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GENETIC STRUCTURE OF NATURAL POPULATIONS OF STERLET (*Acipenser ruthenus* L.) IN THE CATCHMENT BASINS OF THE KAMA AND OB RIVERS BASED ON POLYMORPHIC ISSR MARKERS

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Abstract

Starlet (*Acipenser ruthenus* L.) is included in the Red Data Books of the Russian Federation, Perm Krai and Kirov Province. Inter-microsatellite DNA polymorphism analysis of sterlet populations of the Kama and Ob rivers has not been performed until now. This paper reports on genetic diversity and genetic structure of five natural sterlet populations of the Kama, Ob and Vyatka rivers based on polymorphism of ISSR-PCR markers. The study was carried out in 2015-2916. DNA was extracted from fragments of pectoral fins of fishes aged 3 to 4 years. DNA samples from 195 individuals were analyzed with five effective ISSR primers. POPGENE 1.31 and GenAlEx6 software was used for statistical processing. Basic genetic parameters were proportion (P_{95}) of polymorphic loci, expected (H_e) heterozygosity, number of alleles per locus (Na), effective number of alleles per loci (Ne), and number of rare alleles (R). The Bayesian method of population structure analysis was performed using STRUCTURE 2.3.4 software. Genetic structure of a population was characterized by proportion of heterozygous genotypes (H_T) in the entire population, the expected proportion of heterozygous genotypes (H_S) in the subpopulation, and the proportion of interpopulation genetic diversity (G_{ST}). As a result, a total of 128 ISSR-PCR markers were identified. The number of amplified ISSR-PCR markers ranged from 7 to 23 depending on the ISSR primer. It was found that the portion of polymorphic loci in *A. ruthenus* populations was high and amounted to 0,938. Genetic diversity was the highest in the Vyatka sterlet population (P_{95} = 0.876; H_e = 0.232; Ne = 1.402; R = 10) and the lowest in the Ob sterlet population (P_{95} = 0.634; H_e = 0.100; Ne = 1.175; R = 3). A total of 23 rare ISSR-PCR markers were identified for all the samples studied, and 10 of these markers were characteristic of the Vyatka river sterlets. This indicates the possibility of successful identification of these sterlets by population-specific markers. Genetic structure analysis showed that the expected proportion of heterozygous genotypes (H_T) for the total sample was 0.283, whereas H_S index was much lower making 0.173, therefore, G_{ST} value was high and amounted to 0.386. The studied populations were highly differentiated. The interpopulation component accounted for 38.6 % of genetic diversity, while intrapopulation component was responsible for 61.4 %. In each of the studied populations, the rare ISSR-PCR markers have been determined that can be used for identification of studied populations of this species. Thus, the efficiency of ISSR analysis for the identification of sterlets at population level has been proved. It has been established that polylocus ISSR-PCR markers can be used both for characterizing gene pools and for molecular genetic identification of populations and breeds, including sterlet populations and replacement broodstocks. Recommendations for genetic conservation of the Kama and Ob sterlet populations have been developed. These data should be used to manage replacement broodstocks in sterlet artificial reproduction for further release of the fry in a population with an identical gene pool.

Keywords: genetic diversity, gene pool, genetic structure, ISSR-PCR markers, molecular-genetic identification, *Acipenser ruthenus* L., sterlets

Polymorphism analysis of molecular-genetic markers is a compulsory development stage of programs for preservation of the genetic resources of sturgeons. Gene pool analysis with the use of molecular-genetic markers testifies the

expressed specific properties of genetic structure and the need for further development of genetic-based methods for preservation of biodiversity of rare fish species [1].

Inter-microsatellite DNA polymorphism analysis [inter simple sequence repeats, ISSR] is a method to investigate genetic diversity of plant and animal populations. It has good repeatability and is successfully applied in the global [2, 3] and national practice [4]. ISSR-methods may be used for identification of cross-species and intra-species genetic variability, identification of kinds, populations, lines, and in several cases for individual genotyping [5]. ISSR markers are breed and species specific [6]. ISSR-method was used by Yu.A Stolpovskiy et al. [7-9], L.V. Nesteruk et al. [10], and P.P. Srivastava et al. [11] for genotyping animal populations and breeds, and for identification of silkworm populations [12]. In this regard, it is perspective to apply multi-locus markers in molecular genetic identification of starlet populations and flocks.

