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DESIGN AND STUDY OF SCAR-MARKERS FOR POPULATIONS MYKIZHA *ONCORHYNCHUS (PARASALMO) MYKISS* FROM KAMCHATKA LAND.

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Abstract: The species *Oncorhynchus (Parasalmo) mykiss* has a complex population structure, wide distribution, and high temp of microevolution. Also, this species is the one of the main object of aquaculture and fish farming. That's the one of the reason, why many scientists looking for different studies of mykiss genome.

As have been showed in our previous studies, the kamchatka mykiss has a low level of genome variability. The sequencing and restriction analysis of different genes (*cytb; ND3; ND4; ATP6; ATP8; ITS1; ITS2; GH1; GH2;* and others) of nuclear and mtDNA has obtain the lack of prospects of widespread salmonic gene markers for kamchatka mykiss populations.

All of this turns us to searching of new genetic markers for *Oncorhynchus (Parasalmo) mykiss*. One of methods of search of polymorphic sequences is the RAPD-method (Randomly Amplified Polymorphic DNA). However the RAPD-analysis reveals anonymous sites of genome and has low reproducibility of results. But, the SCAR-markers (Sequence Characterized Amplified Region) has originated from polymorphic RAPD-markers, and there are deprived the majority of this lacks and some of them can be used as unique markers of mykiss genome.

In our study, the searching of polymorphic RAPD-fragments for different mykiss populations has been done (including the resident and anadromous forms from western and east coast of Kamchatka, Shantarian and harvest samples). After, with these fragments, the SCAR-primers we designed.

We received 10 heterogeneous products in the size 100-800 bp, having population character of distinctions. These fragments were cut out, cloned and sequenced. The new SCAR-primers has a specific character and good repeatability. They give a single amplify product and identify the certain sites of DNA. In total 7 polymorphic SCAR-markers have been received.

key words: amplification, markers, polymorphism, genome, species, resident and anadromous forms.

Introduction

Kamchatka mikizha *(Oncorhynchus (Parasalmo) mykiss)* is an object of the Red Data Book, and its resident form (mikizha) is one of the most valuable objects of world aquaculture and fish farming. At the same time, the status of this species is debatable; there is no consensus of opinion among authors regarding its genus; different 12authors assign mikizha to the genera *Salmo, Parasalmo*, or *Oncorhynchus (*Chernavin 1923, Glikman et al. 1973, Dorofeeva 1975, Pavlov and Savvaitova (1996).

Some researchers believe, as before, that Kamchatka steelhead and freshwater mikizha are different species rather than two forms of the same species. This discussion is promoted by an extremely high ecological

flexibility of mikizha. On Kamchatka peninsula, this species forms not only anadromous and resident forms, but also several intermediate forms (estuarine, coastal, etc.). Most American researchers assign mikizha to Pacific salmons (the genus *Oncorhynchus*) (Smith and Stearley 1989, Behnke 1992). However, ecological differences between them are large. Thus, all Pacific salmons spawn only once in their lifetime. In contrast to them, mikizha may spawn six to seven times in its life. All these specific features make the multi-aspected study of the genome of the species *Oncorhynchus (Parasalmo) mykiss* very interesting.

In the natural range, the group of Pacific trout, to which *Oncorhynchus (Parasalmo) mykiss* is assigned, is characterized by an extreme diversity of forms and species. Previously different researchers separated about 50 different species (Behnke 1992); now it is considered that there are four or five the so called "good" species. It was long believed that North American steelhead and rainbow trout are convergent species with respect to Kamchatka steelhead and mikizha. However, genetic studies (Okazaki 1984, Mednikov and Achundov 1975, Berg and Ferris 1984, Philips and Pleyte 1991, Murata et al. 1996, Pavlov et al. 2004) proved that these are different geographic and ecological forms of the same species *Oncorhynchus (Parasalmo) mykiss*.

