

Identification of Bester Hybrid and its Parental Species (♀ *Huso huso* Linnaeus, 1758 and ♂ *Acipenser ruthenus* Linnaeus, 1758) by Nuclear Markers

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Abstract

In Romania, sturgeon farming is gaining advance, different species being raised for commercial purposes and for restocking activities. A correct identification of individuals is imposed since severe ecological damages might occur if non-native species or hybrids are used for restocking. Such identification is required also for commercial reasons, the meat and caviar from different species having different prices. The aim of our study was to analyze two sturgeon species, *Huso huso* and *Acipenser ruthenus* and their interspecific hybrid - bester, using nuclear markers, in order to set up a molecular method for their accurate identification. The genetic pattern of the species was inferred from the analysis of nine microsatellite loci (LS19, LS34, LS39, LS54, AoxD234, AnacC11, LS68, Aox45 and Aox27) amplified by multiplex PCR reactions. The genotype data were analyzed with GENETIX v4.05 and STRUCTURE. The FCA analysis grouped the individuals in three distinct clusters corresponding to each of the pure species and to the interspecific hybrids. The admixture analysis performed in STRUCTURE also assigned three groups, confirming the results highlighted by FCA. We can conclude that the selected microsatellite markers allow the unambiguously identification of the bester hybrid and its genitor species from Romanian farms.

Keywords: sturgeons, bester hybrid, microsatellites, molecular detection.

1. Introduction

The order Acipenseriformes includes primitive species classified in two families and six genera. Within this group, the sturgeons are economically important fish, with biological features that make them particularly vulnerable to extinction due to intensive fishing pressure. Sturgeons are long lived fish, with slow maturation (5-30 years), which do not reproduce annually and require large rivers for spawning [1, 2]. The majority of sturgeon species is in decline because of both habitat destruction and overexploitation and has

been listed in the Appendices to CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) [3]. Moreover, according to the International Union for Conservation of Nature (IUCN) sturgeons are the most threatened group of species in the world, with an extinction risk of 85% [4].

Due to the fact that all sturgeon species are polyploid they tend to hybridize more easily than other species of vertebrates and this phenomenon concerns even the species with a different level of ploidy [5]. The hybrids are relatively rare in the wild and much more frequent in aquaculture. Their number in the wild increases with habitat perturbation (e.g. river dams) and non-native species release [6, 7].

The severe decline of natural populations has brought on an increase of aquaculture programs all

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over the world, with two important directions: the aquaculture for restocking activities in attempt to conserve and restore the wild populations and the aquaculture for production, with emphasis on species that either produce high quality caviar or are characterized by superior growth rates. At present, an important part of caviar production comes from sturgeon hybrids [8].

Morphological characterization is not sufficient for accurate species diagnostic or to demonstrate that a particular individual is a hybrid. The situation is even more complicated when dealing with products like caviar or meat that should be authenticated. Thus, the analysis based on DNA markers represents a reliable tool for sturgeon species and hybrids identification.

The phylogenetic studies in Acipenseriformes find some polyphyletic genera and species complexes, fact that hindered the species diagnostic by mitochondrial DNA (mtDNA) [9]. Moreover, the mitochondrial markers are inefficient for hybrids detection due to the maternal mode of inheritance of the mitochondrial genome. Nuclear markers such as microsatellites, characterized by a high degree of polymorphisms within species and populations, are more useful for molecular diagnostic of species and hybrids because they are co-dominantly inherited. By analyzing the microsatellites, the individual genetic profile is inferred and the relations between different individuals/populations/species can be established. In sturgeons the methods based on nuclear DNA analysis are limited by the polyploidy feature of the group [10], this involving a difficulty in establishing the real genotype of an individual and analyzing the data with specialized software. This drawback can be hurdled by using disomic microsatellite loci.

The aim of our study was to infer the genetic pattern of bester and its parental species by using nuclear markers. Bester is a hybrid between beluga (*Huso huso*) females and sterlet (*Acipenser ruthenus*) males, which produces high quality caviar at a younger maturity age as compared to pure beluga. By using nine microsatellite loci we propose a molecular approach for bester identification.

2. Materials and methods

Sampling and DNA extraction

The biological material was represented by small fin fragments sampled in 20 individuals of bester fish farmed individuals from South-East Romania. The individuals were classified as hybrids based on their morphology and knowing their pedigree. The genomic DNA was isolated by a classic protocol using chloroform–phenol–isoamyl alcohol [10] and its concentration and quality was assessed by using a NanoDrop 8000 spectrophotometer (*Thermo Scientific*).

