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ANIMAL GENETICS

Intraspecific Genetic Polymorphism of Russian Sturgeon *Acipenser gueldenstaedtii*

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Abstract—Three populations (Azov, Caspian, and Black Sea) of Russian sturgeon *Acipenser queldenstaedtii* were tested for polymorphism at nuclear (RAPD and microsatellites) and mitochondrial (PCR identification of two mitotypes) markers. In addition, morphometric analysis of he representatives of Azov population was carried out. According to the morphological characters, the Black Sea population occupied an intermediate position between the Caspian and Azov populations, reflecting the phylogeography of this species. In agreement with the morphometric data, genetic distances (the data of STR analysis) also placed the Black Sea population between the Caspian and Azov populations (F_{ST} = 0.058 and 0.043). The genetic distance between the Azov and Caspian population was somewhat higher (F_{ST} = 0.070). The highest allelic polymorphism at four microsatellite loci was found observed in Caspian population, while the lowest polymorphism was in the Sea of Azov. RAPD analysis distinguished high polymorphism within the populations, although it was not feasible for interpopulation analysis. Using the method differentiating the "baerii-like" and typical "gueldenstaedtii" mitotypes, the absence of the "baerii-like" marker in the Black Sea population was demonstrated. The frequency of this marker in Caspian and Azov populations constituted 31.1 and 1.8%, respectively. Possible evolutionary reasons for the interpopulation differences observed are discussed.

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INTRODUCTION

Russian sturgeon *Acipenser gueldenstaedtii* Brandt, 1833 is an anadromous species, which belongs to the ancient group of cartilaginous ganoid fishes. This speciesis among the most valuable, commercially fishes. Until recently, it was widely distributed in the internal seas of Eurasia, but at present, due to overfishing and habitat destruction, Russian sturgeon acquired the status of rare species [1]. Conservation of Russian sturgeon and maintenance of its optimal population number implies reconstruction of genetic diversity with special emphasis on the species population structure.

Earlier studies demonstrated wide variability of morphological characters in Russian sturgeon [2, 3], along with high polymorphism of serum proteins [4] and mitochondrial DNA [5, 6]. Furthermore, based on behavioral, ecological, and meristic characters, some authors subdivided the species into three or four geographically isolated subspecies with several spawning populations in each [2, 3]. However, in other studies only populations are distinguished within the species [1]. In addition, identification of individual fishes in the absence of distinct phenes, specific to intraspecific groups, requires testing of large samples.

According to modern views, polyploidy was the main speciation mechanism within the order Acipenseriformes. Karyotypes of sturgeon fishes contain the chromosome sets ranging from 4*n* to 16*n* [7] (according to other interpretations, from 2*n* to 8*n* [8, 9], at most represented by microchromosomes [8]. High genome ploidy level makes difficult identification of individual chromosomes, as well as the elaboration of molecular markers for nuclear DNA. Polymorphism of some sturgeon species was evaluated using the AFLP and STR loci [10–13]. Population structure and polymorphism of *Acipenser queldenstaedtii* is examined to a lesser extent.

The present study was focused on comparative analysis of interpopulation variation of Russian sturgeon using standard morphometry, as well as polymerase chain reaction (PCR) with random primers (RAPD PCR), analysis of microsatellites (STR), and PCR identification of two mitochondrial haplotypes with a set of species-specific primers.

MATERIALS AND METHODS

Russian sturgeon *Acipenser gueldenstaedtii* Brandt, 1833 from three populations (Azov, Caspian, and Black

Table 1. Characteristic of microsatellite loci used in the STR analysis of Russian sturgeon

Locus	Allele size, bp	$MgCl_2$, mM	Range of the annealing temperature reduction at stage I (no. of cycles)	Annealing temperature at stage II (no. of cycles)
Afug41	265–173	2.2	63–58°C (10)	58°C (28)
Afug51	278–188	2.0	–	58°C (35)
An20	187–129	2.0	58–54.5°C (7)	54°C (28)
AoxD165	208–140	2.0	63–58°C (10)	58°C (28)

Table 2. Comparative characteristic of the populations of *A. gueldenstadtii* at morphological characters