In water bodies of Asia, representatives of this species have been almost not studied for a long time. Now, thanks to studies of mainly Soviet Russian scientists (Savvaitova and Lebedev 1966), the ecological diversity of Asian populations of mikizha has been studied rather comprehensively. They are mainly localized on Kamchatka peninsula; besides, single findings are known from water bodies of the continental coast of the Sea of Okhotsk and in Amur liman (Berg 1948, Koganovsky 1949). A small relic population of freshwater mikizha was found on Bolshoi Shantar Island (Alekseev and Sviredenko 1985), far beyond the range of the anadromous form. Anadromous Kamchatka steelhead inhabits mainly the western coast of Kamchaka and freshwater mikizha, the eastern coast.

During recent years, parallel to typical freshwater mikizha, trout with characters of North American redband trout of the species *O.(P.) mykiss* were first found on Kamchatka. They are numerous on the western coast of Kamchatka and scarce, on the eastern coast. In addition, trout with characters of the North American species (cuttroat trout *(O. (P.) clarkia*)) were found on Western Kamchatka, in the area of the Sedanka and Krutogorova rivers (Savvaitova et al. 1998).

Thus, the phenetic diversity of freshwater trout of Kamchatka represents several forms between which there are transitions - individuals with different combinations of indistinctly pronounced characters. This situation may result from the secondary integration of two species; however, another hypothesis that the forms of the trout found on Kamchatka belong to the population of one species *P. (O.) mykiss* or, more precisely, to its more primitive representatives - redband trout is more probable. It is not excluded that Kamchatka primitive trout represent a relic population of the species with characters of *P. (O.) mykiss* and *P. (O.) clarkii* that diverged less from each other than the American forms. This fact, as well as the presence of the relic Shantar population indicate that it is early to finish the issue of origin Asian (Jordan, 1892) or North American (Behnke 1992) and dispersal of the genus *Oncorhynchus (Parasalmo)* .

Wild Pacific salmon populations have been preserved only in Asia since their habitats are in practically inaccessible places. This fact and everything taken together make them a unique object for the study of the problems of the group origin, microevolution, species structure, and species formation.

Previous studies performed on the basis of comparative assessment of external morphology, osteology, allozyme variation, and molecular-genetic differentiation of the coding part of the genome demonstrated a very low divergence of representatives of

this fish species. Neither of the previously used biochemical or genetic markers provided particular results. Therefore, for the study of problems of interrelationship between Pacific salmon at different hierarchical levels, the search for marker systems specially for this fish species was made in our laboratory. Polymorphic DNA sequences that may be present in the coding and noncoding parts of the genome seemed most attractive to us.

Materials and methods

One of the most widely known methods of search for polymorphic DNA sites is the RAPD method (Randomly Amplified Polymorphic DNA) - PCR method using a short random primer or its modification with two primers (Williams et al 1990, Welsh and McClelland 1990, 1991). This method is technically simple in use, efficient, and rather cheap. According to the electrophoretic patterns of reaction products, it is possible to record differences between the genomes of closely related organisms in a great number of loci throughout the genome. However, RAPD analysis reveals anonymous sites of the genome whose nature is not completely understood and has a low reproducibility of results determined by an increased sensitivity to the reaction conditions.

Sequence Characterized Amplified Region (SCAR) markers (Paran and Michelmore 1993) originating from polymorphic RAPD markers are devoid of most their shortcomings and may be applied for various research. For producing locus-specific markers, a fragment of interest is extracted from gel, cloned, and sequenced. On the basis of the obtained sequence, long highly specific primers are selected that amplify a single fragment with a high degree of reproducibility. Many of them may be used as unique markers for genotype "passportization".

To isolate specific markers, we studied DNA from different forms of *Oncorhynchus (Parasalmo)* mykiss inhabiting the largest rivers and water bodies of Kamchatka, around the Shantar Islands, as well as from American populations. Taking into consideration the fact that this species is represented by different epigenetic variations, we included into the analysis individuals of riverine (resident) and anadromous (typically anadromous) forms.

The belonging to anadromous or resident form was detected with analysis of Sr/Ca contents in otolits (Zimmerman et al. 2003). We tested 7 populations of mykiss: resident\anadromous forms from Sopochnaya river; resident mykiss from Shantarian islands, harvest sample from Ohotskoe sea. The samples of north American steelhead (coastal and inland forms) were used as a mark samples.

