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DEVELOPMENT OF MULTIPLEX PANEL OF MICROSATELLITES FOR GENETIC STUDIES OF SIBERIAN STURGEON (*Acipenser baerii*) BRED IN COMMERCIAL AQUACULTURE

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Abstract

The Siberian sturgeon (*Acipenser baerii* Brandt, 1869) is one of the most important aquaculture fish species in Russia. Due to the high demand for valuable commercial products, breeding of Siberian sturgeon is promising in the industry. However, breeding this species is significantly complicated by because of its tetraploid genome, which, together with the need to mix fidh roe and sperm from several producers when obtaining offspring, prevents the introduction into practice of well-proven molecular genetic methods, e.g. microsatellite analysis. In this work, for the first time, the known microsatellite loci in the Siberian sturgeon are characterized from the point of view of the possibility of effectively accounting for the doses of their alleles in the tetraploid genome. Seven loci were found that met this criterion. The goal of our work was to create a panel of microsatellite markers adapted for use in the selection of Siberian sturgeon from the Lena population. The research was carried out in 2023. As biological material, we used sections of fin tissue of the Siberian sturgeon of the Lena population, taken from the fish of an experimental herd kept in a closed water supply installation of the Ernst Federal Research Center for Animal Husbandry. The experimental herd contained fish from the Mozhaisk production and experimental fish hatchery (Goretovo village, Mozhaisk urban District, Moscow Province; group I, $n = 42$) and fish obtained from RTF Diana LLC (village Kaduy, Kaduysky District, Vologda Province; group II, $n = 47$). DNA was isolated using the DNA-Extran-2 kit (NPK Synthol, Russia) according to the manufacturer's protocol. Qualitative assessment of DNA was carried out by electrophoresis in 1.2 % agarose gel. PCR was performed in a Thermal Cycler SimpliAmp amplifier (Thermo Fisher Scientific, Inc, USA). Electrophoretic separation of amplification products was carried out in a Nanofor 05 capillary electrophoresis system (NPK Synthol, Russia). Allele sizes were determined using GeneMarker software (Version 3.0.1). For each locus, the dose of each allele was determined. Twenty seven microsatellite markers known for sturgeon fish species were used (Ls 19, Aox 45, Aox 9, Ls 68, Agu 38, Ag 49a, Agu 37, Agu 41, Agu 15, Agu 51, Agu 59, Agu 34, Agu 36, Agu 46, Agu 56, Agu 54, AoxD 161, AfuG 63, AfuG 51, AfuG 112, An 20, Aru 13, Aru 18, Afu 68 b, Spl 163, AfuG41, and Ls 39). Of these, seven were selected for multiplex panels (Agu 38, An 20, Aru 18, Ls 19, Ag 49a, Agu 37, Agu 41). Based on the polymorphism of seven microsatellite loci for the two studied groups of Siberian sturgeon individuals, classical population genetic indicators were calculated, the average number of alleles per locus (Na = 6.86), the number of effective alleles (Ne = 3.61), observed ($H_o = 0.839$) and expected ($H_e = 0.6535$) heterozygosity. In the studied groups, inbreeding was not revealed ($F_{IS} = -0.340$ and -0.173) while a significant genetic differentiation occurred (Nei's GD = 0.1340, Fst = 0.0796). The groups formed two clear, practically non-overlapping PCA clusters despite the fact that the ancestors of Siberian sturgeon individuals in both groups were of related origin. The contribution of the allele dose of the tetraploid locus to the efficiency of microsatellite analysis was assessed. On average, the information content of each locus increased by 32 %. A comparison of the results of genetic analysis with the available research publications allows us to assume that in aquaculture

herds of Siberian sturgeon from the Lena population, processes associated with changes in allele frequencies of microsatellite loci occur, which gradually enhance their genetic differentiation. As a result of the work, the high efficiency of the created panels of microsatellite markers and their potential suitability for use in genetic certification were confirmed, each individual Siberian sturgeon had its own genetic profile. The distribution of alleles at seven microsatellite loci indicated a unique genetic structure in Siberian sturgeon stocks in each of the two fish hatcheries that were sources of fish seeding material.