Character		Populations		
		Caspian	Azov	Black Sea
Number of rays in dorsal fin (D)	Mean value	41.0	35.7	**36.3**
	limits	33–51	27–41	**30–43**
Number of rays in anal fin (A)	Mean value	25.8	21.9	**23.9**
	limits	21–33	18–25	**20–28**
Number of gill rakers (GR)	Mean value	23.5	21.5	**21.8**
	limits	19–29	16–26	**17–27**
Number of dorsal scutes (DS)	Mean value	12.1	**11.9**	12.0
	limits	9–18	8–15	9–15
Number of lateral scutes (LS)	Mean value	39.0	30.5	**34.2**
	limits	30–50	25–36	27–38
Number of ventral scutes (VS)	Mean value	9.8	9.6	9.6
	limits	7–12	8–11	7–12
C/AL, %		18.1	16.4	17.5
AL (16 years), cm	Mean value	**121.6**	129.8	117.7
	limits	–	115–166	–

Note: The intermediate values upon comparison of the three populations are in bold type. The sample sizes of Caspian and Black Sea populations varied from character to character and are presented in accordance to [3].

Sea) was examined. The fish were caught in 1999 through 2005 in northwestern part of the Black Sea (86 fish), in the Sea of Azov (92 fish), and in the northern part of the Caspian Sea (360 fish). The cuts of the first ray of pectoral fin from ichthyological collection of the Azov Research Institute of Fisheries (AzNIIRKH) gathered in the Sea of Azov during 1960 to 1971.

Morphometric analysis of sturgeons from the Sea of Azov was carried out using the traits generally accepted in sturgeon systematic: number of rays in dorsal fin (D), number of rays in anal fin (A), number of gill rakers (GR), number of dorsal scutes (DS), number of lateral scutes (LS), number of ventral scutes (VS), head length (C), and absolute body length (AL) [1]. For comparative analysis, the same morphometric characters of sturgeons from Caspian and Black Sea populations were taken from [2, 3].

Total DNA was isolated from the fin fragments and cuts using the method of salt extraction [14] with our modifications for the fin ray cuts, collected by the ichthyologists from AzNIIRKH for a long [period of time for registration of the population age composition. The samples of cuts were incubated in 50 mM EDTA for 24 h at room temperature. Then the samples were dried, homogenized, and placed in lysing buffer containing proteinase K (0.05 mg/ml). Lysis was carried out for 18 h at 56°C in temperature-controlled shaker. Further isolation steps were the same as in the procedure described in [14]. A total of 345 fin ray cuts were examined, and from 265 of them it was possible to isolate DNA feasible for PCR diagnostics. DNA samples from the remaining cuts were degraded due to long period of storage in increased humidity.

The DNA samples were used for RAPD analysis (by 50 fishes from each population), STR analysis (from 80 to 360 fishes in each sample, see Table 5), and identification of "baerii-like" (BL) and "gueldenstaedtii" (GUE) mitotypes (sample sizes are listed in Table 7).

Table 3. Variability of RAPD loci in Russian sturgeon

Primer	Primer sequence 5'–3'	No. of fragments examined	No. of polymorphic fragments	No. of fragments per individual	
				mean value	limits
OPA-05	AGG-GGT-CTT-G	47	47	23.2	17–31
OPA-07	GAA-ACG-GGT-G	66	62	30.5	24–41
B-3	CAT-CCC-CCT-G	62	60	24.6	17–34
B-11	GTA-GAC-CCG-T	51	47	23.9	20–28
P-8	CCT-GAC-CAG-GCA-CTG-GCA-GA	69	66	29.2	19–38
Total	–	295	282	–	–

Table 4. Indices of variation over RAPD loci in the groups of Russian sturgeon examined obtained with the help of five primers

Population	Mean value q_j^i	t test ($P < 0.001$)	Polymorphism level P, %	Mean heterozygosity value H_j^i	Variance $Var\,(H_j^i)$
Azov	0.813	5.809	92.3	0.379	0.060
Caspian	0.799		93.6	0.396	0.059

RAPD. In preliminary experiments, 30 random primers differing in size and GC content were tested. Five of these primers (Table 3) producing distinct, stably reproducible polymorphic profiles were selected for further analysis (Fig. 1). PCR was carried out in standard conditions [15] using the Tetrad PTC-225 (MJ Research, United States) thermal cycler. Amplification products were fractionated in 6% polyacrylamide gel (PAAG).