Microsatellite genotyping

All individuals from this study were genotyped at nine microsatellite loci (LS19, LS34, LS39, LS54, AoxD234, AnacC11, LS68, Aox45 and Aox27). The primer sequences used in the experiment were already described by Dudu et al., 2011 [11].

The forward primers used to amplify each locus were labeled with a specific fluorochrome (6-FAM, NED, VIC or PET). The amplification conditions were optimized by gradient PCR reactions in order to establish the optimum annealing temperature for each set of primers. After this step, the nine microsatellite loci were amplified by three multiplex reactions, as follows: LS19, S34, LS39 and LS54 in a 4-plex reaction, AoxD234, AnacC11 and LS68 in 3-plex reaction and Aox27 and Aox45 in 2-plex reaction.

PCR amplifications were performed in GeneAmp 9700 PCR System (*Applied Biosystems*) under the following conditions: denaturation at 95°C for 10 minutes; 30 cycles of denaturation at 95°C for 30 seconds, annealing at 50-59°C for 30 seconds and elongation at 72°C for 60 seconds; final extension at 72°C for 60 minutes. The amplifications were carried out in 25 µL reaction volumes containing 20 ng of DNA template, 1X AmpliTaq Gold PCR Buffer, MgCl₂ 1.5 mM, dNTPs 0.8 mM, AmpliTaq Gold DNA polymerase 0.04-0.06 U/µL. All PCR reagents were supplied by *Life Technologies*. The amplification products were loaded with the GeneScan-500 LIZ Size Standard into the ABI Prism 310 DNA Genetic Analyzer (*Applied Biosystems*) and the raw data were analyzed with the GeneMapper 3.0 Software (*Applied Biosystems*).

Genotype data analysis

In order to setup a reliable molecular method for better identification the genotype data obtained for the hybrid individuals included in the study were analyzed alongside with genotype data from their parental species. Beluga and sterlet individuals caught in the Lower Danube River between 2001 and 2008 or coming from fish farms from Romania were previously genotyped and the results are found in the database of our laboratory. The genotype data of 112 individuals (including 50 beluga, 42 sterlet and 20 bester) were analyzed with two specialized software. A Factorial Correspondence Analysis (FCA) based on the multilocus genotypes was carried out by using GENETIX 4.05 software [12]. The Bayesian clustering approach implemented in STRUCTURE 2.3.4 [13] was used for identifying the most likely number of clusters (K) as well as to assign individuals to these clusters. Thus, we completed ten replicate runs (burn-in period of 100000 steps and 500000 MCMC iterations) at each value of K from 1 to 4. The analysis was done assuming correlated allele frequencies and admixture. The highest delta K value corresponding to the best fit K value was determined with Structure Harvester [14].

3. Results and discussion

All nine loci showed a disomic pattern both for bester and its parental species. This means that a maximum of two alleles were observed for each individual at each locus. This demonstrates that the selected microsatellites are suitable for molecular diagnostic of species, as is very difficult to establish the correct genotype for an individual that has more than two alleles. In addition, the specialized software selected to be used in the analysis of microsatellites data can analyze only diploid data.

The number of alleles varied from 1 to 12. The most polymorphic microsatellites were represented by LS54 and AoxD234 for *H. huso* with a number of 11 alleles, Aox45 for *A. ruthenus* and LS68 with 12 alleles and Aox45 and LS68 for bester with 7 alleles. Instead, the loci Aox45 in *H. huso* and Aox27 in *A. ruthenus* were characterized by a fixed allele for the analyzed individuals belonging to these two species (Table 1). For bester hybrid the lowest number of alleles (2) was recorded for the microsatellites LS19 and LS34 (Table 1).

