

MOLECULAR METHODS FOR THE DETECTION OF NATURAL HYBRIDS IN STURGEON POPULATIONS

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ABSTRACT

Due to construction of the Iron Gates dams, the Lower Danube has suffered a decrease in sturgeon populations. The dams have decreased sturgeon habitat area, which in turn has caused an overlap of reproduction areas for all sturgeon species. The ease with which sturgeon species can create hybrid offsprings gave rise to an increase in the number of hybrid sturgeon species now found in the Lower Danube area. We propose a set of molecular methods for hybrid species using DNA markers represented by microsatellites and mitochondrial DNA. This identification data and methodology is important for use on sturgeon farms due to the need to correctly identify species of sturgeons. Using the proposed methodologies, it is possible to avoid identification errors that might appear when using only morphological criteria to identify sturgeons.

RÉSUMÉ: Méthodes moléculaires pour la détection des hybrides naturelles dans les populations d'esturgeons.

En raison de la construction de barrages aux Portes de Fer, le Danube inférieur a subi une diminution des populations d'esturgeon. La diminution de l'aire géographique a induit le chevauchement des sites de reproduction de toutes les espèces d'esturgeons. La facilité d'hybridation caractéristique de ces espèces a conduit à la situation actuelle qui est une augmentation du nombre d'hybrides d'esturgeons. Nous proposons plusieurs méthodes moléculaires pour l'identification des hybrides utilisant deux types de marqueurs d'ADN. Les données sont importantes pour les fermes d'esturgeons de part la nécessité d'une identification correcte des individus. Ainsi, il est possible de pallier les difficultés potentielles liées à une simple identification morphologique.

REZUMAT: Metode moleculare de detecție a hibrizilor naturali în populații de sturioni.

Datorită construcției barajelor de la Porțile de Fier, Dunărea Inferioară a suferit o descreștere a populațiilor de sturioni. Restrângerea arealului a condus la suprapunerea zonelor de reproducere pentru toate speciile de sturioni. Acest fenomen, împreună cu ușurința hibridizării în cazul acestor specii, a condus la situația actuală, în care există o creștere a numărului hibrizilor naturali de sturioni. Propunem un set de metode moleculare pentru identificarea hibrizilor de sturioni, folosind două tipuri de markeri ADN. Datele obținute sunt importante pentru fermele piscicole datorită necesității identificării corecte a indivizilor. Astfel, se pot evita eventualele dificultăți apărute în identificarea indivizilor exclusiv pe baza criteriilor morfologice.

INTRODUCTION

Sturgeons are represented by the order Acipenseriformes, which contains 27 species divided in two families: *Acipenseridae* with 25 species, and *Polyodontidae* with two species (Zhang et al., 2013). This group of “living fossils” is undergoing a dramatic population decline in Eurasia, with fish populations currently at historically low levels (Ludwig, 2006; Ludwig et al., 2009). This decline is due to wrong harvesting, poaching and loss and degradation of habitat, specifically because of dams along rivers (Ludwig et al., 2009; Havelka et al., 2011).

Natural hybridization is one of the effects of habitat degradation and loss, especially in response to hindered migration to spawning areas that occurred in Romania after the construction of the Iron Gates dams. The dam installations, altered parts of the Lower Danube spawning habitats creating favorable conditions for two sturgeon species to overlap. This change in the ecological relationship between the two species left room for “habitat hybridization” (Tranah et al., 2004). Hybridization not only occurs in the wild under natural conditions but also in artificial breeding. Cases of both interspecific and intergeneric hybridization have been observed (Havelka et al., 2011), confirming the rate of this event, especially under the right circumstances.

Cross-breeding sturgeons have the ability to produce fully fertile hybrid offsprings (Havelka et al., 2011) more easily than other vertebrates. Due to possible crossbreeding that may have occurred, using a morphological description to identify sturgeon that may be hybrids is not enough to conclusively identify the sturgeon species. Because first-generation of hybrids are not morphologically intermediate to the genitor morphotypes (Tranah et al., 2004), coupled with the difficulty of identifying sturgeon hybrids in early growth stages such as fry and sub-adults (Zhang et al., 2013), there is a need for molecular hybrid identification and characterization.

