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PRIMER NOTE

Isolation and characterization of *Brachymystax lenok* microsatellite loci and cross-species amplification in *Hucho* spp. and *Parahucho perryi*

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Abstract

We isolated and characterized eight polymorphic microsatellite markers for *Brachymystax lenok* (Pallas, 1773) from genomic libraries enriched for $(GATA)_n$, $(GACA)_n$ and $(ATG)_n$ microsatellites. The number of alleles per locus ranged from two to 17. Heterozygosity ranged from 0.2 to 0.95. In addition, cross-species amplification was successful for seven loci in *Hucho hucho*, eight in *H. taimen* and seven in *Parahucho perryi*.

Keywords: *Brachymystax lenok*, *Hucho hucho*, *Hucho taimen*, microsatellite, *Parahucho perryi*, Salmonids

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Brachymystax lenok is a freshwater resident salmonid present throughout eastern Siberia and portions of northern Mongolia, China and Korea. Despite its wide distribution, populations of this species are currently declining through overexploitation, environmental pollution and other causes, and information about them is still very scarce. Currently, two forms of lenok are distinguished — blunt and sharp-snouted. These two forms differ significantly in their morphology, spawning grounds, and other characters (Alekseyev *et al*. 1986; Mina 1991). In sympatry, the two forms are reproductively isolated, although hybridization sometimes occurs, and, based on protein variation, low levels of introgression were reported (Osinov 1993). Based on sequences of the mtDNA control region, the two forms were represented as monophyletic lineages with 1.4% sequence divergence (Froufe *et al*. 2003). The molecular markers reported here will be used to examine population structure and gene flow within and between these two forms of lenok.

Two *B. lenok* genomic DNA libraries, enriched for tri-$(ATG)_n$ and tetranucleotide motifs [$(GATA)_n$, $(GACA)_n$] were produced using a magnetic bead capture method, adapted from Carleton *et al*. (2002) and the Kocher Laboratory protocol (http://tilapia.unh.edu/WWWPages/carleton/protocol%20pages/Microsat%20library). Genomic DNA from a sharp-snouted individual was digested with *Sau*3AI and fragments in a size range of 500–1000 bp were isolated from a 2% agarose gel using the Nucleospin kit (BD Biosciences, Clonetech). Oligonucleotide adaptors (RBgl24, 5′-AGCACTCTCCAGCCTCTCACCGCA-3′, and RBgl12, 5′-GATCTGCGGTGA-3′) were ligated to the genomic DNA fragments using T4 DNA ligase (Promega) overnight at 4 °C. The resulting DNA was hybridized to 5′-biotinylated probes, which consisted of a 3′-ATAGAATAT tail and microsatellite motifs $(GATA)_8$, $(GACA)_8$ and $(ATG)_9$. Hybridized fragments were captured with Streptavidin Magnesphere Paramagnetic Particles (1 mg, Promega) at 68 °C (tetranucleotide motifs) or 60 °C (trinucleotide motifs). The enriched DNA was amplified by polymerase chain reaction (PCR), digested with *Sau*3AI to remove the adaptors, ligated into the plasmid PUC19 (MBI) and cloned in *E. coli* strain pBluescript II KS.

Thirty-two inserts from the trinucleotide library and 46 inserts from the tetranucleotide library were sequenced (BigDye, Perkin Elmer) and electrophoresed on an ABI 3100 capillary sequencer (Perkin Elmer). After elimination of duplicated sequences, primers were designed for four of the six identified trinucleotide loci and for nine of the 16 identified tetranucleotide loci using OLIGO 6.8 (Table 1). Some emphasis was placed on choosing shorter perfect repeats, as opposed to longer, complex imperfect repeats, which appear to be common in salmonid fishes, and are more prone to cause problems in scoring and inferences based on simple models of mutation.

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Table 1 Primer sequences, repeat motifs, PCR annealing temperatures (T_a) and GenBank accession numbers of the *Brachymystax lenok* microsatellite loci. Fluorescent labels were attached to the forward (F) primers and a GTTT tail to the 5′ of the reverse (R) primer