Keywords: Siberian sturgeon, microsatellites, tetraploids, sturgeon breeding, null alleles

Siberian sturgeon (*Acipenser baerii* Brandt, 1869) is one of the most important fish species grown in commercial aquaculture. The main advantages of the Siberian sturgeon are rapid weigh gain and high survival rate in farm conditions [1, 2]. The high-quality caviar produced by Siberian sturgeon is of special interest. Due to the economic importance and high demand for Siberian sturgeon products, its breeding has significant potential for the development of aquaculture [3].

In the wild, the Siberian sturgeon is in the status of an endangered species because of environment pollution, uncontrolled fishing, and degradation of the natural habitat. The species is listed in the IUCN (International Union for Conservation of Nature and Natural Resources,) Red List [4]. In addition, the Siberian sturgeon is included in Appendix 1 and Appendix 2 of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) [5]. The unsatisfactory state of wild populations dictates the need to preserve the biodiversity of the Siberian sturgeon population at fish hatcheries and wisely use the genetic potential in aquaculture [6].

Modern breeding methods rely on molecular genetics data to significantly speed up and simplify improvemrnt of agricultural animals and plants in turmes of productivity [7-9]. However, in sturgeon farming, the introduction of these technologies into everyday breeding practice has slowed down. Today, molecular genetic markers, primarily microsatellites and mitochondrial DNA, are used to control species identity for further using juveniles to replenish natural populations [10]. Developing molecular genetic panels for individual identification of Siberian sturgeon faces difficulties since the species has a tetraploid genome [11], while certain loci can exhibit a diploid or even hexaploid character [12, 13].

The direct use of molecular genetic methods in breeding Siberian sturgeon is also hampered by the long maturation period characteristic of this species, and by the mixing of reproductive material from several individuals practiced at fish hatcheries. This significantly reduces waste, increases the percentage of fertilized eggs, but also prevents the exact correlation of parents and their offspring, which is necessary in breeding. The mass individual tagging of breeder fish with electronic chips at sturgeon hatcheries promotes using molecular genetic methods in breeding.

In this work, for the first time, the known microsatellite loci in the Siberian sturgeon are characterized in turms of effective accounting for their allele doses in the tetraploid genome. Seven loci were found that met this criterion.

The goal of our work was to create a panel of microsatellite markers adapted for use in the selection of Siberian sturgeon from the Lena population.

Materials and methods. Sections of fin tissue of the Siberian sturgeon from the Lena population were collcected from the fish of an experimental herd kept in a closed water supply installation (the Ernst Federal Research Center for Animal Husbandry — VIZh, 2023). The herd consisted of fish from the Mozhaisk production and experimental fish hatchery (Goretovo village, Mozhaisk urban district, Moscow region; group I, $n = 42$) and fish from RTF Diana LLC (working Kaduy village, Kaduysky District, Vologda Province; group II, $n = 47$).

DNA was isolated using the DNA-Extran-2 kit (NPK Synthol, Russia) as described in the manufacturer's protocol. DNA quality was assessed by 1.2% agarose gel electrophoresis. PCR reaction mixture was 1.5 μl of 10× Turbo buffer

(Evrogen, Russia), 1.5 μl of a 2 mM dNTPs solution, 0.3 μl of a 10 mM primer mixture, 1 unit Smart Taq polymerase (JSC Dialat Ltd., Russia), ~ 50-100 ng of the studied genomic DNA. The reaction mixture was added with deionized water to the final volume of 15 μl. PCR was performed on a Thermal Cycler SimpliAmp amplifier (Thermo Fisher Scientific, Inc, USA) in the following mode: 10 min at 94 °C (primary denaturation); 30 s at 95 °C (denaturation), 40 s at 58 °C (annealing of primers on the DNA template), 35 s at 72 °C (chain elongation) (38 cycles); 5 min at 72 °C (final elongation). Amplification products were electrophoretically separated (a Nanofor 05 capillary electrophoresis system, NPK Synthol, Russia). Allele sizes were determined with GeneMarker software (Version 3.0.1). For each locus, the dose of each allele was determined [14].