Using the Phoretix 1D Database software program (Nonlinear Dynamics, United Kingdom), RAPD profiles were presented in the form of general binary

Table 5. Indices of interpopulation variation of Russian sturgeon over microsatellite loci

Locus	N	No. of alleles	Mean no. of alleles	Heterozygosity, observed, H_O	Heterozygosity, expected, H_E	Intrasample coefficient, F_{IS}	Coefficient for the total sample, F_{IT}
Azov population							
An20	86	11	2.55	0.940	0.969	0.033	0.042
Afug41	92	15	3.1	0.986	0.998	0.010	0.012
Afug51	81	13	2.4	0.730	0.999	0.267	0.267
AoxD165	80	10	2.6	0.911	0.957	0.035	0.072
Mean value						0.096	0.099
Caspian population							
An20	379	13	2.78	0.971	0.971	–0.000	0.011
Afug41	378	23	3.35	0.995	0.999	0.005	0.004
Afug51	380	18	2.78	0.837	0.998	0.155	0.160
AoxD165	373	16	2.85	0.962	0.992	0.027	0.020
Mean value						0.046	0.049
Black Sea population							
An20	85	14	2.53	0.871	0.944	0.068	0.113
Afug41	78	15	2.50	0.971	0.997	0.025	0.028
Afug51	86	20	2.44	0.885	0.998	0.108	0.113
AoxD165	82	9	2.12	0.796	0.934	0.080	0.193
Mean value						0.109	0.111

Table 6. Matrix of the F_{ST} values between the populations of Russian sturgeon

	Caspian	Azov	Black Sea
Caspian	–		
Azov	0.070	–	
Black Sea	0.058	0.043	–

matrix of the object–character type. The program was also used for calculation of the coefficient of cross-correlation of individual RAPD profiles, and for cluster analysis.

Analysis of microsatellite DNA (STR) was performed using four microsatellite loci, An20, Afug41, Afug51, and AoxD165, initially elaborated for *A. naccarii* [16], *A. fulvescens* [17], and *A. oxyrinchus* [11]. PCR conditions optimized for the species of interest are listed in Table 1.

Based on the band electrophoretic mobility, allele numbers and their sizes were determined for each microsatellite locus.

Statistical analysis. Taking into consideration the dominant nature of RAPD loci and tetraploidy of the species examined, the frequency of the recessive null allele, q_j^i, unbiased heterozygosity estimate, H_j^i, and its variance, $Var(H_j^i)$ were calculated as described in [19, 20].

Microsatellite loci allele frequency, p_i, was calculasted using two methods. Tetraploid species, like Russian sturgeon, are characterized by the presence of up to four alleles of a single locus in an individual fish. In case that electrophoregram contained all four bands of different size (heterozygote for all four alleles0, or there was only one band (homozygote), interpretation of allele composition presented no difficulties. In case of the presence of two to three bands, no unambiguous evaluation of the allele composition can be made, since it is not always clear, which particular band is represented by a number of copies of one allele. In most of the cases, determination of individual allele composition was possible taking into consideration inequality of band intensity upon the so-called dose effect. Simultaneously, we used a method of allele frequency estimation that was based on assuming Hardy--Weinberg equilibrium and did not take into account the intensity of amplified bands (minus=allele method). The allele frequencies were calculated as follows: $P_i = 1 - (N/N_j)^{1/4}$, where N is the number of individuals with genotypes lacking allele i and N_j is the number of tetraploid individuals in sample j. In our earlier studies it was demonstrated that computation of the haplotype frequencies with the help of traditional method, taking into consideration the dose effect, and the minus allele method, gave similar results. In a series of experiments on artificial mating the distribution of tetraploid alleles in the progeny was characterized as Mendelian, while null alleles were absent (data not shown). The unbiased intragroup heterozygosity H_E value was calculated according to the formula:

$$H_E = (4N/(4N-1))(1-\sum p_i^4).$$

To test whether microsatellite analysis was feasible for attribution of each individual fish to certain population, the assignment test was performed. Each fish was tested for belonging to a certain population based on the observed and expected allele frequency distributions estimated using the maximum likelihood approach as described in [21].

Wright's F_{ST} statistics and Nei's genetic distance D [22] was calculated as described in [19] and using the ARLEQUIN software program.

