

# Analysis of Genetic Diversity in Beluga Sturgeon, *Huso huso* (Linnaeus, 1758) from the Lower Danube River using DNA Markers

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## Abstract

The beluga sturgeon (*Huso huso*) represents an anadromous species native to the Ponto-Caspian region, which has been severely affected by anthropic intervention. To take appropriate conservation measures for the wild population of *Huso huso* from the North-Western of the Black Sea that is reproducing in the Lower Danube is necessary to infer the genetic diversity and the structure of population by using DNA markers. The current study represents a preliminary attempt to evaluate the genetic diversity of beluga population by using microsatellites markers. Seven microsatellite loci (LS19, LS34, LS39, LS54, AoxD234, AnacE4, AnacC11) were analyzed and the obtained genotypic data were used to calculate the estimated heterozygosity ( $H_E$ ) and observed heterozygosity ( $H_O$ ) with Genetix Software. The Hardy-Weinberg was tested using GenePop Software. The average value of  $H_O$  is  $0.4760 \pm 0.2989$ , while the  $H_E$  is  $0.6049 \pm 0.2843$ . With the exception of LS19 and LS39, the other loci showed departures from Hardy-Weinberg equilibrium. The deviation from the expected values might be the consequence of reduced population, inbreeding or the presence of null alleles that might cause a false excess of homozygous. Our preliminary results pointed out reduced level of genetic variation and might be first indication about the fragile status of this population.

**Keywords:** beluga sturgeon, genetic diversity, Lower Danube River, microsatellites.

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## 1. Introduction

Sturgeons represent extremely important fish species. This group is among the oldest group of fishes, which appeared 200 millions year ago and constitutes a model of vertebrate evolution [1]. Apart of their scientific value the sturgeons have a remarkable socio-economical importance since they are the caviar producers, one of the most expensive gourmet delicacies in the world. The majority of sturgeon species are anadromous, migrating from the sea, where the feeding cycle of

their life is taking place to fresh water environment for breeding [2]. After reproduction, the adults and their progeny are returning to the sea. This type of life cycle made sturgeons more susceptible to different anthropic factors like overfishing, poaching, pollution, damage of the reproduction habitats, construction of dams that are obstruct the accession of adults to the breeding sites in the river. All these threats severely affected the sturgeon population all over the world and this group of fish is at the brink of its extinction. Nowadays, the greatest diversity of sturgeon species is found in the Ponto-Caspian region.

The beluga sturgeon, *Huso huso* is an anadromous species that is considered to be "Critically Endangered" according to IUCN Red List.

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The current native wild distribution of this species is restricted to the Black Sea (in the Danube River only) and the Caspian Sea (in the Ural River and Volga River). The species is found in the Azov Sea also, but these are stocked fish [3].

Males reproduce for the first time at 10-15 years, females at 15-18 years, at a body size of two meters, with an estimated generation length of 20-25 years [4]. These biological aspects make beluga sturgeon populations particularly vulnerable to anthropic factors and difficult to monitoring and study. To take appropriate conservation measures for the wild population of *Huso huso* from the North Western of the Black Sea that is reproducing in the Lower Danube is absolutely necessary to infer the genetic diversity and the structure of population by using DNA markers.

Microsatellites, also named VNTR-Variable Number of Tandem Repeat are short repetitive sequences of 2-9 bp, heterogeneous distributed in the genome and possessing a high degree of hypervariability within species and populations [5]. Due to their characteristics like the co-dominant inheritance and the significant level of polymorphism, these molecular markers are recommended to assess the genetic differences on individual and population level and appear to be very suitable to investigate the genetic diversity within species, to reconstruct pedigrees, and to compare wild and captive populations [6].

The present study represents a preliminary attempt to evaluate the genetic diversity of beluga sturgeon population from Lower Danube by using microsatellites markers.

## 2. Materials and methods

### Sample collection and DNA extraction

Samples representing small fin of fragments were collected from 33 *Huso huso* individuals from Lower Danube River between 2001 and 2007. The collected samples belong to three different groups (Spring 2001- 11 individuals, Spring 2004 – 12 individuals and Spring 2007 – 10 individuals), classified according to the collection date. Genomic DNA was isolated using a standrad phenol/ chloroform/ isoamyl alcohol protocol. The quality and concentration of DNA was determined by using a spectrophotometer.

### Microsatellites amplification and genotyping

In this study we used seven pairs of primers to amplify microsatellite loci with di-, tri- and tetranucleotide repetitive motifs: LS19, LS34, LS39, LS54, AoxD234, AnacE4, and AnacC11.