The assembled panels were tested on groups I and II of the experimental herd of Siberian sturgeon. For each group, classical population genetic indicators were calculated, namely, expected (H_e) and observed (H_o) heterozygosity, average number of alleles per locus (Na), average number of effective alleles (Ne), coefficient of inbreeding (F_{IS}), genetic distances by M. Nei method [15], index F_{st} [16, 17].

Microsatellite analysis data were processed using R package Polysat (Version 1.7) [18], STRUCTURE (Version 2.3.4) [19], and SPA Ge Di1-5d [20] software. PCA (Principal Coordinate Analysis) plot was constructed according to R. Bruvo et al. [21] based on the genetic distances.

Results. Since we needed to create multiplex microsatellite panels with a uniform ploidy pattern of loci and the absence of null alleles, the microsatellite markers had to strictly comply with certain parameters. The locus should not have more than four alleles in one sample, should not have zero alleles, should have polymorphism and stable PCR amplification subject to multiplexing, and the dose of each allele should be visually well determined.

To create a panel of microsatellite markers for individual identification and control of the Siberian sturgeon origin, we tested 27 microsatellite loci, the Ls 19, Aox 45, Aox 9, Ls 68 [22, 23], Agu 38, Ag 49a, Agu 37, Agu 41, Agu 15, Agu 51, Agu 59, Agu 34, Agu 36, Agu 46, Agu 56, Agu 54 [24], AoxD 161, AfuG 63, AfuG 51, AfuG 112, An 20, Aru 13, Aru 18, Afu 68 b, Spl 163, AfuG41, and Ls 39 [25, 26]. A number of loci (e.g., AoxD 161, AfuG 41, Agu 59) where more than 4 alleles were identified (Fig. 1) we excluded from further analysis.

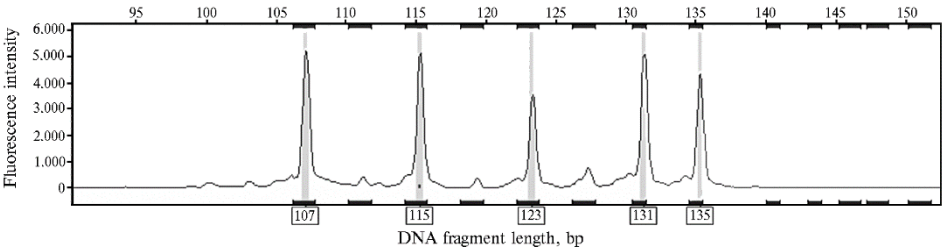

Fig. 1. Genetic profile of the domesticated Siberian sturgeon (*Acipenser baerii* Brandt, 1869) individuals of the Lena population at the AoxD 161 locus with five identified alleles (Ernst Federal Research Center for Animal Husbandry — VIZh, 2023).

Null alleles are known to distort statistical calculations, overestimating homozygosity [27, 28]. Species with a polyploid genome acquires a higher risk of having null alleles [29]. Polyploidization can be caused by the fusion of genomes that are polymorphic at the primer annealing site. In addition, it is believed that the polyploidization itself stimulates transposon activity and loss of DNA sections due to genomic rearrangements [30], which can also destroy primer binding sites [31]. Therefore, determining allele dosage was a critical requirement in loci selection. Determining the dose of each allele (Fig. 2) identified loci AfuG 51, Aru 13,

Agu 15 that presumably had null alleles.