Table 7. Number of individuals with "baerii-like" and species-specific (GUE) mitotypes in populations of Russian sturgeon

Population	No. of individuals tested	No. of individuals with species-specific (GUE) mitotype		No. of individuals with "baerii-like" (BL) mitotype	
		absolute	%	absolute	%
Caspian	527	362	68.69	165	31.31
Azov (generations of 1931 to 1956)	102	102	100.00	0	0.00
Azov (contemporary, generations of 1980 to 2005)	759	745	98.12	14	1.88
Black Sea (generations of 1945 to 1970)	163	163	100.0	0	0.00
Black Sea (contemporary, generations of 1975 to 2000)	86	86	100.00	0	0.00

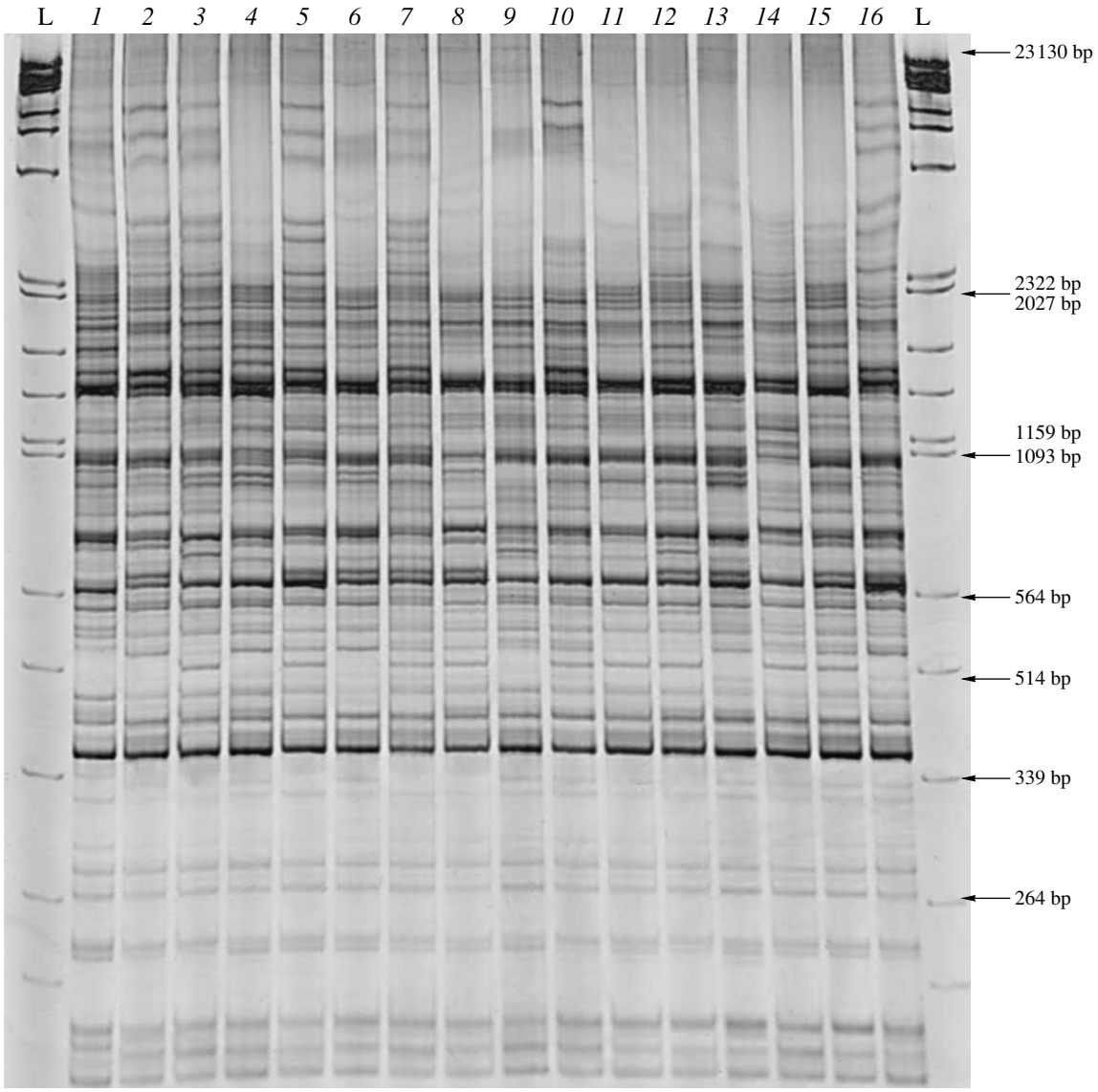

Fig. 1. RAPD profiles of Russian sturgeon obtained with the help of primer OPA-07. *1* to *8*, individuals from Azov population; *9* to *16*, individuals from Caspian population; L, molecular size marker, DNA λ/*Pst*I *Hind*III (Fermentas).