Each year, sturgeon breeding in Russia becomes more extensive as one of the most important agricultural industries. Artificial reproduction allows compensating damages caused to water resources and taking measures for reintroduction of sturgeons [13]. Starlet (*Acipenser ruthenus* L.) is one of the mostly known sturgeon representatives in Russia with long evolutionary history [14]. Certain populations of such species are included in the Red Book of the Russian Federation [15], Perm Territory [16] and Kirov Region [1] Commercial value of sturgeons promotes intensive catching of such fishes. Construction of hydrotechnical facilities and location of industrial zones near water objects had resulted in sharp decrease of the number of natural starlet populations [18]. For its recovery and for compensation of damage caused by economic humankind activity, species raised at fish breeding farms are released to the natural environment. Nowadays, there is no information on effectiveness of such measures since it lacks information on recovery of populations which is one of the most important components of nature protection reacclimatization measures. Accounting of returns to the populations of the species raised at fish breeding farms requires conduction of studies involving identification of young fish at farms, as well as species being members of the natural population in the studied water reservoirs [19]. Due to the need for protection of wild capture and endangered fishes, it is especially important to study populations undergoing antropogenic loads [20]. Inter-microsatellite DNA polymorphism analysis of starlet populations of the Kama River and Ob River basins has not been performed until now.

Use of Inter-microsatellite DNA polymorphism analysis enabled us for the first time to obtain information on starlet populations in Kama, Ob, and Vyatka rivers. We have identified ISSR makers and proposed two approaches to identification of starlet populations. i.e. by unique markers and combination of polymorphous markers.

Purpose of present paper is studying genetic diversity of the natural starlet populations for ISSR marker polymorphism.

Techniques. The natural starlet populations (*Acipenser ruthenus* L.) aged 3-4 years were studied. Samples were collected in 2015-2016 from 195 individuals caught in five places: Vi — Vyatka River near Vishkil Urban Area (middle course), Sh — Vyatka River near Shurma Urban Area (lower course, 236 km from Vi sample down the stream), Vp — Vyatka River in area of Vyatskie Polyani city of Kirov Region (lower course, 138 km from Sh sample down the stream), Km — Kama River lower the stanch of Votkinsk Hydroelectric Station, CHM — Ob River in confluence region of Irtysh and Ob rivers.

Flipper fragments were collected in vivo. Afterwards, fishes were returned to basin. Flippers were fixed in 96 % alcohol. Total DNA was extracted from

100 mg samples by S.O. Rogers method [21]. DNA quality and concentration were controlled spectrophotometrically (NanoDrop 2000, (Thermo Fisher Scientific, USA) and diluted to 10 ng/μl.

Inter-microsatellite DNA polymorphism analysis protocol was as described [5]. A 25 μl PCR (polymerase chain reaction) mixture contained 2 units of Tag DNA-polymerase, 2.5 μl standard 10× PCR buffer, 25 pM of primer, 2.5 μM Mg^{2+}, 0.25 μM dNTPs, 5 μl of total DNA. DNA probes of fishes were analyzed with five earlier selected starlet-effective ISSR primers [22]. PCR (an amplifier GeneAmp Biosystem, Applied Biosystems, USA) was carried out as per usual ISSR protocol: 2 minute preliminary denaturation at 94 °C; 20 second denaturation at 94 °C, 10 second primer annealing at 6 °C, 10 second elongation at 72 °C (5 cycles); 5 second denaturation at 94 °C, 5 second primer annealing at 56 °C, 5 second elongation at 72 °C (30 cycles); 2 minute final elongation at of 72 °C. Annealing temperature depended on primer GC content and varied from 56 to 64 °C. In lieu of DNA, 5 μl de-ionized water was added to reaction mixture as negative control (K^-) to check purity of the reagents. To prove repeatability of the results, PCR analysis was repeated trice. Amplification products were separated by electrophoresis in 1.7 % agar gel with 1× TBE buffer. DNA fragment lengths were determined with molecular weight marker (100 bp + 1.5 + 3 Kb DNA Ladder, SibEnzyme-M LLC, Moscow) using Quantity One software in Gel-Doc XR system (Bio-Rad, USA).