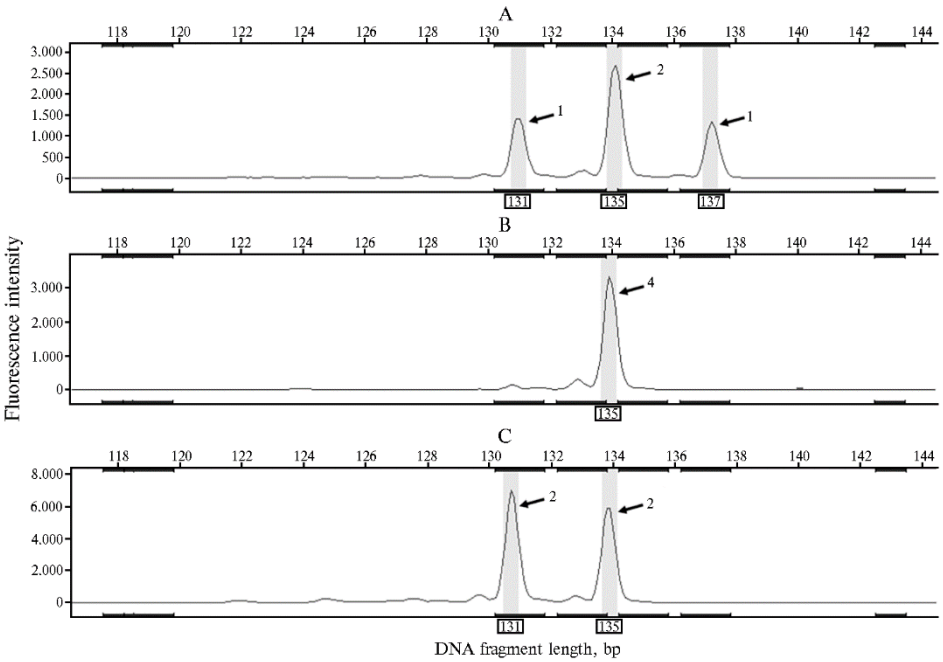

Fig. 2. Assessment of the each allele dose at the microsatellite locus Ls 19 in individuals of the domesticated Siberian sturgeon (*Acipenser baerii* Brandt, 1869) of the Lena population: A — with allele ratio of 1/2/1, B — with allele ratio of 4/0, C — with allele ratio of 2/2. Arrows indicate peaks corresponding to alleles. 1, 2, 3, 4 indicate allele doses (the number of chromosomes in a tetraploid genome carrying a given allele) (Ernst Federal Research Center for Animal Husbandry — VIZh, 2023).

Thus, for the AfuG 51 microsatellite locus, three alleles with approximately the same amount of the resulting PCR product were identified (Fig. 3). Since the Siberian sturgeon genome is tetraploid, the fourth allele should most likely be null, but we observed the presence of a fourth peak with a weak signal which was probably the fourth allele with a modified primer annealing site.

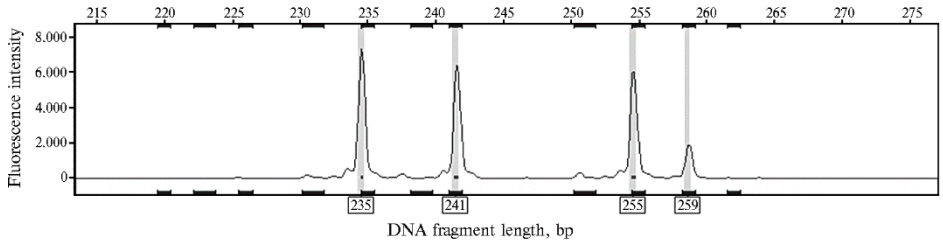

Fig. 3. Identification of the null allele on the example of the AfuG 51 locus in individuals of the domesticated Siberian sturgeon (*Acipenser baerii* Brandt, 1869) of the Lena population: 235, 241, 255 are three alleles with approximately the same efficiency in PCR, 259 is the fourth peak with a low fluorescence that probably corresponds to an allele with a modified primer annealing site (Ernst Federal Research Center for Animal Husbandry — VIZh, 2023).