One of the techniques for identifying hybrids and pure species sturgeons is PCR-RFLP (Polymerase Chain Reaction - Restriction Fragment Length Polymorphism). This technique is based on the amplification of the region tRNA^{Glu}/cytochrome b of 462 bp from the mitochondrial DNA and endonuclease restriction of this fragment. The restriction fragments observed on the electrophoresis gel are analyzed to show the maternal genitor species of an individual sturgeon. This technique is useful for confirming purespecies fish or finding out the maternal genitor of a hybrid because of the small intraspecific variations in sequence for the analyzed region (Wolf et al., 1999). The analysis is based on restriction of species-specific sites resulting in a species-specific band pattern with the use of a single universal primer pair (Ludwig, 2006).

Along with using PCR-RFLP as an identification technique, the DNA barcoding was also used for species identification, respectively for maternal genitor identification in the case of hybrids. The advantage of using DNA barcoding for sturgeon species is that it overcomes the possibility of incorrect identification due to using the phenotype only. Additionally, the DNA method is effective at any life-cycle stage and regardless of gender. From a molecular standpoint, DNA barcoding for the mitochondrial gene COI (Cytochrome Oxidase I) is good because of the haploid mode of inheritance, a lack of introns, and limited exposure for recombination (Hebert et al., 2003; Hubert et al., 2008). The DNA analysis is centered on genes because universal primers are very robust and because changes in the amino-acid sequence change slowly in respect to other mitochondrial genes (Hebert et al., 2003).

In the case of natural sturgeon hybrids, these two techniques lack precision due to the maternal inheritance of mitochondrial DNA (Havelka et al., 2011). For this reason we use nuclear microsatellites markers that are short-tandem repeats of two to nine nucleotides with a high-degree of length polymorphism. Because microsatellites are markers with great

variability and due to the fact that they have co-dominant inheritance, unbiased by sex-specific differences (Nelson et al., 2013), microsatellites are used for various types of population studies, and in this case for hybrid identification and characterization. This is possible using the factorial correspondence analysis (FCA) test that shows the relationship of individual samples with respect to a microsatellite dataset of known individual samples.

MATERIAL AND METHODS

Molecular methods were tested using samples harvested without endangering the life of individual fish. Small fin fragments were collected from individual sturgeon captured in the Lower Danube and labeled as either pure species or hybrids based on their morphology. For total genomic DNA extraction from fin tissue we used a standard phenol/chloroform protocol modified from Taggart et al. (1992; 2004).

PCR-RFLP

The PCR reaction was made in a total volume of 25 µL which contained: DNA template, 1X PCR Gold Buffer (AppliedBiosystems), 1.5 mM MgCl₂, 200 µM of each dNTP (AppliedBiosystems), 0.48 µM of each primer (forward primer sequence: 5'-AAAAACCACCGTTGTTATTCAACTA-3', reverse primer sequence 5'-GCCCCTCAG AATGATATTTGTCCTCA-3'), 1U AmpliTaq Gold DNA Polymerase (AppliedBiosystems), and nuclease-free water. The reaction mixes were amplified on GeneAmp 9700 PCR System (AppliedBiosystems) using the following cycling conditions: 95°C for 10 minutes, 40 cycles of 95°C for 30 seconds, 60°C for 45 seconds, 72°C for a minute and a final extension at 72°C for 10 minutes. For the enzymatic restriction we used a total volume of 20 µL with: 2 µL Reaction Buffer, 0.4 µL Bovine Seric Albumine, 16.5 µL PCR product and 1.1 µL restriction enzyme: *RsaI* or *SspI* (Promega). The mix was incubated for three hours at 37°C and then visualized consequently electrophoresis in a 3% agarose gel alongside 50 bp DNA Step Ladder (Promega). Table 1 shows the enzymes we used in this study and the restriction fragment length attributed to the species of sturgeon still found in the Danube River.