Portion of polymorphic loci (P_{95}), expected heterozygosity (H_e), mean allele number (Na), effective allele number (Ne) per locus, and number of rare alleles (R) were calculated with the use of POPGENE 1.31 software [23] and special macros GenAlEx6 [24] for Microsoft Excel.

The following parameters were used to describe genetic structure of population [25, 26]: expected level of heterozigous genotypes across the entire population (H_T) as a measure of total genetic diversity; expected level of heterozigous genotypes in the subpopulation (H_S) as a measure of interpopulation diversity; proportion of interpopulation genetic diversity in total diversity, or population subdivision indicator (G_{ST}). Bayesian method of analysis of the population structure was carried out with the use of STRUCTURE 2.3.4 software. Reliability assessment of possible cluster groups and their visualization was carried out by STRUCTURE HARVESTER software [27]. Population structure was estimated by allocation of the studied species in the most probable number of clusters according to the algorithm of G. Evanno et al. [28]. Cluster number probability was determined within the range from 1 to 10.

Results. Total 128 ISSR-PCR markers of which 120 were polymorphic (P_{95} = 0.938) were revealed in five samples of the natural starlet populations. Number of amplified ISSR-PCR markers varied from 7 (CR-212 primer) to 23 (X9 primer) depending on the primer. The highest level of polymorphic loci was in sample Vp (P_{95} = 0.876), the least value was in CHM (P_{95} = 0.634). The expected heterozygosity (H_e) for the total sample was low (0.173) (Table 1). The expected heterozygosity was the highest in Vp population (H_e = 0.232), and the least in CHM (H_e = 0.100). We assume that high expected heterozygosity in Vp is due to systematic releases of young fish from the neighboring fish breeding farms where individuals from the Vyatka river natural populations are the basis for breeders' stock. Presence of unique ISSR-PCR markers (R) in only one of the studied populations is important to successfully affiliate starlet with certain population or to control geographic origin. We found 23 unique ISSR-PCR markers in the total sample of natural populations, provided that 10 of them were in Vp sample. This testifies on possibility of successful identification of

starlet belonging to such population.

1. Genetic diversity of the natural starlet (*Acipenser ruthenus* L.) populations (2015-2016)

Populations	P_{95}	H_e	Na	Ne	R
Vp	0.876	0.232 (0.018)	1.625 (0.486)	1.402 (0.381)	10
Km	0.768	0.162 (0.018)	1.476 (0.501)	1.282 (0.374)	5
CHM	0.634	0.100 (0.016)	1.258 (0.439)	1.175 (0.328)	3
Vi	0.835	0.198 (0.017)	1.625 (0.486)	1.325 (0.344)	5
Sh	0.805	0.174 (0.017)	1.523 (0.501)	1.295 (0.364)	0
Total	0.938	0.173 (0.110)	2.000 (0.000)	1.468 (0.340)	23

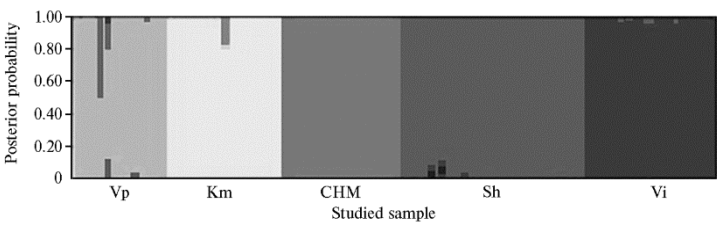
N o t e. P_{95} — portion of polymorphic loci, H_e — expected heterozygsity, Na — number of alleles per locus, Ne — effective number of alleles per locus, R — number of unique ISSR-PCR markers. Values are statistically significant at $P \leq 0.05$; standard deviations (SD) are in parenthesis. See description of populations in the section "Tehniques".

