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ANIMAL GENETICS

A Panel of Microsatellite Loci for Population Studies of Sakhalin Taimen *Parahucho perryi* (Brevoort)

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Abstract—Using DNA samples of Sakhalin taimen and a set of microsatellite loci, earlier reported for other salmonid fishes (Salmonidae), successful cross-species amplification was performed. A total of 56 Sakhalin taimen (*Parahucho perryi*) samples from the Daga, Nabil, Poronai, and Agnevo rivers (Sakhalin Island) were examined at 36 microsatellite loci, most of which were described for other species and first tested in taimen. Among the 21 loci first tested in taimen, two loci produced no amplification products. The remaining 19 loci were successfully amplified (for some loci, new primers were generated). Thirteen of these loci were monomorphic, while six loci were polymorphic and used in further population genetic analysis. In addition, with the purpose of modification of the allele sizes and optimization of the research technique, new primers for the already known 12 loci of Sakhalin taimen were designed. Three more loci were included in analysis without changes. As a result, a joint panel consisting of 21 polymorphic microsatellite markers was suggested for analysis of Sakhalin taimen. This panel was tested with four population samples from the rivers of Sakhalin Island.The results showed that this panel of markers could be used in detailed population studies for evaluation of the level of genetic differentiation, inbreeding, and migrations in Sakhalin taimen, an endangered species with a fragmented range. Using this approach in further studies will make it possible to isolate basic populations, which is necessary for conservation of this rare species.

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INTRODUCTION

Sakhalin taimen *Parahucho perryi* (Brevort) is a narrow-range species, endemic to the Russian Far East. Taimen is in the Red Book of Russia (under category 2, endangered Far East endemic species) [1] and IUCN Red List of Threatened Animals as critically endangered. Commercial and recreation fishing, illegal catching, habitat degradation, and active economic development of the currently existing limited habitats of Sakhalin taimen (forest cutting in Primorye and Khabarovsk krai, oil and gas projects on Sakhalin Island) lead to the reduction of the species number and range fragmentation, resulting in reproductive isolation of Sakhalin taimen individuals [2]. In turn, reproductive isolation in the species with the low number can be the major cause of the population genetic differentiation. The latter can be identified through analysis of the DNA marker polymorphism. Using these markers facilitates evaluation of the degree of species genetic differentiation, the level of inbreeding, migrations, and other population indices, important for elaboration of the rare species conservation and monitoring programs [3–6].

Population studies performed with the purpose of conservation and monitoring of the rare species require the use of a large number of DNA markers. This is because in the conditions of low species number it is problematic, and in some cases impossible, to get a sufficient number of samples for investigation. The number of individuals in some isolated populations of Sakhalin taimen usually constitutes several tens of individuals [2]. Thus, the only way to reduce statistical errors of the experiment is the application of a large number of DNA markers. In the previous studies of European and Japanese researchers, two sets of microsatellite loci were suggested for analysis of Sakhalin taimen. One of these sets included eight moderately polymorphic loci with dinucleotide repeats [7]. Another set was comprised of six loci with di- and tetranucleotide repeats [8]. These sets, however, were not tested together on the same samples to assess their compatibility in population studies.

In the present study, the previously suggested locus panels were supplemented with six additional microsatellite loci, discovered in Sakhalin taimen with the help of cross-species amplification and primer modification for some of the loci. As a result, a set of 21 microsatellite loci is suggested. The use of this set will make it possible to reveal the population genetic structure of Sakhalin taimen.

MATERIALS AND METHODS

A total of 56 Sakhalin taimen samples were examined. The samples represented four populations of the Sakhalin Island, including 31, 11, 11, and 3 samples from the Daga River, Nabil River, Poronai River, and Agnevo River, respectively. All samples were collected in the autumn 2009. Tissue samples (fin pieces) were fixed in 96% ethanol. Genomic DNA was extracted using the Diatom DNA prep 200 kit (Isogene Laboratory, Russia).

A total of 36 microsatellite loci were tested with the taimen samples described. A part of these loci (21) were previously described for other Asian salmonid species (Salmonidae), and in the present study, were first tested on taimen samples.

Two out of 21 loci first tested in taimen produced no amplification products, while the remaining 19 loci were successfully amplified (for some of them, new primers were designed). Thirteen of the amplified loci were monomorphic. The other six loci were polymorphic and used in further population studies. In addition, new primers were designed for 12 Sakhalin taimen loci, previously described by other authors [7, 8]. The primer design was conducted with the purpose of the allele size modification and the research technique optimization (Table 1).