Table 1: PCR-RFLP of the amplified cytb gene of four sturgeon species (Wolf et al., 1999).

	<i>Huso huso</i>	<i>Acipenser gueldenstaedtii</i>	<i>Acipenser stellatus</i>	<i>Acipenser ruthenus</i>
<i>RsaI</i>	317 bp	317 bp	462 bp	341 bp
	112 bp	112 bp		88 bp
	33 bp	33 bp		33 bp
<i>SspI</i>	277 bp	277 bp	462 bp	277 bp
	185 bp	185 bp		185 bp

DNA sequencing

For DNA barcoding we used a PCR mix with a total volume of 25 µL containing 50 ng of DNA template, 1X PCR Gold Buffer (AppliedBiosystems), 1.5 mM MgCl₂, 200 µM of each dNTP (AppliedBiosystems), 0.4 µM of each primer (forward: 5'-TCAAGCCAGCCGCATAAC-3', reverse: 5'-CGCTATTCCCTATTAGCTTCT-3'), 1U AmpliTaq Gold DNA Polymerase (AppliedBiosystems), nuclease-free water. The cycle conditions were as follows: 95°C for 10 minutes, then 35 cycles of 95°C for 30 seconds, different annealing temperatures for different hybrids for 30 seconds, 72°C for a minute and a final extension at 72°C for 10 minutes. The PCR products were visualized on 2% agarose gel and purified using Wizard SV Gel and PCR Clean-Up System (Promega). The sequencing of amplicons was done with BigDye Terminator v3.1 Cycle Sequencing Kit, the sequencing products were purified using BigDye XTerminator Purification Kit (AppliedBiosystems) and loaded on 3130 Genetic Analyzer (AppliedBiosystems) for capillary electrophoresis. We

sequenced the forward and reverse strands for more accurate data; we edited the sequences using BioEdit Sequence Alignment Editor 7.1.9. (Hall, 1999) and then we aligned the resulting data with known sequences from various sturgeon species using the BOLD (Barcode of Life Data) system (Ratnasingham and Hebert, 2007) for identification.

Microsatellite amplification

For microsatellite genotyping, we selected a set of eight microsatellite markers presented in table 2, first isolated from North American sturgeon species and used for native species by cross-amplification. The reaction mixes were prepared in a total volume of 25 µL with: 30 ng of DNA template, 1X PCR Gold Buffer (AppliedBiosystems), 1.5 mM MgCl₂, 200 µM of each dNTP (AppliedBiosystems), 0.24 µM of each primer with the forward primer fluorescently labeled, 1U AmpliTaq Gold DNA Polymerase (Applied Biosystems), nuclease free water. The reaction mixes were amplified on GeneAmp 9700 PCR System (AppliedBiosystems) using the following cycling conditions: 95°C for 10 minutes, then 35 cycles of 95°C for 30 seconds, specific annealing temperatures for the different primer pairs due to the different amplified microsatellite markers for 30 seconds, 72°C for a minute and a final extension at 72°C for 60 minutes. The amplified fragments were loaded with the GeneScan-500 LIZ Size Standard into ABI Prism 310 DNA Genetic Analyzer.

The statistical analysis of microsatellite genotype data was performed by FCA implemented in the program GENETIX (Belkhir et al., 2002).

Table 2: Microsatellite genotyping primer sequence.