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	GenBank Accession no.
BleTri1	F: CTAAAAACCATGACCATTCAG R: CATACCATCTCTGTCTAATGT	$(CAT)_7$	55	AY48447
BleTri2	F: CCAGGACATATTCCCTTCTAG R: CCACAGCTCAGGGCAGGGAGT	$(CAT)_{11}$	55	AY48448
BleTri3	F: CAGACGTGGCGCTTGTTTGGT R: CTAGTCAGGAAGCAAGTGATG	$(CAT)_7C(ATT)_7$	57	AY48449
BleTri4	F: CTCCTGGAGAGGACACCACTG R: CCAGCTTCCTCTGGTGGGATG	$(CAT)_5$	57	AY48450
BleTet1	F: TCTTAAAGGGCCAGCGCAGAG R: CAGAGGGCATAGCGGGACTTC	$(TCTA)_{11}$	x	AY48451
BleTet2	F: TGTCAGAGGCCTTGACTGCGT R: GCTAGGCTGTTTACTCTAGGT	$(CAGA)_5$	57	AY48452
BleTet3	F: TCCGCACCACACTTGGCACCG R: AGTAGAAGACTGACACAGACA	$(TGTC)_7$	x	AY48453
BleTet4	F: CAAAAAAACAGAAACGAGCAG R: GCAAACAGACAACCAGCCACG	$(TGTC)_7$	x	AY48454
BleTet5	F: CTTCTTCACCCGCCTGAGTGT R: TTGAATGGGCTATCTGGCTGT	$(TGTC)_5$	57	AY48455
BleTet6	F: AGACAGCATGACAGCACAACG R: GGCAGACAGACAGGCAAACAG	$(CCTG)_7$	57	AY48456
BleTet7	F: CCCAATAACCCCTAACCCTAG R: TAATAAAAAACGTTCGCCAGG	$(CAGA)_5(CA)_9$	60*	AY48457
BleTet8	F: ACCTGAGGGCAAAGGTCAGAG R: GCTGCTCTCATCTGGGGACAG	$(TGTC)_4$	60*	AY48458
BleTet9	F: ACTGGATAGAAAGACCTGTGG R: AGATTCTTGGTAAAAGTGAAG	$(TATC)_2(TGTC)_{12}(TATC)_3$	57	AY486103

x, nonspecific amplification.
*Presumable duplicated locus yielding up to four amplification products.

PCR amplifications of microsatellite loci were done in 25 μL volumes in a GeneAmp 9700 thermal cycler (Perkin Elmer). Each reaction contained 19 μL H_2O, 2.5 μL 10 × PeqLab Buffer B, 0.5 μL 10 mM of each primer, 1.5 μL 25 mM $MgCl_2$, 0.5 μL 10 mM dNTP mix, 0.1 μL PeqGOLD Taq-Polymerase (Peq Laboratory) *Taq* DNA polymerase, and 50 ng DNA template. The PCR conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation for 40 s at 94 °C, annealing for 40 s at locus specific temperatures given in Table 1, extension for 40 s at 72 °C, and a final extension at 72 °C for 5 min. Fragments were separated on an automated sequencer (ABI 377, Perkin Elmer) using an internal size standard [TAMRA (Perkin Elmer)] and analysed using GENESCAN software (Applied Biosystems).

Of the 13 tested primer pairs, three had to be discarded due to nonspecific amplification (BleTet1, BleTet3 and BleTet4). Two primer pairs (BleTet7 and BleTet8) amplified up to four fragments per individual, the sizes of which were consistent with a microsatellite polymorphism of the cloned locus. Given the tetraploid ancestry of the salmonid genome (Ohno 1970), we presume that the loci are duplicated.

The remaining eight loci were characterized in 20 individuals of each form of *B. lenok* (blunt-snouted: Amur River, Russia; sharp snouted: Khor River, Russia). Locus BleTet9 could not be amplified in the sharp-snouted population from Khor River, but amplified successfully in several sharp-snouted individuals (data not shown) from the same location as the original clone. Allele size ranges, number of alleles per locus, expected and observed heterozygosities and tests for Hardy–Weinberg equilibrium are reported in Table 2. Tests of heterospecific amplification were carried out on one individual each of *Hucho taimen* (Gramna Lake, Russia), *Parahucho perryi* (Amur River, Russia) and *Hucho hucho* (Drau River, Austria) using the PCR conditions and annealing temperatures described above. Cross-species amplification was highly successful, as only one locus, BleTet9, could not be amplified in the *Hucho hucho* and *Parahucho perryi*.

These results indicate the utility of the new markers for genetic studies in *B. lenok*, and their potential applicability to the genera *Hucho* and *Parahucho*.

Table 2 Characterization of the new markers in blunt (B) and sharp (S) snouted forms of *Brachymystax lenok* and results of cross-species amplification in *Hucho taimen* (HT), *Parahucho perryi* (PP) and *Hucho hucho* (HH). For *B. lenok*, allele size ranges are given, whereas for *Hucho* and *Parahucho* the single genotype is given. *P*-values refer to tests of Hardy–Weinberg equilibrium

	Allele size range (bp)					No. of alleles		Heterozygosity					
								Observed		Expected		*P*-value	
Locus	B	S	HT	PP	HH	B	S	B	S	B	S	B	S
BleTri1	102–115	102–135	105	115	105	6	9	0.95	0.8	0.74	0.81	0.135	0.367
BleTri2	109–142	73–118	121	94	127, 130	12	8	0.75	0.65	0.85	0.71	0.104	0.021
BleTri3	109–130	97–133	109	124, 127	106, 109	5	10	0.2	0.75	0.59	0.83	0	0.45
BleTri4	81–84	69–138	72	75	72, 93	2	6	0.25	0.3	0.21	0.51	1	0.003
BleTet2	111–127	119–131	139, 143	119, 127	127	4	4	0.45	0.55	0.52	0.64	0.075	0.092
BleTet5	167–183	155–167	151, 159	151	159	5	4	0.6	0.8	0.63	0.72	0.228	0.205
BleTet6	232–260	208–220	208, 216	216	212	8	4	0.5	0.75	0.55	0.66	0.299	0.115
BleTet9	211–291	x	203, 207	x	x	17		0.95		0.92		0.379	

x, no amplification.

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