On a small sample of Siberian sturgeon DNA (n = 16), we preliminarily tested if microsatellite loci might be multiplexed, and whether there was a clear deviation from tetraploid inheritance. One more requirement was to retain the opportunity of accounting the dose of alleles in PCR multiplexing. Of 27 microsatellite markers, 7 loci, the Agu 38, An 20, Aru 18, Ls 19, Ag 49a, Agu 37, and Agu 41 met the requirements and was chosen for multiplex PCR (Table 1, Fig. 4). The loci were arranged into two multiplex panels, Agu 38, An 20, and Aru 18 (panel 1), Ls 19, Ag 49a, Agu 37, and Agu 41 (panel 2).

1. Microsatellite loci for testing individuals of the domesticated Siberian sturgeon (*Acipenser baerii* Brandt, 1869) of the Lena population (Ernst Federal Research Center for Animal Husbandry — VIZh, 2023)

Locus	Dye	Allele length expected, bp [22-26]	Allele length observed, bp	Primer sequence
		Panel 1		
Agu 38	6-FAM	108-114	90-112	F: ACTGGGGTTGAAGGACAGTG R: TCCGTCTCATGTCCAAGGGTA
An 20	6-FAM	151-207	143-185	F: AATAACAATCATTACATGAGGCT R: TGGTCAGTTGTTTTTTTATTGAT
Aru 18	R6G	138-154	137-145	F: CCTGGAACACGTCCAGTTTT R: TGGGTGAATGTTTTGGTGTG
		Panel 2		
Ls 19	6-FAM	118-145	119-137	F: CATCTTAGCCGTCTGGGTAC R: CAGGTCCCTAATACAATGGC
Agu 37	R6G	128-136	124-128	F: ACATGGTAGCAAAATCCCAA R: CAGCAAGCTTAGATGCATGG
Agu 41	ROX	178-218	177-229	F: AAGACAAACAGTGGCCCAAC R: CAATGGCAGGTGCTACTGAA
Ag49a	6-FAM	198-219	192-218	F: TGTTATCTGCTCTGATATTGATTCG R: CGTTTTAAAGTTTGAACGGCA

Table 2 submits results of testing these panels in two groups of individuals from the experimental Siberian sturgeon.

2. Allele frequency for 7 microsatellite loci in individuals of the domesticated Siberian sturgeon (*Acipenser baerii* Brandt, 1869) of the Lena population (calculated in the SPAGeDi1-5d program; Ernst Federal Research Center for Animal Husbandry — VIZh, 2023)

Locus	Group	Na	Ne	H_e	H_o	F_{IS}	PIC
All (*M*±SEM)	I + II	6.86±1.506	3.61±0.731	0.6535±0.07441	0.839±0.0976	–0.191±0.0778	
	I	4.43±0.519	2.90±0.476	0.5770±0.10235	0.816±0.1413	–0.340±0.1106	
	II	6.14±1.300	3.63±0.657	0.6744±0.05663	0.860±0.0653	–0.173±0.0590	
Agu 38	I + II	6	3.45	0.7102	0.989	–0.366	0.639
	I	5	3.13	0.6809	1.000	–0.460	0.609
	II	5	3.32	0.6993	0.979	–0.359	0.608
An 20	I + II	13	6.17	0.8379	0.989	–0.167	0.813
	I	6	3.98	0.7486	1.000	–0.335	0.688
	II	12	6.11	0.8365	0.979	–0.156	0.803
Aru 18	I + II	4	1.45	0.3122	0.337	0.231	0.396
	I	3	1.05	0.0471	0.048	0.330	0.301
	II	4	1.98	0.4943	0.596	0.091	0.447
Ls 19	I + II	5	2.47	0.5953	0.854	–0.158	0.526
	I	3	2.01	0.5028	0.857	–0.397	0.429
	II	5	2.67	0.6258	0.851	–0.172	0.526
Ag 49a	I + II	6	3.56	0.7193	0.966	–0.291	0.653
	I	4	3.05	0.6723	0.976	–0.411	0.591
	II	5	3.98	0.7491	0.957	–0.233	0.687
Agu 37	I + II	4	2.33	0.5703	0.753	–0.078	0.502
	I	4	2.56	0.6090	0.833	–0.184	0.519
	II	3	2.01	0.5036	0.681	–0.028	0.449
Agu 41	I + II	10	5.86	0.8295	0.989	–0.180	0.793
	I	6	4.51	0.7781	1.000	–0.282	0.724
	II	9	5.33	0.8123	0.979	–0.185	0.767