Statistical significance of the differences between the population-averaged values was estimated using Fisher's *F*, *t*, and χ^2 tests [19, 23].

Mitochondrial DNA markers, "baerii-like" (BL) and "gueldenstaedtii" (GUE) mitotypes were determined using the method of species identification of sturgeon fishes described in [24].

RESULTS

Morphological Polymorphism of the Populations of Russian Sturgeon

The results of morphometric analysis by eight criteria are demonstrated in Table 2. The prevailing in number Caspian group was characterized by wider variation range at all characters examined. Azov population was characterized by the lowest value of the *C/AL* ratio along with more intense individual growth rate. The values of most morphometric indices showed that population of the Black Sea occupied an intermediate position relative to the Caspian and the Azov samples.

Comparative interpopulation evaluation of the averaged values performed with the help of Fisher's *F* test revealed substantial differences only in the individual growth rates ($p < 0.05$). Thus, the distribution pattern of the values of morphometric indices, characterizing Russian sturgeons, requires analysis of the large samples, and in practical work, makes difficult the population identification of the fish.

Interpopulation Differences of Russian Sturgeon over RAPD Markers

Characteristics of RAPD profiles obtained for two spawning populations (Caspian and Azov) of Russian sturgeon are presented in Table 3. The sizes of the fragments analyzed varied from 250 to 1000 bp (Fig. 1). The total number of RAPD loci identified by five primers used constituted 295 fragments with 282 of these, which were informative.

According to the data from Table 4, the samples compared had high polymorphism levels. However, cluster analysis (UPGMA) based on the RAPD profiles, revealed no interpopulation differences. The fish formed separate clusters irrespective of their ranges. At the background of high interpopulation polymorphism values (H_j^i, P), the level of interpopultion differentiation was found to be statistically no insignificant (F_{ST} = 0.011).

Interpopulation Differences of Russian Sturgeon over STR Loci

The results of microsatellite analysis of three populations of Russian sturgeon at four loci are presented in Table 5 and 6, and in Fig. 2.

In the sturgeons examined (n = 540), a total of 58 alleles were identified. The averaged number of alleles per locus varied from 11.5 (AoxD165) to 17.5 (Afug51). Each of the populations examined had specific set of alleles with clearly different frequencies ($p < 0.01$). The exclusion was locus AoxD165, for which no differences between the Caspian and the Azov populations were identified. It should be noted that alleles, typical of individual population or basin, could not serve as population markers because of low p_i values ($P < 0.01$).

The values of observed heterozygosity (H_O) over microsatellite loci were close to the expected (H_E) values (Table 5). The exclusion was locus Afug51, for which the deficit of heterozygotes was observed in all groups examined ($H_O < H_E$). The highest allelic diversity over all loci was observed in the Caspian sample. Since in this population, the mean F_{IS} values were close to 0, its state corresponded to the conditions of Hardy–Weinberg equilibrium. On the contrary, the Azov and the Black Sea populations demonstrated a deficit of heterozygotes (F_{IS} = 0.096 and 0.106, respectively).

Table 6 demonstrates pairwise genetic distance values (F_{ST} ststistics) averaged over four loci, for the analysis of Russian sturgeon populations. High variability within the populations along with the tetraploid nature of the loci determined low interpopulation variation (1.9%). Nevertheless, the F_{ST} values, estimated for all group pairs examined were statistically significant.

To evaluate the effectiveness of individual identification of Russian sturgeon with the help of STR technique over four loci in the probability test [21], the allele frequency database identified for each of the populations, was used. Maximum identification accuracy was obtained for the Black Sea population, 94%. For the Caspian population, the value of this index was 86%, and only 64%, for the Azov population. The precision level was sufficient for population identification of the fish from the Black Sea and the aspian Sea. Apparently, additional analysis of other microsatellite loci is required for typing of the Azov population.

Identification of the BL and GUE Mitotypes

In earlier studies, the presence of the so-called BL mitotype ("baerii-like") in 30% of the fishes from Caspian Sea was repeatedly reported. This mitotype was clearly different from the other species-specific mitotypes [24, 25], and characterized by high homology to mtDNA of Siberian sturgeon (*A. baerii*).

Table 7 presents the data on identification of two mtDNA markers, the BL mitotype and Russian sturgeon-specific mitotype GUE, in the samples examined.