PCR was performed using the Gen Pack PCR Core kit (Isogene Laboratory, Russia), containing the Hotstart *Taq* DNA polymerase, deoxynucleoside triphosphates, and magnesium chloride in the final concentrations of 1 unit, 200 μM, and 2.5 μM, respectively, and the optimized buffer system. Final concentration of primers in the reaction mixture was from 0.1 to 0.5 μM, and the DNA amount, from 50 to 100 ng. The reactions were run in a final volume of 20 μL. Amplification was performed using the Applied Biosystems 9800 Fast Thermal Cycler and the Applied Biosystems Veriti 96 Well Fast. The amplification conditions included initial denaturing at 95°C for 5 min; followed by eight cycles of denaturing for 1 min at 94°C; annealing for 30 s at 50 to 60°C; extension for 30 s at 72°C; and 25 cycles of denaturing for 30 s at 94°C; annealing for 30 s at 50 to 60°C; and extension for 15 s at 72°C. Primer annealing temperatures for each locus are shown in Table 1.

Analysis of the fragments (excluding the products of the *Ots*102, *OMM*1037, *Oki*10, and *OtsG*68 loci) was carried out in vertical block (21-cm long) of 6% nondenaturating polyacrylamide gel in 0.5× TBE buffer, pH 8.0, at 300 V during 2–4 h. The fragments generated were visualized under UV light in the presence of ethidium bromide (5 μmL). The pBr322 plasmid DNA (SibEnzim, Russia), digested with either *Hae*III, or *Hpa*II, or *Fsp*4HI restriction nuclease (SibEnzim, Russia), served as molecular size marker. The fragment sizes at each locus were determined using the Molecular Imaging Software, Version 4.0 (Kodak).

Fragment analysis of the *Ots*102, *OMM*1037, *Oki*10, and *OtsG*68 alleles was performed using the QIAxcel capillary electrophoresis device (QUIAGEN). The allele sizes were determined using the DNA fragments of standard size from 52 to 292 bp, and the step of 24 bp, as implemented in the BioCalculator Software (QUIAGEN).

RESULTS

New markers for Sakhalin taimen. Nineteen out of 21 microsatellite loci, described for other Asian salmonid species (Salmonidae) were successfully amplified with the Sakhalin taimen samples. Thirteen loci were found to be monomorphic, while the remaining six loci were polymorphic with the number of alleles from two to 17 and gene diversity from 0.38 to 0.90 (Table 1). Among these markers, the most variable were *OMM*103 and *Oki*10 loci, and the least variable locus was *Omy*301 with two alleles and gene diversity of 0.43.

Microsatellite loci with new primers. In this study, we used 15 polymorphic microsatellite loci identified in Sakhalin taimen [7, 8]. For 12 of these loci, new primers were designed with the purpose of the allele size modification and optimization of the research technique (Table 1).

Additional markers. In addition, three more loci were tested with original primers without modifications. These loci were *Hper*-5 and *Hper*-15 [7], and *Pper*_5 [8].

DISCUSSION

Kopun et al. [8] reported that due to strong phylogenetic deviation of Sakhalin taimen from other Asian salmonids (Salmonidae), the only way for identification of the new microsatellite primers for Sakhalin taimen was a construction of species-specific genomic library and its analysis. The same approach was used in the study of Hatakeyama et al. [7]. In the present study, we suggest a simpler approach to search for microsatellite markers of Sakhalin taimen, which consists in cross-species amplification. For the study, the loci previously described in other salmonids were used. However, in some cases success of the work required constructing new primers, based on the known locus sequences in salmonids. Due to relative conservatism of flanking regions, some primers were also effective with the Sakhalin taimen samples. In particular, among the six new polymorphic markers, suggested for Sakhalin taimen (the *Salvelinus malma* – *Smm*5 and *Smm*17; *Oncorhynchus mykiss* – *Omy*301 and *OMM*1037; *Oncorhynchus* sp. *Ots*102 and *Oki*10 loci), three loci were successfully amplified with new primers (*OMM*1037, *Ots*102, *Oki*10).