Total DNA was isolated from the frozen muscle tissue by a standard method including cell lysis by 3% Na sarcosyl, lysate incubation with proteinase K (100[mu]g/ml) for 3 h at 60C, and deproteinization with mixtures phenol--chloroform (1 : 1) and chloroform--isoamyl alcohol (24 : 1). DNA was amplified in 25 µl 0.01 M Tris-HCl buffer, pH 8.3, containing 0.05 M KCl, mix of dNTP (0.1-0.2 mM), $MgCl_2$ (5mM), two random primers in free compose, Tag- polymerase (Thermus aquaticus), including monoclonic antibodies for “hart start” (“Smart Tag”) 2unit for sample, and 100 ng of tested DNA. All needed controls for clean test of primers by matrix and matrix by primers were done. The polimeraze chain reaction conducted by two progamms:

1. “Hart start” 94^0 C – 5 min, (94^0 C-42 sek, 50^0 C- 30 sek, 72^0 C -30 sek) x 3 repeat.
2. “Hart start” 94^0 C – 5 min, (94^0 C-42 sek, 60^0 C- 30 sek, 72^0 C -30 sek) x 32 repeat.

The product of reaction were tested for repeatability.

A portion of the amplified DNA fragments was analyzed by electrophoresis in horizontal 1.5% agarose gel (GenePure LE, Bio Express) in TBE buffer for 1.5--2 h (160 V). The 1-kb DNA ladder from “SibEnzim” served as the fragment length marker. On completion of electrophoresis,

the gel was stained with ethidium bromide and photographed in transmitted UV light using an image analyzer device presenting the results of electrophoresis in the form of a computer file.

Polymorphic fragments were detected and cut out from agarose gel. The DNA was extracted and cleaned (by RAPD fragments) with QIAquick Gel Extraction Kit (QIAGEN).

The eluted RAPD fragment was liquated with pGEM-T vector system II ("Promega"). After transformation from recombinant clones, plasmid DNA was isolated, verified for the presence of cloned RAPD fragments, and sequenced from two sides using primers present in the plasmid.

SCAR primers having a length of 24--30 bp and usually including the entire RAPD primer sequence or its fragment were selected for the obtained sequences. Amplification was performed once again.

Results

Totally, 150 pairs of 23- and 20-mer primers were used for the RAPD analysis. Some of them has no products of amplification. Others has no polymorphism.

The primers and there combinations, giving stainable polymorphism, were used in following analyses (table 1, 2). After amplification, we had patterns with some fragments (figure.1). Polymorphic fragments were cut out from gel, cleaned and sequenced. In cases of variable sequences we picked up specific SCAR-primers, which were used for SCAR-markers amplification (Table 3) We reviled polymorphism in seven pairs of SCAR primers, designed by us (marked in table). With other heterogenic fragments, we have no success. In some cases, we reviled a quantitative polymorphism instead of qualitative one. For calculating of quantitative data and for the control of amplification, we used primers for the marker sequence of an similar size (700 bp) of the gene 18 S RNA of acipenserids. This reference sequence was also frequently used for the control of gene expression in course of real-time PCR (Carson J. et al 2002) The amount of DNA was counted using Phoretix 1 D program (version 2003.02). The example of variable SCAR-marker (bottom) and its sequence (top) is represented in figure 2.

Discussion

Disappearance of polymorphism by SCAR-markers creation was recorded in literature previously. It happens usually, when original polymorphism was created by a point mutation in site of primer annealing. It can be explained, that single replacement of base in site of annealing prevents seating shorter RAPD-primer, but no stopping annealing of longer SCAR-primer. In addition, in cause of a point mutation of SCAR-primer (which is base of original RAPD-polymorphism) using, the former moves in a middle portion of the latter. Normally, it does not influence on specificity and effectiveness of amplification. (Koveza and Gostimskii 2005).

From all common methods of polymorphism reparation, only increasing of annealing temperature of primers was used.

Using the aforementioned SCAR primers in our studies, a single fragment with a size slightly smaller than the initial polymorphic RAPD fragment was amplified. For verifying of the obtained experimental data, we sequenced some previously obtained SCAR markers. Their sequence completely coincided with the corresponding RAPD product.