In the Black Sea sample, which comprised 249 fish, complete absence of mitotype BL was observed. The frequency of BL individuals in the Caspian population was close to the expected one and constituted 0.313. In the Azov population, two groups were distinguished. In one of the samples comprised of the fish from generations of 1970 to 2005, among 759 individuals, 14 BL-carrying fish were detected. In the second group, represented by 102 fish from completely substituted generation of 1931 to 1956, BL marker was not detected.

DISCUSSION

Russian sturgeon is an endemic of the Ponto-Caspian pool. It belongs to ancient, relict fish group known from the Upper Jurassic period (more than 240 Myr ago).

Literature [2, 3] and our data point to high plasticity and variability of the main morphometric characteristics of Russian sturgeon. Because of this, it is problematic to use these characters as biological markers for species population differentiation (Table 3).

RAPD analysis of genomic DNA revealed a high variation (Table 3 and 4) along with the absence of the differences between the geographically isolated populations. Polyploid origin of *A. gueldenstaedtii* generally determines the increased heterozygosity of the species, as well as the high number of polymorphic loci, and the number of alleles per locus [26].

In our preliminary experiments, tetrasomic inheritance of microsatellite loci of Russian sturgeon was demonstrated, which was typical of tetraploids. Species with increased ploidy are characterized by low population divergence due to weak selection and gene drift, caused by a low number of homozygotes [27]. Nevertheless, the use of STR succeed in demonstrating low albeit statistically significant interpopulation differ-