Table 1. Characterization of microsatellite loci examined in Sakhalin taimen

Locus [Literature source]	Primers (5'–3')	Repeated motif	Annealing temperature (T_a), °C	Number of alleles/expected heterozygosity/number of samples	Allele sizes (bp) amplified with	
					new primers	original primers (only for the polymorphic loci from) Hatakeyama et al. [7] and Kopun et al. [8])
New polymorphic markers for Sakhalin taimen						
*Smm*5 [9]	F: agatgtgtgataaactcagcctc R: agttgtttaaatagggcggatag	$(CA)_n$ + $C(CA)_m$	52	2/0.45/56	~80–82	–
*Smm*17 [9]	F: aaggatggtgaggacaataca R: accttgagaaatctatatgtggtcta	$(CA)_n$	52	5/0.38/56	~132–140	–
*Omy*301 [10]	F: acttaagactggcaacctt R: ctacacggccttcgggtgaga	Unknown	52	2/0.43/56	~70–72	–
*OÌÌ*1037 [11]	F: gaacggcgactggatttaatact[a] R: ccgctcaccctcgtctcttaa[a]	$(GAAA)_n$	60	17/0.87/46	~207–263	–
*Ots*102 [12]	F: ggatccaataaggagtgatatagtag[a] R: tatccccttaccatttcccttgcta[a]	$(GTCT)_n$ + $(GCCT)_m$	60	4/0.67/46	~256–272	–
*Oki*10 [13]	F: gagtgctggacagattggatt[a] R: gggagctacagctttttacaaatc[a]	$(CTGT)_n$	60	16/0.90/47	~146–222	–
Polymorphic markers from Hatakeyama et al. [7], amplified with new primers						
Hper-4	F: cacactcatgaccagatataaca[a] R: tcagctgatgtaagaaaggctt[a]	$(GT)_n$	56	4/0.19/55	~98–106	186–188
Hper-6	F: gtaatagcagtcctttgtgaaagtg[a] R: gtgcatttatccagggtgact[a]	$(CA)_n$	56	2/0.25/49	~101–103	192–194
Hper-8A	F: caaaccacacacactgagct[a] R: atggagtgggctacaatgct[a]	$(CA)_n$	56	6/0.73/56	~162–178	223–229
Hper-16	F: tgggggaggtggcggttt[a] R: gagaagcacgtattgttttagtgcaa[a]	$(GT)_n$ + $(GT)_m$	56	5/0.56/55	~134–146	215–219
Hper-25	F: gcaggctaaactctaaatgtg[a] R: catgtatatgtattgcaatctcac[a]	$(GA)_n$	56	2/0.42/55	~106–112	197–203
Polymorphic markers from Kopun et al. [8], amplified with new primers						
*Pper*_1	F: gtacccacactactttgtgcttt[a] R: cacagtcagtaaggcagtcta[a]	$(TGTC)_n$	53	29/0.94/55	~126–246	156–211
*Pper*_2	F: caagtagggtgaatcaacatgtt[a] R: atgtggccattgttctgatgat[a]	$(GACA)_n$	53	14/0.89/56	~162–230	292–348
*Pper*_3	F: ccactctctcctgtattatgt[a] R: tgacacacaccggagctagt[a]	$(GTCT)_n$	53	17/0.90/55	~146–226	174–310
*Pper*_6	F: gatgtaatttaccttgtgttgactaca[a] R: gtaaagtttcattgccaccacaatca[a]	$(CA)_n$	53	10/0.73/54	~105–129	151–177
*Pper*_7	F: ggaatcgctgctcaatg[a] R: tgcagtatgtgtgggtgctct[a]	$(TG)_n$	54	4/0.39/55	~128–146	179
*Pper*_8	F: gagggattaagagatagagatataaa-ga[a] R: gtcaatggcaaaaagtatctaagtc[a]	$(AG)_n$	53	5/0.65/55	~111–121	118
*Pper*_11	F: tgtcaggaggacacactgta[a] R: gttttgttcagcaccaaatcac	$(ATGG)_n$	53	15/0.87/54	~130–210	151–277

Table 1. (Contd.)