2. Genetic structure and differentiation of the studied starlet (*Acipenser ruthenus* L.) populations o Kama, Ob, and Vyatka rivers (2015-2016)

ISSR-primer	Nucleotide sequence (5′→3′)	H_T	H_S	G_{ST}
CR-212	$(CT)_8TG$	0.302 (0.028)	0.225 (0.017)	0.252
CR-215	$(CA)_6GT$	0.314 (0.033)	0.159 (0.085)	0.492
ISSR-9	$(ACG)_7G$	0.220 (0.022)	0.159 (0.012)	0.277
X9	$(ACC)_6G$	0.277 (0.022)	0.155 (0.008)	0.442
X11	$(AGC)_6G$	0.312 (0.022)	0.182 (0.009)	0.417
Per total sample		0.283 (0.026)	0.173 (0.011)	0.386

N o t e . H_T — expected level of heterozygous genotypes in the total sample, H_S — expected level of heterozygous genotypes in a separate population, G_{ST} — interpopulation genetic diversity in total diversity (population subdivision indicator). Values are statistically significant at $P \leq 0.05$; standard deviations (SD) are in parenthesis.

Analysis of the genetic structure and differentiation of the studied starlet populations (Table 2) revealed the expected heterozygosity H_T of 0.283 for the total sample. The expected level of heterozygous genotypes in certain population (H_S) was 0.173. Accordingly, in average 17 % of heterozygotes were in each population. G_{ST} value for population subdivision is 0.386, i.e. interpopulation genetic diversity component accounted for 38.6 % of diversity, intrapopulation diversity accounted for 61.4 %. That is, the studied starlet samples are highly differentiated.



Allocation of starlet (*Acipenser ruthenus* L.) genotypes from Kama, Ob, and Vyatka rivers (at K = 5) (2015-2016). STRUCTURE software. See description of populations in section "Methodology".

We have identified five genetically segregated groups and determined probability of affiliation of each of the 195 studied individuals with any of the proposed clusters (see Table).

The identified ISSR-PCR markers are stable and reproducible. Obtained data result in two approaches to identification of starlet population, i.e. by unique markers and by combination of polymorphic markers. Illustration of the first way that we suggest is the Vi sample with unique ISSR-PCR 780 bps marker $Ac_{un}780_{X9}$ which is amplified with primer $(ACC)_6G$ (denoted as X9) and present in Vi with frequency of 0.660. Simultaneous presence of two polymorphic markers, the $Ac_p640_{CR\text{-}212}$ (640 bps, frequency 0.706) amplified with primer $(CT)_8TG$, and Ac_p520_{X9} (520 bps, frequency 0.735) amplified with $(ACC)_6G$, implies the affiliation with Km population.

Earlier], ISSR-PCR markers were used to control genetic structure of agricultural mammal species and to identify breed-specific properties of their

gene pools [29]. Simple sequence repeats (SSR) loci were used to identify specific affiliation of sturgeons and to identify fish species of hybrid origin [30]. In this paper we have shown effectiveness of inter-microsatellite DNA polymorphism analysis (ISSR-PCR markers) in studying genetic structure and molecular identification of starlet populations. This is necessary to form replacing breeding stocks in which artificial starlet reproduction occurs with further release of young fish to population with identical gene pool. *A. ruthenus* population from Vyatka river (Vp) with high genetic diversity is recommended for such stocks. Molecular genetic formulae, bar codes, and genetic passports based on molecular markers, including rare ISSR-PCR markers, will provide control of affiliation of individuals to certain population and/or their geographic origin.

Therefore, we have found 128 ISSR-PCR markers in the studied populations of *Acipenser ruthenus* of Kama River and Ob River basins. Polymorphic loci revealed with the use of these markers in the total sample are very frequent (0.938). The *A. ruthenus* sample from Vyatka river (Vyatskie city) shows the highest genetic diversity indicators (P_{95} = 0.876; H_e = 0.232; Ne = 1.402; R = 10) compared to the sample from the same river close to headwaters. Sample from Ob river shows low genetic diversity (P_{95} = 0.634; H_e = 0.100; Ne = 1.175; R = 3). The expected heterozygosity (H_T) in total sample is 0.283, whereas in a separate population this indicator (H_S) is significantly lower (0.173). Accordingly, interpopulation genetic diversity component in starlet populations accounts for 38.6 %, and intrapopulation component accounts for 61.4 %. Studied populations are strongly diversified. Unique molecular markers are identified in each sample of *A. ruthenus*. Combinations of polymorphic markers can be used in molecular genetic identification of the starlet populations.

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