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TECHNICAL NOTE

Eight new polymorphic microsatellite DNA markers for Sakhalin taimen *Parahucho perryi*

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Abstract We developed eight polymorphic microsatellite markers for the endangered salmonid *Parahucho perryi* from genomic libraries enriched for (GACA)n and (GATA)n repeat sequences. Emphasis was placed on developing highly polymorphic, perfect repeats that could be scored with confidence. Six tetra- and two di-nucleotide loci were screened in 49 individuals from two populations on Sakhalin Island, Russia. Allelic variation was high with eight to 23 alleles per locus and expected heterozygosity ranging from 0.48 to 0.93. These highly variable markers should prove useful in evaluating inbreeding, gene flow and population structure in Sakhalin taimen throughout its range.

Keywords Salmonidae · Sakhalin Island · Tetra-nucleotide repeats · Endangered species

Sakhalin taimen (*Parahucho perryi*) is a large primarily anadromous salmonid fish, distributed along the coast of the Russian Far East as well as Sakhalin, Kuril, and Hokkaido Islands (Zolotukhin et al. 2000). Formally considered a member of the genus *Hucho* (taimen of Europe and Asia), a range of molecular based studies support its placement into a unique genus (Oakley and Phillips 1999; Crespi and Fulton 2004; Matveev et al. 2007), the name for which was first proposed by Vladykov (1963). Indeed, analyses in both Crespi and Fulton (2004) and Matveev et al. (2007) support *Salmo* as the genus most closely related to *Parahucho*. This perspective is further supported by our lack of success in applying microsatellite primers on *Parahucho*, which were developed from other Asian salmonids including the sister genera *Hucho* and *Brachymystax* (results not shown). This failed effort motivated our need to develop species-specific primers for *Parahucho*.

Other than their systematic placement, little is known about the genetic architecture of this increasingly endangered salmonid. The species was recently added to the IUCN Red List of Threatened Species as critically endangered in 2006 (IUCN 2007). In order to evaluate gene flow, inbreeding and potential geographic structure in this species, we aimed to develop highly polymorphic nuclear markers, which could be typed reliably and efficiently at the population level.

For the identification of new microsatellite DNA loci for *Parahucho perryi* two specimens from two different populations on Sakhalin Island were chosen. Whole genomic DNA was extracted from ethanol preserved liver tissue or fin clips using a high salt (Ammonium acetate) protocol, modified from Miller et al. (1988). The template DNA of both isolations was pooled and then partially restricted with *Sau*3AI to obtain two libraries of DNA fragments with a size range from 300 to 1,000 bp length. Enrichment was conducted with the two tetranucleotide motifs GACA and GATA. Further isolation steps followed the magnetic bead capture method described in Carleton et al. (2002) and the protocol of Winkler and Weiss (2008). After conducting the colony PCRs and sequencing of 268 positive clones on an ABI 3100×l automated genotyper, a total of 38 primer pairs were designed using the program oligo 6.8 (Rychlik and Rhoads 1989). Standard PCR conditions included an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 57°C for 10 s, elongation at 72°C for 30 s and a final elongation step at 72°C for 7 min. Based on the resolution of the PCR

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Table 1 Eleven new microsatellite loci for *Parahucho perryi*

Locus	Primers (5′–3′)	Repeat motif	Allele size range (bp)	Ta (°C)	Number of alleles	Genebank access number
Pper_1	F:ATGGAGGTGGAGAAAGGTAC	$(TGTC)_{23}$	156–211	57	23	EU598773
	R:GTTTACACAGTCAGTAAGGCAGTC					
Pper_2	F:GCATTGCCGTTTCTCTAC	$(GACA)_{12}$	292–348	58	11	EU598774
	R:GTTTCTCCACTATTCCAGCACC					
Pper_3	F:TAGCCCTCAGTCAACACC	$(GTCT)_{14}$	174–310	57	7	EU598775
	R:GTTTTATCCCCACAGAAAGGTC					
Pper_4	F:TAGGGAAGGGCGAGCAGG	$(TATC)_{39}$	316–404	62	20	EU598776
	R:GTTTCTTACGACTGAGGGAGGATACACAC					
Pper_5	F:GCTCTGGACCTTTCTGTG	$(AC)_{17}$	147–167	55	27	EU598777
	R:GTTTAAAGGGTAAAGGTGTGTGAG					
Pper_6	F:GATGTAATTTACCTTGTGTTGAC	$(CA)_{20}$	151–177	55	10	EU598778
	R:GTTTTGAACACTAATAACAATGAGG					
Pper_10	F:GCTGGCACTGTCTGACTATC	$(TAGA)_{26}$	218–294	57	16	FJ158613
	R:GTTTGCTCGCTCGCTCATCTAC					
Pper_11	F:GGTGCCATTCGGATTAG	$(ATGG)_{16}$	151–277	57	16	FJ158614
	R:GTTTTGTTCAGCACCAAATCAC					
Monomorphic loci						
Pper_7	F:GGAATCGCTGCTCAATGG	$(TG)_{19}$	ca.179	55	2	EU598779
	R:GTTTGGTTGGGTATTTTGGTAGG					
Pper_8	F:GGGGAGAGGGATTAAGAGATAG	$(AG)_{11}$	ca.118	55	3+	EU598780
	R:GTTTAGGGTCAATGGCAAAAAG					
Pper_9	F:AACGACGACCGCTGCTGTAC	$(CT)_{16}$	ca.258	55	2	EU598781
	R:GTTTGCACTCTCCAGCCTCTCACC					