It should be noted that transition from complex RAPD patterns to the amplification of one specific fragment considerably facilitates studies with a large samples for the presence of the given fragment. In addition, in case of a distinct standardization to the concentration and quality of the studied DNA , to pass immediately to the stage of determining the presence or absence of SCAR primer is possible (Higuchi R. et al 1992). That is achieve verifying the amount of amplification of the base sequence of the gene 18 S RNA.

Table 1. Nuclear sequences of primers, used by PCR-RAPD DNA reaction of mykiss.

№	Sequence 5'-3'	bp
1	Tgg cct ggc tgc cct gag cag	21
2	Gat cat gcc att gca ctc ta	20
3	Atg ctt tgg wac aca cct tcg t	22
4	gag gat tgt ggc ctt ctt tg	20
5	Taa tac gac tca cta tag gg	20
6	Gca cat tt acg att cct agt gg	20

Table 2. Primers combinations in PCR-RAPD DNA reaction of mykiss and length of mykiss, used by analyses.

Primers combinations	Common length of markers, according1 kb "Ladder"
1+2	500 bp
2+3	400bp
4+2	500 bp; 600 bp
4+5	200 bp
4+6	300 bp; 400 bp и 500 bp

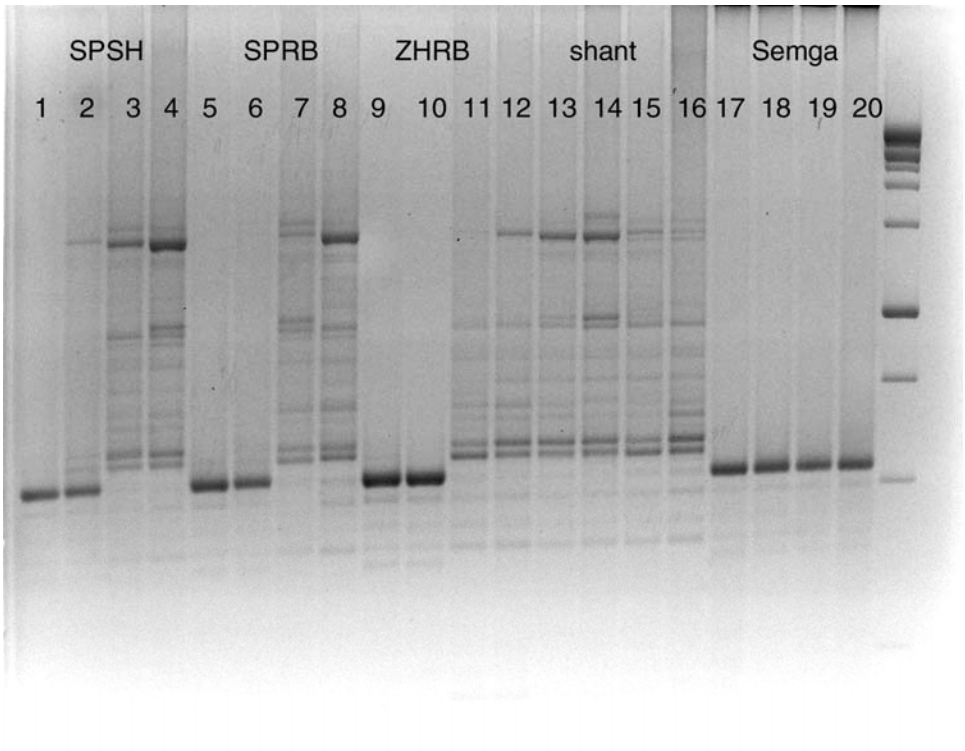

Figure 1. Example of polymorphic RAPD-profile for mikizha. SPSH, SPRB, resident and anadromous forms from the Sopochnaya River (Western Kamchatka); ZHRB, mikizha from the Zhupanova River (Eastern Kamchatka); shant, mikizha from the Shantar Islands; Kamchatka steelhead, the Sea of Okhotsk steelhead sample; marker 1 kb ("SibEnzim").

Table 3. SCAR primers for amplification of SCAR markers of Kamchatka mikizha.