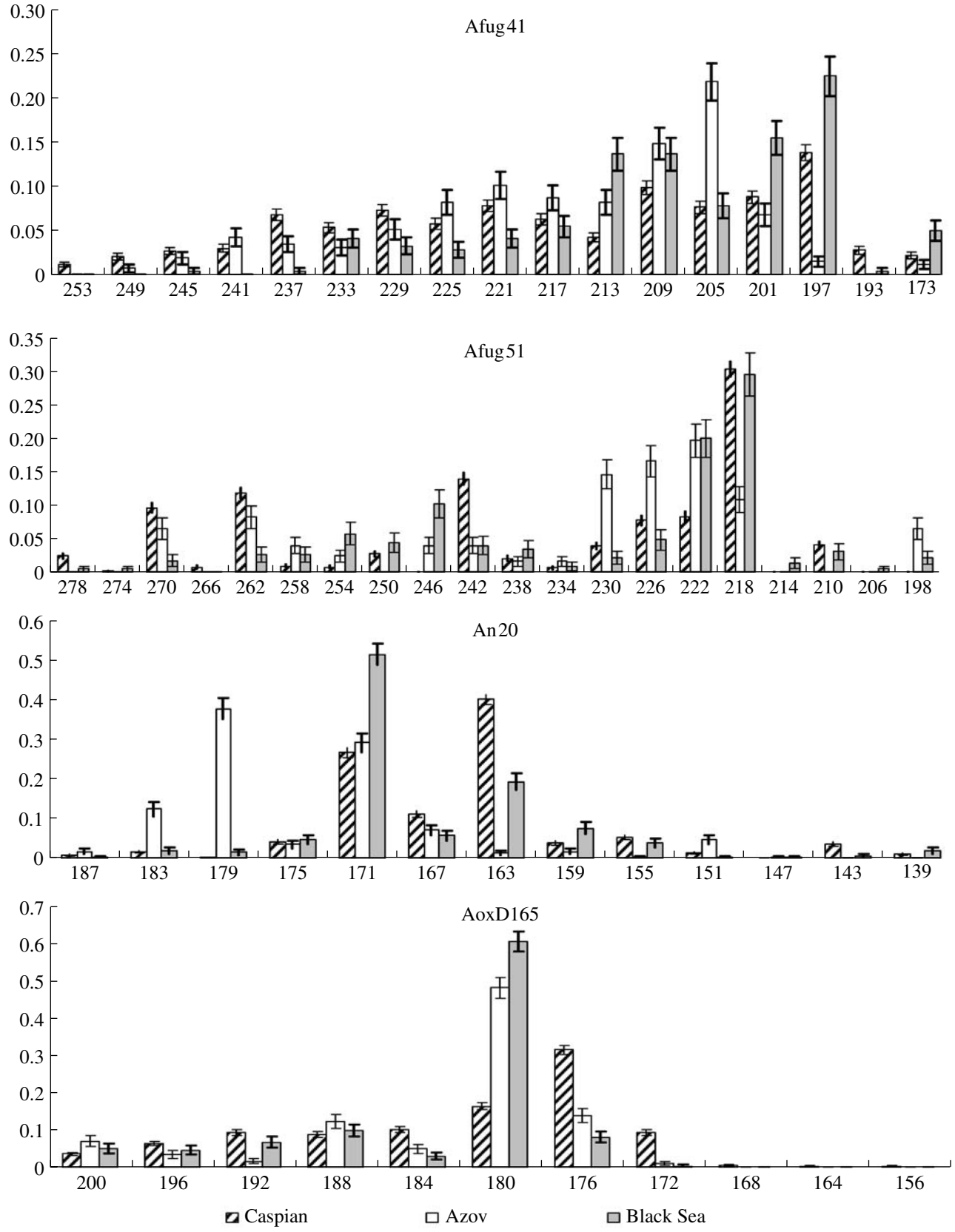

Fig. 2. Microsatellite allele frequency distributions in different populations of Russian sturgeon. On abscissa, allele sizes, bp; on ordinate, allele frequencies in parts.

ences of Russian sturgeon over four microsatellite loci (Table 6). It should be noted that the highest values of genetic distances were observed between the Caspian and the Azov populations, while the Black Sea population occupied an intermediate position.

In the Black Sea and the Azov populations, STR analysis revealed a deficit of heterozygotes (Table 5). In the Black Sea population this phenomenon can be explained by the Wahlund effect [19], since this population includes two spawning subpopulations, Dnieper and Danube. Since the samples were collected in the sea, identification of individual fish by descent was impossible. It seems likely that deficit of heterozygotes in Azov population was associated with a dramatic decrease of its number during the last decade, as well as by inbreeding, as a result of the limited number of the producers upon artificial breeding [28]. On the other hand, in the tetraploid genome, the deficit of heterozygotes can be explained in terms of asymmetric structure of homologous chromosome quadruplets during the first meiotic division, associated with the amphiploid (allotetraploid) nature of multichromosomal sturgeon species.

The history of sturgeon dispersal is strongly associated with the geologic history of large Eurasian water basins. At present, Russian sturgeon inhabits the seas with different ecological features, and the sturgeon intraspecific dedifferentiation is associated with the evolution of the three seas. It is known that during Late Pleistocene, when the connections with the Mediterranean Sea were lost due to the water-level fall, in Azov–Black Sea basin there was desalinated body of water (15 to 40 thousand years ago) [29]. Furthermore, during that period, the Sea of Azov was disappearing, converting into the lowland valley, crossed by the Don River. The absence of mitotype BL in the Black Sea population suggests insignificance, or absence of the Russian sturgeon migrations from the Caspian Sea into Black Sea, along the Kumo–Manych spillway, which shortly existed at the end of the Ice Age. The following Black Sea transgression, caused by inrush of the Mediterranean salt waters through the Bosporus, and the reappearance of the Sea of Azov due to a water rise, provided the formation of the Sea of Azov population of Russian sturgeon from the part of the Black Sea population. This situation fixed the isolation of the Russian sturgeon populations in the northwestern part of the Black Sea and the Sea of Azov. It can be thus suggested that the Black Sea population descended from the Caspian one. Azov population, in turn, originated from the Black Sea population, which explained the intermediate position of the latter population, on the basis of morphometric indices and microsatellite analysis. The distribution of mitochondrial BL marker (Table 7) in contemporary populations along with the absence of this marker in the Black Sea suggest that the appearance of sturgeons with BL mitotype in the Sea of Azov occurred in the 20th century and was associated with the introduction of Caspian sturgeons into the Sea of Azov [28]. This suggestion is supported by the absence of the marker in the sample of Azov sturgeons until the 1960s (before systematic introduction of sturgeon from the Caspian Sea, the fin cut data).

Thus, the history of the dispersal of Russian sturgeon and isolation of its local populations, as a result of geological rearrangements of inner Eurasian water bodies, had an effect on its genetic structure. The appearance of the mtDNA BL marker in the Sea of Azov points to the changes in the formed population structure and gene diversity in the history of Russian sturgeon. These changes are actively introduced by humans via artificial reproduction and intense fishery. The use of microsatellite and mitochondrial DNA markers investigated in the present study makes it possible to determine the population affiliation of Russian sturgeon with high degree of accuracy.

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