Table 1. The characteristics of nine microsatellite loci used in the study

Locus	Allelic range (bp)			Number of alleles		
	<i>H. huso</i>	<i>A. ruthenus</i>	Bester	<i>H. huso</i>	<i>A. ruthenus</i>	Bester
LS19	133-163	130-142	133-154	10	5	2
LS34	142-148	136-151	139-142	2	5	2
LS39	115-133	115-127	124-133	6	4	3
LS54	212-280	152-168	168-280	11	4	3
LS68	144-152	168-232	152-216	3	10	7
AnacC11	145-173	161-201	145-185	5	8	4
Aox27	118-134	118	114-138	5	1	3
AoxD234	195-251	219-271	207-247	11	5	4
Aox45	154	170-218	154-214	1	12	7

The FCA based on microsatellite genotypes highlighted a clear segregation between the pure sturgeon species, *H. huso* and *A. ruthenus* and the bester hybrids. The individuals were assigned to three different clusters in concordance with their

taxonomic classification. The hybrid cluster occupies an intermediate position between the parental species according to the multidimensional analysis of microsatellite genotyping data (Figure 1).

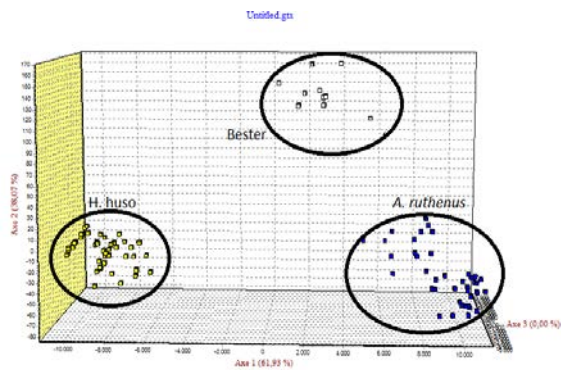


Figure 1. The Factorial Correspondence Analysis based on nine microsatellites loci in interspecific hybrid bester and its genitor species.

The axis 1 is the most informative, with 61.93% of total genetic variation, followed by axis 2 with 38.07%.

In order to investigate the assignment of individuals in different clusters according to their species, the admixture model implemented in the STRUCTURE software was used to analyze the microsatellite genotype data. The assignment test was conducted with K ranging from 1 to 4. The best value of K according to highest delta K value determined by STRUCTURE HARVESTER is equal to 2. The results of the assignment test highlighted the separation in three distinct groups congruent with the two pure species *H. huso* and *A. ruthenus* and their interspecific hybrid, confirming thus the results of FCA (Figure 2). The cluster in which bester individuals were assigned presented composite bars signaling mixed genotypes of these individuals. Knowing that the bester is a F1 hybrid between beluga female and sterlet male we might expect the membership coefficient (Q values) for the analyzed individuals to be around 0.5.

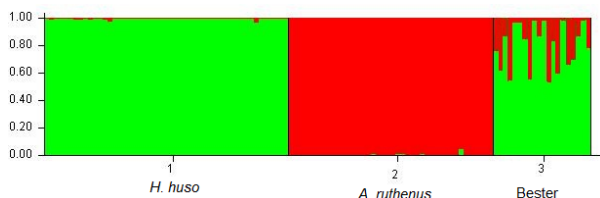


Figure 2. Assignment test with STRUCTURE based on nine microsatellite analysis. Histogram represent the estimated membership coefficients (Q). Composite bars are characteristics to hybrid individuals.

This situation is observed for the most of the individuals, but there are some exceptions when the Q value is much lower and the proportion of genotypic contribution from the beluga parental

species is prevalent. This situation occurs when the majorities of alleles composing the genotype of a hybrid individual are originating mostly from one of the genitor species and can be resolved by including more microsatellites into the analysis.

Our molecular approach come to complete other attempts made for sturgeon hybrids identification. Similar studies based on microsatellite loci analysis was proposed for several sturgeon species and hybrids (including bester) and aimed at finding specific alleles for diagnostic [15, 16]. The method seems to be precise only if a screening of genotypes of thousands individuals is completed. Other method proposed for bester detection is based on AFLP (Amplified Fragment Length Polymorphism) technique [17]. Despite the fact that a clear pattern of bands were observed consequently the electrophoresis for the bester individuals, the low number of analyzed specimens might be considered a limitation in sustaining the accuracy of the technique. Moreover, it is consider that the AFLP power of inter species discrimination may decrease for closely related species and the resolution depends on among other factors by the genome complexity [18, 19], which is the case of bester and its parental species.

Conclusions

The genotype data resulted from the analysis of nine disomic microsatellite loci with two specialized software allowed the molecular diagnostic of bester hybrids evaluated alongside with the parental species. Both FCA and assignment test differentiate between hybrid and pure species categories. For a higher degree of confidence for the diagnostic based on nuclear markers, the method can be improved by including a higher number of loci and individuals into the analysis.

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