N o t e. Group I is fish from the Mozhaisk production and experimental fish hatchery (Goretovo village, Mozhaisk urban District, Moscow Province; $n = 42$), Group II is fish from RTF Diana LLC (working village Kaduy, Kaduysky District, Vologda Province; $n = 47$). H_o — observed heterozygosity, H_e — expected heterozygosity, Na — average number of alleles per locus, Ne — average number of effective alleles per locus, F_{IS} — inbreeding coefficient, PIC— locus polymorphic information content index.

Of the seven microsatellite loci, An 20 and Agu 41 were the most effective as followes from the locus polymorphic information content index (PIC) values. Despite the fact that the domesticated fish originated from the wild Siberian sturgeon of the Lena population, we revealed a clear genetic differentiation between the studied groups. Given the fixation index $F_{st} = 0.0796$, 7.96% of the variability was due to intrapopulation differences, and 92.04% to interpopulation differences. The genetic distance calculated by M. Nei based on the allele frequencies of the

seven microsatellite loci was 0.1340. The genetic differentiation of Siberian sturgeon groups was clearly identified when clustering using the STRUCTURE program (Fig. 5) and by PCA analysis (Fig. 6).

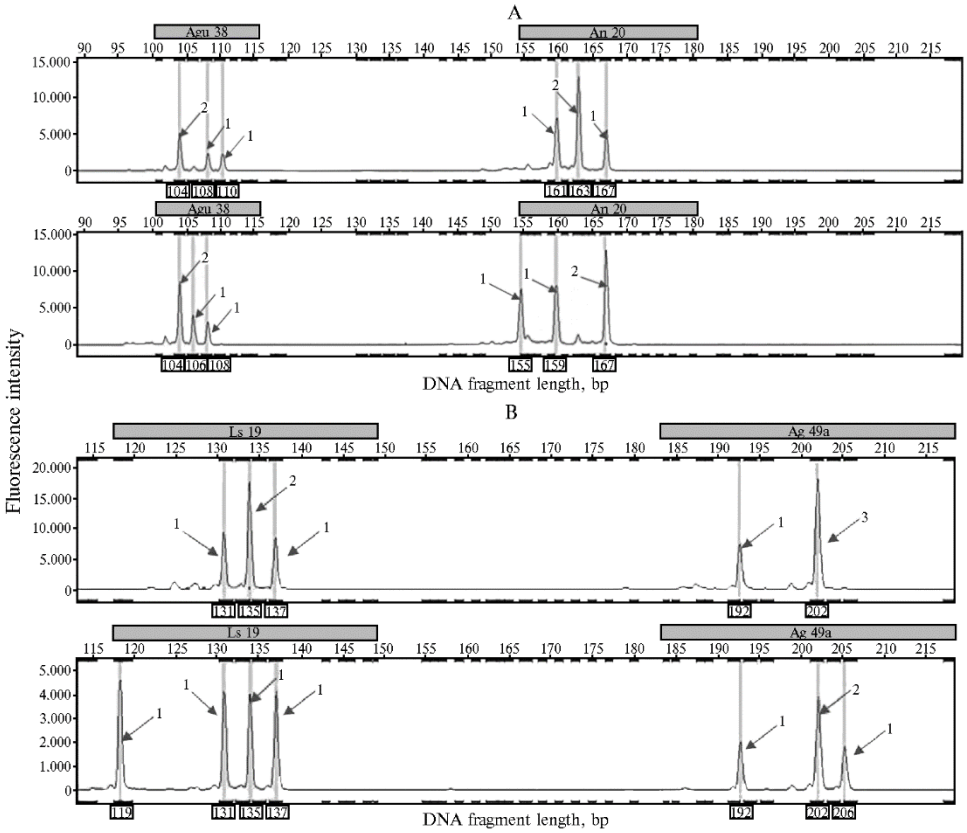

Fig. 4. An example of multiplexing loci with preservation of the allele dose effect for individuals of the domesticated Siberian sturgeon (*Acipenser baerii* Brandt, 1869) of the Lena population: A — panel 1 (loci Agu 38, An 20; FAM channel), B — panel 2 (loci Ls 19, Ag 49a; FAM channel). The arrows indicate the peaks corresponding to the alleles, 1, 2 and 3 indicate the allele doses of the (Ernst Federal Research Center for Animal Husbandry — VIZh, 2023).

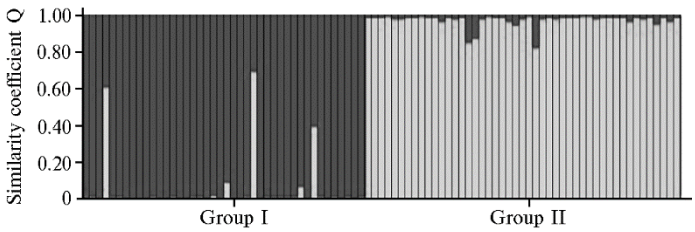