Initially a PCR reaction in temperature gradient was performed in order to establish the optimum annealing temperature for each set of primers. Based on the obtained results the loci LS19, LS34, LS54 were selected to be amplified in a multiplex PCR reaction, while the other four were amplified by monoplex reactions.

PCR were carried out using fluorescent labeled forward primers. Amplifications were performed in a GenAmp PCR System 9700 (*Applied Biosystems*) thermocycler with a 25 µL reaction volume containing 30-50 ng of DNA template, 1X PCR Buffer, 1.5 mM of MgCl<sub>2</sub>, 0.4 µM of each primer, 0.8 mM of each dNTP and 0.5U of AmpliTaq Gold Polymerase (*Applied Biosystems*). The thermal cycling conditions were set as follows: initial denaturation (95°C, 10min); 30 cycles of denaturation (95°C, 30s), annealing (specific annealing temperature, 30s) and extension (1min, 72°C), and a final extension (72°C, 60min). The amplifications conditions are described in table 1. The products PCR were loaded with the GeneScan-500 LIZ Size Standard into ABIPrism 310 DNA Genetic Analyzer and the results were visualised with the GeneScan 3.1.2 software. The alleles were scored with Genotyper 2.5.2 Software (*Applied Biosystems*).

**Table 1.** Primer sequences and annealing temperature for seven loci analyzed in this study.

Locus	Primer sequence (5' → 3')	Annealing temp. (°C)
LS19	F: 6-FAMcattcttagccgtctgtgtac R: caggtcctaataacaatggc	55
LS34	F: VICtatacattcttcaacg R: gatcccttctgtatcaac	55
LS39	F: PETtctgaagttcacacattg R: atggagcattattggaagg	55
LS54	F: NEDctctagtctttgtgattacag R: caaaggactgaaactagg	55
Aox D234	F: 6-FAMaactggctttgtgattgatcc R: tgaagcaaagggtattatttgag	52
Anac C11	F: VICaattccattgggggtgt R: cttcgtttgagaacccg	50
Anac E4	F: 6-FAMtcagctacagggttctggg R: gttgtactcattggaactc	55

**Statistical analysis**

The estimation of genetic diversity within *Huso huso* population was completed by the calculation of estimated heterozygosity ( $H_E$ ) and observed heterozygosity ( $H_O$ ) using Genetix v4.5 Software [7]. The Hardy-Weinberg was tested using GenePop v1.2 Software [8]. The genetic differentiation between the three groups based on F-statistics and determination of statistical indices of differentiation ( $F_{st}$ ,  $F_{is}$ ,  $F_{it}$ ) were computed using FSTAT software v. 2.9.3.2 [9].

The phylogenetic relationship among the analyzed groups of beluga was inferred by Neighbor Joining method implemented in PHYLIP 3.67 software [10]. The phylogenetic tree was visualized with TreeView [11].

**3. Results and discussion**

In this study, we have successfully amplified all seven microsatellite loci for all the individuals of *Huso huso*. All the analyzed loci presented a disomic pattern and we identified a total number of 44 alleles in the population, while the number of alleles per locus ranged from 1 to 11.

The most polymorphic locus was AoxD234 with 11 alleles, while LS34 exhibited one fixed allele. Thus, a unique allele was identified on the studied loci. For the other five loci we identified an intermediate number of alleles: 10 for LS54, 9 for LS19, 6 for AnacE4, 4 for LS39 and 3 for AnacC11. The characteristics of loci in terms of number and size of alleles are presented in table 2.

**Table 2.** Characteristics of the analyzed loci in *Huso huso* population from Lower Danube.

Locus	Size (bp)	Number of alleles
LS19	113-163	9
LS34	142	1
LS39	105-114	4
LS54	152-252	10
AoxD234	195-251	11
AnacC11	145-173	3
AnacE4	332-360	6

The estimation of genetic diversity within *Huso huso* population was accomplished by the

inference of different statistical indices, like estimated heterozygosity ( $H_E$ ), observed heterozygosity ( $H_O$ ) and Mean Number of Alleles (MNA) (Table 3).

**Table 3.** Estimated heterozygosity ( $H_E$ ), observed heterozygosity ( $H_O$ ) and Mean Number of Alleles (MNA) for the beluga sturgeon from Lower Danube.

Locus	$H_E$	$H_O$	MNA
LS19	0.8033	0.6667	
LS34	0.0000	0.0000	
LS39	0.5492	0.3889	
LS54	0.7758	0.4800	
AnacE4	0.6540	0.3889	
AnacC11	0.6372	0.4259	
AoxD234	0.8146	0.9815	6.2857
Average	0.6049± 0.2843	0.4760± 0.2989	

It is accepted that microsatellites are useful in inferring the genetic variation if they present values of heterozygosity between 0.3 and 0.8 [12]. With one exception, the microsatellites analyzed in this study accomplish this requirement and we appreciate that they are suitable to be used in the genetic diversity of beluga sturgeon population.