Locus [Literature source]	Primers (5'–3')	Repeated motif	Annealing temperature (T_a), °C	Number of alleles/expected heterozygosity/number of samples	Allele sizes (bp) amplified with	
					new primers	original primers (only for the polymorphic loci from) Hatakeyama et al. [7] and Kopun et al. [8])
Monomorphic markers						
*BleTri*4 [14]	F: ctcctggagaggacaccactg R: ccagcttcctctggtgggatg	$(CAT)_n$	52	1/40	~78	–
*BleTri*2 [14]	F: ccaggacatattcccttctag R: ccacagctcagggcagggagt	$(CAT)_n$	56	1/43	~95	–
*OMM*1050 [11]	F: accaacctgaacacagcctaat[a] R: gctgtaacatttcagggatcat[a]	$(GATA)_n$	60	1/47	~156	–
*OtsG*68 [15]	F: tatgaactgcagcttgttatgttagtttg[a] R: catgtcggctgctcaagttataa[a]	$(GATA)_n$ + $(TAGA)_m$	60	1/47	~129	–
*Oki*1 [13]	F: aggatggcagagcaccact R: caccataatcacatattcaga	$(CTGT)_n$	51	1/50[b]	~118 and ~136	–
*Ots*3 [16]	F: cacactctttcaggag R: agaatcacaatggaag	$(TC)_n$	51	1/50	~90	–
*One*103 [17]	F: aatgttgagagctatttcaatcc R: gattgatgaatgggtggg	$(ATCT)_nN_q$ + $(ATCT)_m$	51	1/50	~78	–
*One*109 [17]	F: agggagagaagagagggaga R: cctcagaagtagcatcagctc	$(TACA)_n$	51	1/50	~105	–
*Ssa*20.19 [18]	F: tcaacctggtctgcttcgac R: ctagtttccccagcacagcc	$(AC)_n$	52	1/50[b]	~73 and ~81	–
*Ssa*197 [19]	F: gggttgagtagggaggcttg R: ctagtttccccagcacagcc	$(GT)_nC(TG)_p$+ $TC(TG)_qA$ + $(GTGA)_m$	52	1/50	~105	–
*Oke*11 [20]	F: caaggtgatgcgtgcatacac R: tcatttattttgcctgtttctaccc	$(CA)_nGA$ + $(CA)_m$	52	1/50	~80	–
*Oke*3 [20]	F: accctgagagcaatcaac R: tcagggatatgcagtaaatagta	$(TCCCTCT-CGTCTC)_n$	50	–	No product	–
*Smm*3 [9]	F: tggctcaaattaagatcctac R: agccattatgcattacttgttc	$(CA)_nC_g$ + $(CA)_m$	53	1/50	~110	–
*Smm*24 [9]	F: cattgatcaagaagccagtgc R: tgtattttgccaatataacacagc	$(TATC)_n$	50	–	No product	–
*Ssosl*456 [21]	F: cttcccaggagtcatcataaatct R: taaaccccactgcttgttgagtgt	$(AC)_nAG$ + $(AC)_m$	54	1/50	~150	–

Note: [a], primers designed in the present study; [b], in each sample, two fragments were amplified with no polymorphism between the samples; *n* and other indices designate the number of repeats.