Рис. 5. Cluster analysis of the domesticated Siberian sturgeon (*Acipenser baerii* Brandt, 1869) individuals of the Lena population based of allele frequencies of seven microsatellite loci: group I — fish from the Mozhaisk production and experimental fish hatchery, Goretovo village, Mozhaisky urban District, Moscow Procine, group II — fish obtained from RTF Diana LLC (working village of Kaduy, Kaduysky District, Vologda Province). The STRUCTURE program for the number of clusters K = 2 (Ernst Federal Research Center for Animal Husbandry — VIZh, 2023).

Considering the obtained values of population genetic indicators, it can be argued that the analyzed groups of Siberian sturgeon from the Lena population are not inbred. Taking into account the relatively lower values of Na, Ne, H_o and H_e, we can conclude that there is less genetic diversity of group I from the Mozhaisk production and experimental fish hatchery. This effect could result from

the fish hatchery ecomovic model targereted to the release of juvenile sturgeon to replenish natural populations and more stringent control for the producers' origin.

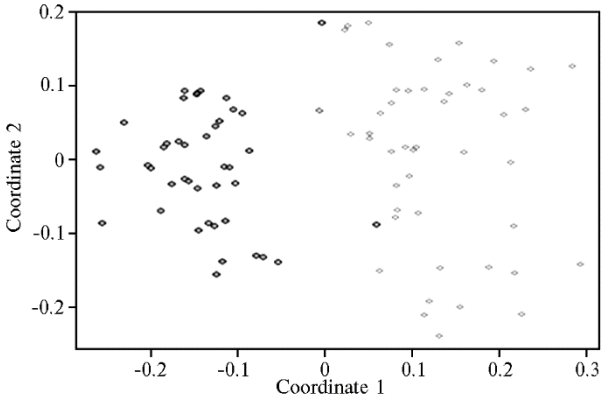

Fig. 6. Principal component analysis of the domesticated Siberian sturgeon (*Acipenser baerii* Brandt, 1869) individuals of the Lena population based on genotyping for 7 microsatellite markers. Group I **(black color)** — fish from the Mozhaisk production and experimental fish hatchery, Goretovo village, Mozhaisky urban District, Moscow Procine, group II **(gray color)** — fish obtained from RTF Diana LLC (working village of Kaduy, Kaduysky District, Vologda Province). PCA plot is built based on the calculation of genetic distances accridng to R. Bruvo et al. [21] (Ernst Federal Research Center for Animal Husbandry — VIZh, 2023).