Shown is the locus name, the primer sequence, the repeat motif, annealing temperature (Ta), and the Genebank Accession number. Forward primers were fluorescently labeled with FAM or HEX. Additional "pigtails" (GTTT or GTTTCT) were attached to the reverse primers

Table 2 Characterization of the eight polymorphic microsatellite loci in two Sakhalin Island populations of *Parahucho perryi* (Nabile and Dagi river). Given are the number of individuals (*N*), the allele size range, the number of alleles and size range in each population, the expected and observed heterozygosity, the F_{IS} per population. Statistically significant F_{IS} values are marked with an asterisk "*". Significance was based on 1200 randomizations and adjusted for multiple testing at alpha equal to 5%

Locus	*N*		Allele size range		No. of alleles			H. exp.		H. obs.		F_{IS} per pop.	
	Nab	Dag	Nab	Dag	Nab	Dag	Total	Nab	Dag	Nab	Dag	Nab	Dag
Pper_1	29	18	156–235	156–237	20	17	23	0.93	0.89	0.93	0.78	0.01	0.16
Pper_2	30	17	292–348	292–348	10	9	11	0.85	0.84	0.90	0.88	−0.05	−0.02
Pper_3	30	19	227–310	174–297	17	16	19	0.91	0.90	0.90	0.74	0.03	0.21
Pper_4	29	18	345–436	316–428	17	17	20	0.86	0.92	0.79	0.83	0.10	0.12
Pper_5	29	19	151–167	147–167	5	8	8	0.48	0.64	0.45	0.42	0.08	0.37
Pper_6	29	17	151–173	153–173	9	9	10	0.75	0.62	0.72	0.65	0.05	−0.01
Pper_10	27	11	218–290	230–294	13	8	16	0.86	0.83	0.63	0.27	0.29*	0.70*
Pper_11	31	18	151–277	151–251	14	9	16	0.86	0.86	0.90	0.83	−0.00	0.06
All	30	19						0.81	0.81	0.78	0.68	0.09	0.21

fragments, the 5′-end primer for 12 of these loci was labeled with fluorescent dyes (HEX, FAM) and the subsequent amplifications analyzed in relation to an internal size standard (ROX500, Applied Biosystems) on the ABI 3100×1 genotyper, using GeneMapper Software version 3.7. PCR amplification was first tested with unlabeled

primers. After initial amplification, 23 of the tested primer pairs were chosen for fluorescent labeling. Of these, 14 revealed fragments that could be analyzed with confidence. These loci were used to type two Sakhalin populations (Dagi and Nabile rivers) of *Parahucho perryi*, consisting of a total of 49 individuals. Three loci were clearly duplicated and thus discarded whereas three additional loci were monomorphic. Table 1 summarizes information of each locus. For the eight polymorphic loci, observed and expected heterozygosity was computed in the program GENETIX v. 4.03 (Belkhir et al. 2004) and deviations from Hardy-Weinberg equilibrium (F_{IS} per population) as well as linkage disequilibrium were evaluated using FSTAT v.2.9.3.2 (Goudet 2002). Probability of null-alleles at each locus was evaluated by using the MicroChecker software v.2.2.3 (van Oosterhout et al. 2004).

Analyses of linkage equilibrium showed no significant association among the eight loci. Allelic diversity was moderately high ranging from eight (locus Pper_5) to 23 (Pper_1) alleles per locus (Table 2). There was significant deviation from HWE at one locus (Pper_10) in both the Dagi and the Nabile populations. A general excess of homozygotes for most allele size classes at this locus implies that null alleles may be present. Characteristics of microsatellite polymorphism across the two populations are summarized in Table 2. The loci and protocols presented here should provide for an efficient and reliable population genetic screen for Sakhalin taimen throughout their range. As noted above, we discourage cross-species application of *Parahucho*-specific loci with other Asian salmonids.

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