Locus	Species	Primer sequence from 5' to 3'
LS19	<i>Acipenser fulvescens</i>	F: CATCTTAGCCGTCTGTGGTAC R: CAGGTCCCTAATACAATGGC
LS34	<i>Acipenser fulvescens</i>	F: TACATACCTTCTGCAACG R: GATCCCTTCTGTTATCAAC
LS39	<i>Acipenser fulvescens</i>	F: TTCTGAAGTTCACACATTG R: ATGGAGCATTATTGGAAGG
LS54	<i>Acipenser fulvescens</i>	F: CTCTAGTCTTTGTTGATTACAG R: CAAAGGACTTGAACTAGG
Aox27	<i>Acipenser oxyrinchus</i>	F: AATAACAATAACGGCAGAACCT R: TGTGTTGCTCAAGACAGTATGA
AoxD234	<i>Acipenser oxyrinchus</i>	F: AACTGGCTTTGTGATTGATCC R: TGAAGCAAAGGGTATTATTTGAG
AnacE4	<i>Acipenser naccari</i>	F: TCAGCTACAGGGTTCTGGG R: GTTGTTACTCATTGGAAGTC
AnacC11	<i>Acipenser naccari</i>	F: AAATTTCCATTGGGGTGT R: CTTCGTTTTGAGAACCCG

RESULTS AND DISCUSSION

The FCA statistical interpretation, based on the eight nuclear microsatellite loci, (Fig. 1) shows three clusters of pure-species individuals surrounding and the analyzed hybrids (marked as 203 and 204) which show up between these pure-species clusters. The position occupied by hybrid 204 indicates that this is a hybrid between *Acipenser ruthenus* and *Acipenser gueldenstaedtii*. The maternal genitor species identified by mitochondrial DNA marker analysis is represented by an individual belonging to *Acipenser ruthenus* species, while the paternal genitor is from *Acipenser gueldenstaedtii*.

In the case of hybrid 203, the genitors are represented by *Acipenser gueldenstaedtii* as paternal genitor and *Huso huso* as maternal genitor, this last one being confirmed by mitochondrial DNA marker analysis (Figs. 2, 3 and 4).

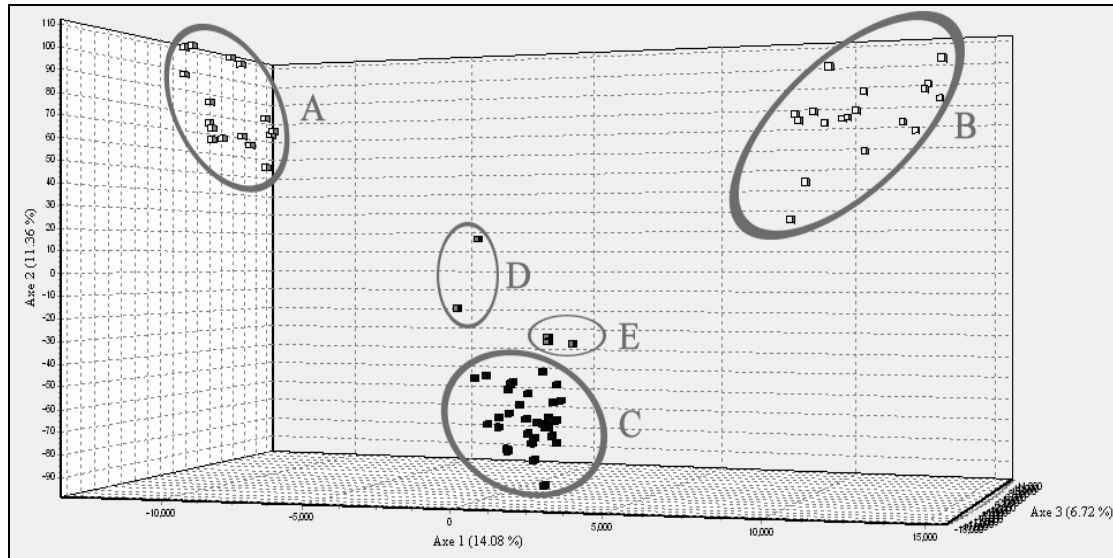


Figure 1: GENETIX - FCA hybrid analysis; A - *Acipenser ruthenus*; B - *Huso huso*; C - *Acipenser gueldenstaedtii*; D - hybrid 204; E - hybrid 203.

