F: TGG-TGA-TGT-TTC-CAC-ACA-G (F) R: ATG-ACT-CAC-AAT-CGT-GTC-TCA	$N_{18}[CA]_5N_{58}[GGT]_5N_{29}$	52	6	162-177	0.604	0.902*
Etr-5	HM594252	F: TGT-TGC-TGC-TGA-TGT-TGT-T (N) R: CAT-TTC-CCG-TGT-TTG-TTT-C	Interrupted $[GCT]_N$, $[GGT]_N$, $[GCG]_N$	55	7	168-189	0.707	0.804*
Etr-6	HM594253	F: CTA-CCG-CTC-CCA-ACA-ATA-C (N) R: GTT-TGC-TAC-ATC-ACC-GAA-TC	Interrupted $[CAT]_N$, $[CAG]_N$	51	13	277-316	0.669	0.686
Lri-2	HM594255	F: GGC-TCT-TAC-CGA-ACA-CCT-G (F) R: CAG-CGT-GCT-AAC-TGC-TAT-CC	$[GT]_5AC[GT]_2T[GT]_6$	52	3[a]	136-144	0.513	0.388
Lri-3	HM594256	F: GAG-GCT-GAC-GAC-CAG-TTG (N) R: CAT-TGG-GAT-GCT-GTG-TCC	$[CA]_6G[CA]_9G[CA]_7G[CA]_5$	53	11	203-241	0.750	0.784
Lri-5	HM594258	F: GCC-GAC-AAC-AAC-CAA-CAT-C (F) R: CAC-GCA-GGT-CAC-CCT-CTA-C	$[CAA]T[CAA]_4T[CAA]_4C[CAA]$	57	4[a]	268-277	0.527	0.551
Lri-7	HM594260	F: TGC-CAA-ACA-TTC-CAA-GTG (H) R: AGG-TCT-TCC-TCC-AAC-AGT-G	$[CAT]_3TAT[CAT]_4$	52	5	142-157	0.632	0.706
Lri-9	HM594262	F: GAG-AGG-AGC-GAG-GCT-CTA-C (F) R: ACA-TCC-ACG-CTT-AAA-TAC-TGG	$[AAC]_5[CAC]_3[AAC]_3$	57	4	246-267	0.543	0.549
Lspn 088	AB209985	F: GGA-TAA-TCG-TCA-GCA-GTG-TT (F) R: TCC-ATC-TCT-CTC-GTT-ACC-AT	$[CA]_4CG[CA]_6$	55	3	160-164	0.515	0.569

Primers for all Lri loci were developed by Luzier et al. (2010). Primers for Lspn 088 were developed by Takeshima et al. (2005). Fluorescent labels: P = Pet; H = Hex; F = 6-Fam; N = Ned. T_a = annealing temperature; N_A = number of alleles; H_E = expected heterozygosity; H_O = observed heterozygosity. *Indicates significant deviation from Hardy-Weinberg equilibrium. [a] = amplified in 49 individuals.

Table 2. Number of alleles and size ranges of 12 microsatellite loci in four other species of *Entosphenus* and in *Lampetra richardsoni*.

Locus	*E. lethophagus*		*E. macrostomus*		*E. minimus*		*E. similis*		*L. richardsoni*	
	N_A	Size range (bp)	N_A	Size range (bp)	N_A	Size range (bp)	N_A	Size range (bp)	N_A	Size range (bp)
Etr-1	1	227	1	227	1	227	2	227-229	1	259
Etr-2	3	243-247	3[b]	243-249	3	243-249	4	245-251	-	-
Etr-3	5	131-165	6	131-173	4	131-163	2[c]	163-173	8	137-163
Etr-4	4	162-171	2[a]	171-174	3[a]	165-171	4	165-171	-	-
Etr-5	2	180-183	3	177-183	1	177	3	177-183	3[a]	159-183
Etr-6	6	280-313	2	286-289	1	286	4	280-298	2	160-175
Lri-2	3	136-142	2	136-142	2[a]	136-142	2	136-142	6[d]	135-161
Lri-3	4	207-231	6	197-235	2	207-229	2[b]	221-229	5[d]	215-231
Lri-5	2[a]	271-274	2[a]	271-274	3[a]	271-277	3	271-277	6[d]	258-274
Lri-7	3	148-154	3	151-157	1	154	2	151-154	6[d]	136-157
Lri-9	2	264-267	3	249-267	2	249-264	5	249-270	2[d]	260-263
Lspn 088	4	160-166	2	160-162	2	160-162	2	160-162	-	-

Unless otherwise indicated, loci were amplified in 10 individuals of each species. - = no amplification. [a] = amplified in 9 individuals. [b] = amplified in 8 individuals. [c] = amplified in 7 individuals. [d] = amplified in 35 individuals, described in Luzier et al. (2010).

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