Fragment number, size of the obtained SCAR product	Combination of RAPD primers	Primers for a fragment (SCAR primers)
(470bp)	1+2	
1	Tgg cct ggc tgc cct gag cag	ccgggcacctacaggctgaattcg*
	Gat cat gcc att gca ctc ta	tagtagagtagtgtcctgagggca*
2		Tagccacgatatccagagaaccag
		Gtctccaacagcaatacctcagtc
3		Acggcatactgggcaacaaacatc
		Attgcactctagcgactgtcatgt
4		Atgctacgttgatgaaatccgact
		Gccacgatatccagagcaccagtt
(358bp)	2+3	
1	Gat cat gcc att gca ctc ta	Cctattcatcccggacctctaact
	Atg ctt tgg wac aca cct tcg t	gtctgcgtctcatacataatactg
2		tgatgtcaccttgtccccttaatg*
		Cactgcactctgggaagcctaact*
(155 bp)	4+2	
1	gag gat tgt ggc ctt ctt tg	Gccttctttgcgaaacattgtaaaacctcc*
	Gat cat gcc att gca ctc ta	Gcaatactagtgtttaagctacttttttgaa*
	4+5	
1 (617 bp)	gag gat tgt ggc ctt ctt tg	ccctagtctttgtggttgaatctg*
	Taa tac gac tca cta tag gg	Actggagtggagcaaatgttagcg*
2 (483 bp)		Ctggaggctaagaggacgagagga*
		Ttcctagtggctcagtgtgggtca*
	4+6	
1 (491 bp)	gag gat tgt ggc ctt ctt tg	Caatcccaaatacatcctctaagg
	Gca cat tt acg att cct agt gg	Catttacaactgagacctggccaa
2 (487 bp)		Actgagacccggccaagataaagc
		Tgttgagctggtacggattaattg
3 (514 bp)		Ccgtctcctctctgatagctgtgc*
		Ggccttctttgtctatttcctcac*
4 (383 bp)		gtgtattctccatctcccctcttg*
		ttctttgtgggttcgactataggc*
5 (293 bp)		Aacgagcttccatgccatacaaca
		Gtttggcagcatgagtgaaggagg