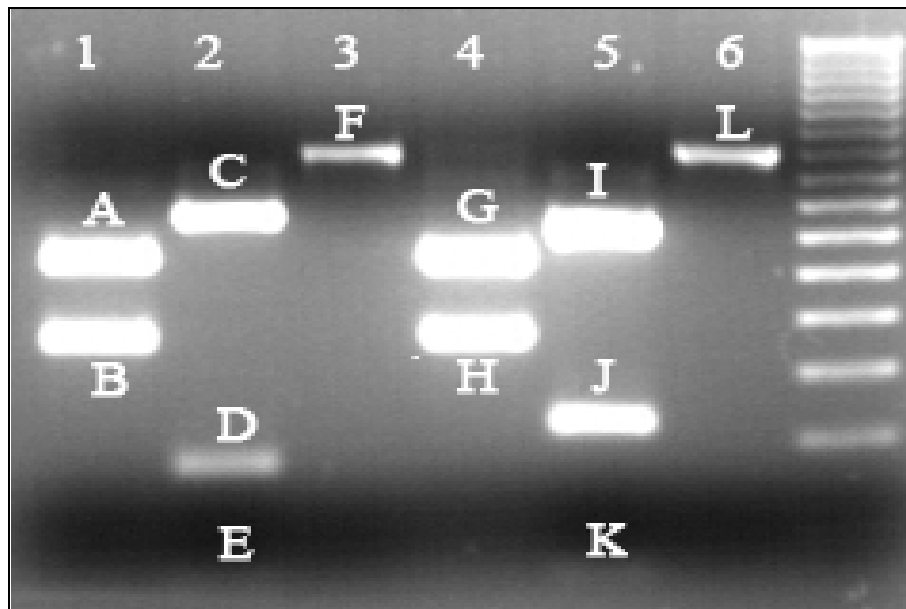


Figure 2: PCR-RFLP electrophoresis gel with 50 bp DNA Step Ladder; 1, 2, 3 - hybrid 204; 4, 5, 6 - hybrid 203; F, L - uncut fragments, 462 bp; A, B - *Ssp. I* restriction fragments, 277 bp and 185 bp; C, D, E - *RsaI* restriction fragments, 341 bp, 88 bp and 33 bp; G, H - *Ssp. I* restriction fragments, 277 bp and 185 bp; I, J, K - *RsaI* restriction fragments, 317 bp, 112 bp and 33 bp.

Using maternal genitor identification by PCR-RFLP analysis, the electrophoretic gel (Fig. 2) shows that for hybrid 204 the *SspI* digested fragments, 277 bp and 185 bp, are common between *H. huso*, *A. gueldenstaedtii* and *A. ruthenus*, but because of the *RsaI* digested fragment length the result is pinpointed to *A. ruthenus* with 341 bp, 88 bp, 33 bp fragments. In case of hybrid 203 the *SspI* digested fragments show the same patterns as for hybrid 204 but the *RsaI* band pattern, indicates as maternal genitor species to *H. huso* or *A. gueldenstaedtii*.

The PCR-RFLP analysis showed that hybrid 204 has maternal lineage from *A. ruthenus* yet hybrid 203 has a lineage from *H. huso* or *A. gueldenstaedtii*. The BOLD system was used to confirm the lineage of hybrid 204 and to clarify the origin of hybrid 203. This BOLD system identification showed that the hybrid 204 barcode sequence (Fig. 3) is a 100% match with *A. ruthenus*. Results for hybrid 203 (Fig. 4) showed a 99.9% similarity to the *H. huso* barcode sequence, thereby excluding thus the possibility of *A. gueldenstaedtii* as a paternal genitor as was previously highlighted by PCR-RFLP band pattern. For both hybrids the top five matches are the species to which they are assigned, raising confidence in results.

Using PCR-RFLP and BOLD system sequence alignment we conclude that for hybrid 204 the maternal genitor is *A. ruthenus* while *H. huso* is the maternal genitor for hybrid 203.