The heterozygosity represent a good index for the diversity and the history of a population. A high value of medium heterozygosity indicates a high level of genetic variation. Instead, if the medium heterozygosity is reduced the genetic diversity is also reduced.

In our analysis the medium value of  $H_O$  is lower than  $H_E$ , indicating a reduced level a genetic variation and the possible existence of inbreeding between the individuals.

The loci were tested for the Hardy-Weinberg equilibrium. With the exception of LS19 and LS39, the other loci showed departures from Hardy-Weinberg equilibrium. The deviation from the expected values might be the consequence of reduced population, inbreeding or the presence of null alleles in population that might cause a false excess of homozygous.

The genetic structure of the population was estimated by F-statistics [13] for each of the seven loci across the three groups of beluga (Table 4).

Mean estimates of F-statistics obtained over loci were:  $F_{is}$  = 0.196;  $F_{st}$  = 0.134;  $F_{it}$  = 0.304. The genetic differentiation among the analyzed groups of beluga ( $F_{st}$ ) was 13.4%.

Thus, was estimated the value of inbreeding index. The value of inbreeding coefficient (Fis) is situated between -0.112 for AoxD234 and 0.648 for LS19, with a mean value of 0.196. In general, the values obtained for Fis range between -1 (no inbreeding) and +1 (complete identity). Positive values of Fis signify that the amount of heterozygous offspring in the population will decrease, normally due to inbreeding. The mean value that we obtained demonstrates a moderate inbreeding for the analyzed beluga sturgeon population. The value of fixation coefficient (Fst) per locus ranged from 0 to 0.311, with an overall loci value of 0.134. This means that 13.4% of the genetic variation observed in the beluga population is due to the differentiation among the analyzed groups.

**Table 4.** F statistics indices at each locus in *Huso huso* population from the Lower Danube

Locus	Fis	Fst	Fit
LS19	0.648	0.182	0.712
LS34	-0.000	0.000	0.000
LS54	0.039	0.156	0.189
LS39	0.325	0.039	0.351
AnacE4	-0.103	0.311	0.240
AoxD234	-0.112	0.089	-0.013
AnacC11	0.520	0.026	0.507
Overall loci	0.196	0.134	0.304

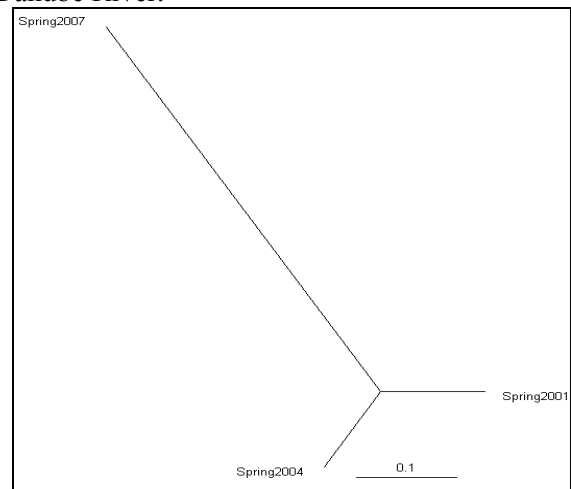
The matrix of Nei's standard genetic distances (D<sub>s</sub>) among groups is presented in Table 5 and the correspondent phylogenetic tree is presented in Figure 1.

**Table 5.** Estimates of Nei's standard genetic distance (D<sub>s</sub>).

	N	Spring 2007	Spring 2001	Spring 2004
Spring 2007	11	0.000	0.370	0.383
Spring 2001	12	0.370	0.000	0.127
Spring 2004	10	0.383	0.127	0.000

The lowest genetic distance was observed between the groups Spring 2001 and Spring 2004 (0.127), while the genetic distance between Spring 2007 and Spring 2001 (0.370) and respectively, Spring 2007 and Spring 2004 (0.383) were similar.

The phylogenetic tree provides a method of visualizing the genetic relationship between the analyzed groups of beluga population from Lower Danube River.



**Figure 1.** Neighbor-Joining phylogenetic tree based on Nei's standard genetic distances

#### 4. Conclusions

The results showed a relatively reduced level of genetic variation and confirmed thus the fragile status of *Huso huso* population in the Lower Danube.

The analysis could be improved by analyzing a larger set of microsatellites in a higher number of individuals, although the last aspect is difficult to achieve taking into consideration the reduced number of specimens that are caught in the river. The analysis of a higher number of loci might provide a better reflection of genetic diversity of *Huso huso* from Lower Danube.

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