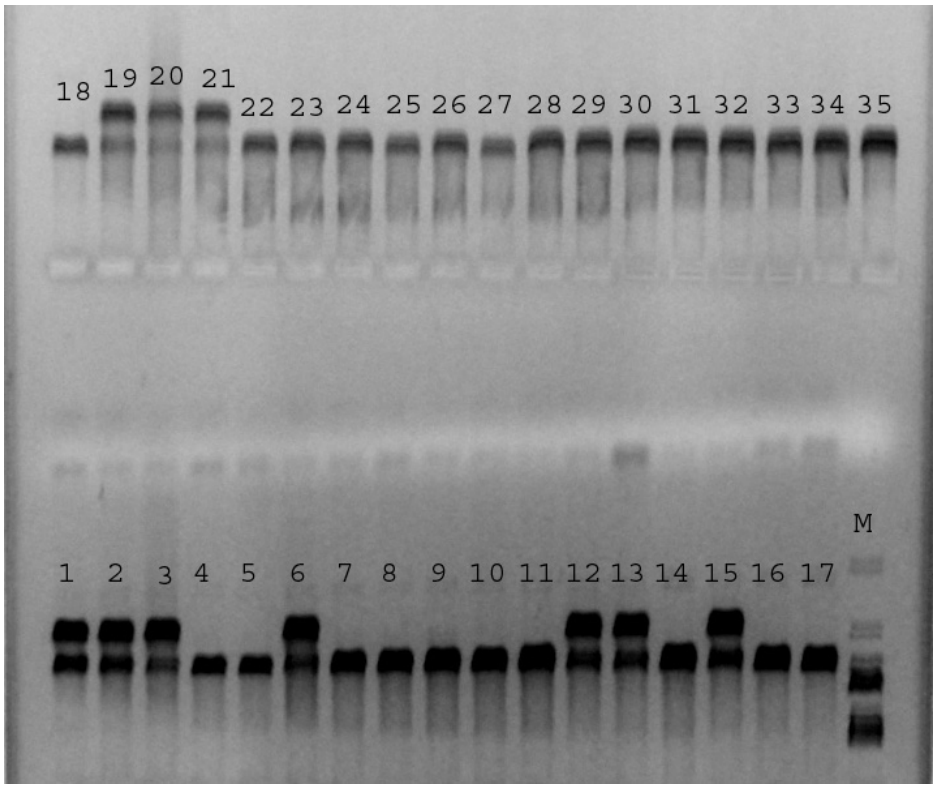

Figure 2. Polymorphic SCAR profile: (1 -21) mikizha from the Kol River; (21-35) *O. masou* from the Kol River; marker 1 kb ("SibEnzim")

The verification of isolated sequences having polymorphism according to Gene Pool demonstrated that only one of sequences (SCAR-marker of 155 bp) has about 32% of similarity (64 bp from 200 bp – 82-86% of identity) with structure genes of *Salmo salar* (*caspase* 3B gene, *pparb*2B gene *for peroxisome proliferator-activated receptor beta*2B, exon 5); *Oncorhynchus (Parasalmo) mykiss SYPG*1 gene. Other SCAR-marker (617 bp) has a fragment of 108 bp (14,4%) identical with *Oncorhynchus (Parasalmo) mykiss SYPG*1 gene in 87 % . Other sequences had a very small percentage of similarity with any sequences deposited to Gene-bank. Therefore, the SCAR-marker (358 bp) had a fragment (32 bp) and was identical with intron D of gene growth I and II from different species of *Coregonus* in 94% . For SCAR-marke (514 bp), only very short fragments (20-23 bp) were identical to sequences of genome zebrafish and *Homo sapiens* .The SCAR-marker (470 bp) was identical with microsatellite CA-repeat of *S.salar* and *16-28 S RNA* bacteria, deposited in Gene bank. The SCAR-marker (483 bp) had small fragments (about 30 bp) similar to different sequences of *Homo sapiens*, dogs and mice. The SCAR-marker (383 bp) had a fragment (120 bp), similar to gene of macroglobulin of *O.mykiss*. All designed sequences were deposed in Gene Bank (№EU805500 - №EU805506).

Therefore, most probably we obtained the fragments of noncoding and accumulating mutations of repeatable sequences of DNA. Number of these repeats is variable in populations of Kamchatka mykiss. It can be regarded as a genetic marker in population analysis of these animals.

Similarity of one of our fragments with a microsatellite repeats demonstrates, that probably we revealed a polymorphic intra-satellite region of DNA. Such markers are traditionally used in genotyping of different organisms.

The number of these repeats varies in populations of Kamchatka mykiss and may be use as a genetic marker in population

analysis of this taxon. We believe that the using of this system of markers will be extremely effective in large-scale studies of large samples of mikizha and will help to elucidate the questions under discussions concerning this species.

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References

Alekseev, S.S., & Sviredenko, M.A. (1985). Mikizha *Salmo mykiss* Walbaum (Salmonidae) of Shantarian islands. J. Ichtyol. **25(1):** 68-73

Behnke, R.I. (1992). Native trout of Western North America. Amer. Fish. Soc. Monogr. **6:** 275.

Berg, L.S. (1948) Fish of fresh waters of the USSR and the adjacent countries. Moscow- Leningrad : AS USSR. **1:** 466 pp.

Berg, W.J., & Ferris, S.D. (1984). Restriction endonuclease analysis of salmonid mitochondrial DNA. Can. J. Fish. Aquat. Sci. **41:** 1041-1047.

Carson, J., Reed, W., Lucier, T., Brighton, L., Gambling, T., Huang, C., & Collier, A. (2002). Pre- and postnatal lung development, maturation, and plasticity axonemal dynein expression in human fetal tracheal epithelium. Am. J. Physiol. Lung Cell Moll. Physiol. **282:** 421-430.