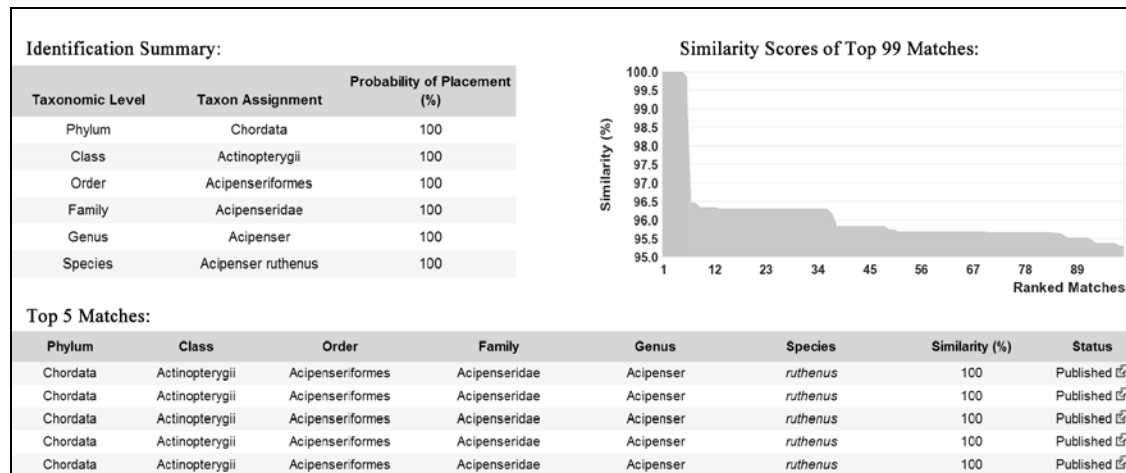


Figure 3: Hybrid 204, BOLD system sequence alignment.

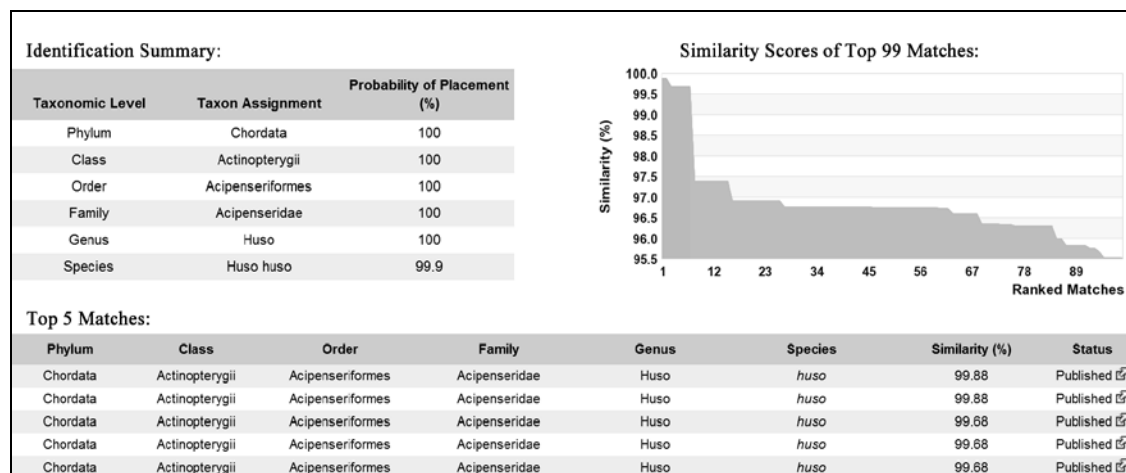


Figure 4: Hybrid 203, BOLD system sequence alignment.

CONCLUSIONS

Based on our analysis we conclude that the methods used here are particularly effective in hybrid characterization, yielding information not only about the identification of a sturgeon hybrid but also about the maternal and paternal ancestry of the individual sturgeon in question.

With the current difficulty in morphological characterization of natural sturgeon hybrids because of their unknown genitors, we propose the methods shown above provide the most reliable methodologies for identifying hybrids and the pure-stock sturgeon species. The specific application of these methodologies at various fish farms would allow people to identify and then avoid using hybrids in reproduction and repopulation programs that might lead to the alteration of the native populations.

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