Table 2. Genetic diversity indices in four populations of Sakhalin taimen

Locus	r. Dagi (sample size = 31)				r. Nabil (sample size = 11)				r. Poronai (sample size = 11)				r. Agnevo (sample size = 3)			
	H_e	H_o	p	n/N	H_e	H_o	p	n/N	H_e	H_o	p	n/N	H_e	H_o	p	n/N
*Smm*5	0.405	0.419	1.00	2/31	0.506	0.273	0.21	2/11	0.524	0.273	0.22	2/11	0.333	0.333	1.00	2/3
*Smm*17	0.348	0.419	0.46	3/31	0.312	0.364	1.00	2/11	0.312	0.182	0.27	2/11	0.933	1.000	1.00	5/3
*Omy*301	0.355	0.323	1.00	2/31	0.247	0.273	1.00	2/11	0.416	0.364	1.00	2/11	0.533	0.000	0.19	2/3
*OMM*1037	0.869	0.952	0.71	12/21[a]	0.636	0.545	0.44	6/11	0.736	0.636	0.27	7/11	0.933	0.667	0.20	5/3
*Oki*10	0.833	0.818	0.28	8/22[a]	0.883	0.727	0.24	9/11	0.866	0.909	0.50	8/11	0.867	1.000	1.00	4/3
*Ots*102	0.650	0.591	0.89	3/22[a]	0.489	0.200	0.08	3/10	0.675	0.636	1.00	4/11	0.533	0.000	0.19	2/3
Hper-4	0.033	0.033	1.00	2/30	0.173	0.182	1.00	2/11	0.485	0.546	1.00	2/11	0.000	0.000	1.00	1/3
Hper-5	0.489	0.613	0.16	2/31	0.505	0.400	1.00	2/10	0.506	0.272	0.23	2/11	0.667	1.000	1.00	2/2
Hper-6	0.230	0.185	0.35	2/27	0.366	0.444	1.00	2/9	0.268	0.300	1.00	2/10	0.000	0.000	1.00	1/3
Hper-8A	0.782	0.580	0.07	6/31	0.662	0.727	0.16	4/11	0.481	0.272	0.25	3/11	0.533	0.000	0.20	2/3
Hper-15	0.266	0.290	1.00	4/31	0.623	0.636	0.58	5/11	0.628	0.636	0.46	5/11	0.733	0.667	1.00	3/3
Hper-16	0.577	0.548	0.40	5/31	0.632	0.636	0.67	5/11	0.195	0.200	1.00	3/10	0.733	1.000	0.61	3/3
Hper-25	0.427	0.467	0.68	2/30	0.416	0.364	1.00	2/11	0.416	0.545	0.49	2/11	0.533	0.667	1.00	2/3
*Pper*_1	0.904	0.833	0.34	18/30	0.931	0.909	0.45	13/11	0.887	0.727	0.16	10/11	1.000	1.000	1.00	6/3
*Pper*_2	0.857	0.871	0.82	10/31	0.844	0.727	0.24	8/11	0.835	0.909	0.93	6/11	0.867	0.667	0.46	4/3
*Pper*_3	0.905	0.871	0.27	14/31	0.784	0.727	0.73	9/11	0.613	0.600	0.37	6/10	0.800	1.000	1.00	4/3
*Pper*_5	0.536	0.433	0.03	5/30	0.541	0.636	0.75	3/11	0.385	0.455	1.00	3/11	0.333	0.333	1.00	2/3
*Pper*_6	0.641	0.767	0.72	5/30	0.866	0.727	0.45	8/11	0.721	0.500	0.17	4/10	0.533	0.667	1.00	2/3
*Pper*_7	0.098	0.100	1.00	3/30	0.481	0.636	0.64	3/11	0.645	0.636	0.43	3/11	0.333	0.333	1.00	2/3
*Pper*_8	0.398	0.333	0.64	2/30	0.645	0.545	0.78	5/11	0.662	0.818	1.00	5/11	0.733	0.333	0.19	3/3
*Pper*_11	0.812	0.897	0.64	9/29	0.918	0.909	0.89	10/11	0.827	0.818	0.68	6/11	0.933	1.000	1.00	5/3
Mean	0.544	0.540	0.83	–	0.593	0.552	0.96	–	0.575	0.535	0.91	–	0.600	0.556	0.99	–

Note: in the sample from the Daga River, only 22 specimens were examined at these loci; H_e, gene diversity (expected heterozygosity); H_o, observed heterozygosity; P, significance level for the Hardy–Weinberg test; n/N, total number of alleles/number of samples examined.

Construction of new primers was also important for optimization of the research technique and modification of the allele sizes (lower allele sizes simplify technical work) (Table 1). As it was mentioned above, we amplified 12 loci described by Hatakeyama et al. [7] and Kopun et al. [8], using new primers (Table 1). Interestingly, in the original study, marker *Hper*-8 had two polymorphism zones (the *Hper*-8A and *Hper*-8B loci). Amplification of this marker with our primers resulted in identification of a single (heavier) zone, *Hper*-8A (Table 1). The polymorphism identification at the second (lighter) zone requires additional primer selection.

Thus, in the present study a panel of 21 microsatellite loci is suggested for population studies of Sakhalin taimen. The genetic diversity indices of the loci examined are demonstrated in Table 2. At all loci and in all samples, no linkage disequilibrium and departure from the Hardy–Weinberg equilibrium was observed. The exclusion was locus *Pper*_5 in the sample from the Daga River. However, after the introduction of the Bonferroni correction, this departure proved to be not statistically significant.

The suggested panel of microsatellite loci contains 13 loci with dinucleotide repeats and eight loci with tetranucleotide repeats (Table 1). Despite of considerable differences in the genetic diversity indices between these two types of loci (allele richness, 4.5 and 14.4; and mean gene diversity, 0.48 and 0.81, respectively), these loci were characterized by similar differentiating ability. The degree of differentiation (F_{ST}) for the loci with di- and tetranucleotide repeats constituted 10.3 and 10.5%, respectively. Thus, the panel of microsatellite loci suggested can be successfully used for the population genetic studies of Sakhalin taimen.

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