Since the allele dose effect for each microsatellite locus was accounted, we compared the resolution of microsatellite analysis when ignoring data on this effect and when taking it into account. When accounting for the allele dose effect, the number of genotypes increased on average by 31.75% for each locus (Table 3).

3. Number of the Lena population Siberian sturgeon (*Acipenser baerii* Brandt, 1869) genotypes as influenced by allele dose effects for 7 microsatellite loci (Ernst Federal Research Center for Animal Husbandry — VIZh, 2023)

Accounting for unique genotypes	Locus							
	Agu 38	An 20	Aru 18	Ls 19	Ag 49a	Agu 37	Agu 41	∑
Without allile dose	11	34	5	11	21	5	39	126
With allile dose	19	48	6	14	27	6	46	166

Unfortunately, it should be recognized that modern software for calculating population genetic indicators is poorly adapted to process microsatellite locus allele dose data. Thus, to convert the data file with genotyping results into the STRUCTURE and SPAGeDi program formats, we used the Polysat R package, specially designed for the analysis of polyploid genotypes. In this case, data on allele dose were removed. The decrease in the quality of the analysis, however, did not lead to critical errors in the calculation of population statistics. However, when comparing the genetic profiles of parents and offspring, this can have a significant impact and lead to the need of a larger number of microsatellite markers involved in the analysis. The situation will be especially noticeable for herds where there is a large proportion of siblings and half-siblings among the sires.

Microsatellite polymorphism of the wild Lena population of Siberian sturgeon has been studied in sufficient detail [10, 14]. Many researchers have investigated the genetic structure of aquaculture stocks of this species, but among similar works we can highlight the report of A.E. Barmintseva et al. [32] who compared the polymorphisms for five microsatellite loci in Siberian sturgeon stocks from the Lena population at nine farms. As in our study, the authors identified two clear genetic clusters, but their formation significantly differed. The authors note an unusual effect that the year of birth had a decisive influence on whether a particular

individual belonged to a cluster. Regardless of their belonging to the farm, individuals of the 1990-1996 generations grouped in a single cluster with wild representatives of the Lena population, and the aquaculture cluster was formed from individuals starting from 2001. In our study, this effect was not repeated. We obtained a clear genetic differentiation of individuals born in 2022 solely depending on their origin. The Siberian sturgeon of the Lena population was domesticated in the recent past (1993) [33]. In the work of A.E. Barmintseva et al. [32] testing was carried out on samples of the generations 1990-2008. If we take into account the rather significant time period as compared to the time of domestication (2008-2022), then a quite likely explanation may be the gradual accumulation of differences in allele frequencies in current broodstocks of the Siberian sturgeon of the Lena population at different farms due to ongoing selection. In addition, the detection of genetic differentiation could be influenced by an increase in the number of microsatellite markers used. However, these assumptions need to be tested on other samples.

Thus, multiplex panels of seven microsatellite loci that we have developed allows us to obtain for each individual Siberian sturgeon its unique genotype even without accounting the dose of each allele. This gives the opportunity to create individual genetic passports. A clear genetic differentiation between the groups of Siberian sturgeon from two different enterprises reflects the influence of the selection carried out. Such genetically differentiated herds may be useful in creation of new breeds and lines. Before releasing juvenile Siberian sturgeon into natural populations, it is advisable to compare the genetic profiles of wild and released fish to control and preserve the genetic structure of native populations. The development of software for processing data that include doses of alleles in polyploid loci is very urgent. Such a tool will significantly improve genetic analysis of polyploid species.

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