Chernavin, V.V. (1923). Experience of a systematic grouping of the some *Salmonoidei*, based on their osteological attributes. News of institute of skilled agronomics. **1 (3):** 103-206

Dorofeeva, E.A. (1975). Systematical relations salmons of *Salmo* genus . Zool. J. **54(4):** 583-589

Glikman, L.S., Konovalov, C.M., & Rassadnikov, O.A. (1973). Directions of evolutionary development salmon chondrocranium of a genus *Salmo, Salvelinus, Oncorhynchus.* DAN USSR. **211 (6):** 1472-1474

Higuchi, R., Dollinger, G., Sean Walsh, P., & Griffith, R. (1992). Simultaneous amplification and detection of specific DNA sequences. Nature Publishing Group.

Jordan, D.S. (1892). A description of the golden trout of Kern river California Salmo mykiss aquabonita. Proc. U.S. Nat. Mus. **15(916):** 62-65.

Koganovsky, A.G. (1949). About a finding of a salmon (*Salmo penshinensis* Pallas) in the Amur estuary. TINRO. **31:** 200-2001.

Koveza, O.V., & Gostimskii, S.A. (2005). Development and study of SCAR markers in pea (Pisum sativum L.). Genetika **41(11):** 1522-1530

Mednikov, B.M., & Achundov, A.D.G. (1975). Systematization of noble salmons *Salmo* (Pisces, Salmonidae) in view of DNA molecular hybridization data. DAN USSR. **222(3):** 744-746

Murata, S., Takasaki, N., Satch, M., Tachida, H., & Okada, N. (1996). Details of retropositional genome dynamics that provide a relation for salmon and trout *(Oncorhynchus)* from the Atlantic salmon and trout (Salmo). Genetics. **142:** 915-926

Okazaki, T. (1984). Genetic divergence and its zoogeographic implications in closely related species *Salmo gairdneri and Salmo mykiss.* Ibid. **31(3):** 297-311

Paran, J., & Michelmore, R.W. (1993). Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor. Appl. Genet**. 85:** 985-993.

Pavlov, D.S., & Savvaitova, K.A. (1996). Modern problems of studying and preservation of the species included in the Red book of Russia – Kamchatka mikizha *Salmo mykiss* Walbaum. J. Ichtyol. **36 (5):** 609-614

Pavlov, S.D., Kolesnikov, A.A., Melnikova, M.N., & Ushakova, M.V. (2004). Genetic divergence of mykizha (*Parasalmo (Oncorhynchus) mykiss*) from Kamchatka inferred from restriction analysis and sequencing of mtDNA cytochrome b gene. Russian Journal of Genetics. **40(12):** 1407-1412.

Philips, R.B., & Pleyte, K.A. (1991). Nuclear DNA and salmonid phylogenetics. J. of Fish Biology. **39 (A):** 259-275.

Savvaitova, K.A., & Lebedev, V.D. (1966). About systematical position of the Kamchatka steelhead (*Salmo penshinensis* Pallas) and mikizha (*Salmo mykiss* Walbaum) and their relations with American representatives of a genus *Salmo*. J. Ichtyol. **6(4):** 593-608.

Savvaitova, K.A., Kuzishin, K.V., & Proebstel, D.S. (1998). Numbers of form of trout genus *Salmo* of the western Kamchatka. To problems of group phenetics variety. J. Ichtiol. **38(3):** 338-346.

Smith, G.R., & Stearley, R.F. (1989). The classification and scientific names of rainbow and cutthroat trout. Fisheries. **14:** 4-10.

Welsh, I., & McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. Nuc. Acid Res. **18:** 7213-7218.

Welsh, I., & McClelland, M. (1991). Genomic fingerprinting using arbitrarily primed PCR and a matrix of pairwise combinations of primers. Nuc. Acid Res. **19:** 5275-5279.

Williams, I., Kubelik, A.R., Livak, K.I., Rafalski, I.A., & Tongey, S.N. (1990). DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nuc. Acid Res. **18:** 6531-6535

Zimmerman, K.E., Kuzischin, K.V., & Gruzdeva, M.A. (2003). Determination of the Life Strategy in Parasalmo mykiss Walbaum (Salmonidae, Salmoniformies) of Kamchatka by Analyzing the Sr/Ca Ratio in Otoliths. Dokl. Akad. Nauk. Ser. Obshch. Biol. **384 (2):** 274-279.

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