Characterization of seven microsatellites in the Black Sea sturgeon (*Huso huso*)

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Abstract:

Romanian sturgeon populations have declined because of anthropogenic influences. Recent developments in molecular biology allow the detection of intra- and interspecific genetic differences and could ensure an increased ability to characterize and quantify the extent of genetic variation in sturgeon populations. In our study we optimized a protocol for microsatellite PCR multiplex in *Huso huso*. We designed seven pairs of primers to amplify seven microsatellites in the same multiplex reaction. We obtained fragments of different sizes which were analyzed by agarose electrophoresis. This technology has the potential to be of great use in monitoring levels of genetic variation within stocks.

Key words: Sturgeon, microsatellites, PCR multiplex.

Introduction:

Sturgeons (order Ancipensiformes) represent a very ancient group of fish who appeared in the Upper Cretaceous [1]. These "living fossils" are endangered because of anthropogenic influences like overexploitation, especially for caviar production, habitat alteration, barriers to migration, loss of spawning habitat and water pollution.

Other threats for this species could be the increasing practices of restocking in the natural basins with artificially reproduced individuals raised in aquaculture conditions. Uncontrolled restocking drastically reduces the genetic diversity of species, especially because of fast decline in number of wild individuals remaining [2].

Huso huso, like other marine migratory sturgeons in Romania, which swim up the Danube River for reproduction, represents an appealing fish species from both scientific and commercial point of view. While the effectives of *Huso huso* has significant decreased in the last decades, the economical interest was constantly increasing due to the incredible value of the roe known commercially as beluga caviar, which represents one of the most expensive food items in the world. In these conditions, it is necessary to develop conservation programs for Romanian sturgeons species, which imply knowledge of its genetic diversity and of the evolutionary relationships among geographic populations.

The recent developments in molecular biology allow the detection of intra- and interspecific genetic differences and could provide the ability to characterize and quantify the extent of genetic variation in sturgeon populations.

Multiplex polymerization chain reaction represents a technique of simultaneous amplification of multiple regions of a DNA template. Recent studies in a number of organisms have shown that the class of variable numbers of tandem repeat (VNTR) loci with simple sequence repeats motifs often termed microsatellite loci and offers the advantages of high allelic variation per locus and simplified tissue sampling requirements for their analysis. Microsatellites are efficient markers as they are evenly distributed across the genome and highly polymorphic. In addition, microsatellites can be scored from tissues non-destructively sampled (e.g., muscle, fin, blood) [3]. They allow the evaluation of intraspecific genetic diversity, thus, microsatellite multiplexing is a powerful technique that can be successfully used in genetic studies.

Materials and methods

DNA extraction

Sturgeons' fins collected in liquid nitrogen were used for DNA extraction. The genomic DNA was extracted from 50 mg fin by a specific method [4]. The DNA concentration and quality was assessed spectrophotometrically at 260/ 280 nm.

Gradient temperature PCR

We used seven pairs of primers to amplify seven microsatellite loci: LS-19, LS-34, LS-54, LS-57, LS-68, Aox 23, Aox 45. The PCR conditions were optimized for each set of primer by varying the annealing temperature between 51-60˚C on a gradient termocycler IQ Cycler (BioRad).

Amplification reactions were carried out in 25 µL final volume and consisted of 1X PCR Buffer, $MgCl_2$, 200 µM of each nucleotide, DNA template, 0.5 µL of each primer (see Table 1), 0.5 units of AmpliTaq Gold DNA Polymerase and nuclease free water. PCR amplifications were performed in 0.2 ml tubes using a program with 35 cycles. Denaturation was performed at 95˚C for 30 seconds, for annealing a temperature

gradient between 51-60˚C for 30 seconds and extension at 72˚C (60 seconds) was set. The first denaturation step was of 10 minutes at 95˚C and the final extension was of 10 minutes at 72˚C. The PCR products obtained were analyzed by electrophoresis in 2% agarose gel.

Table 1: Primers sequence

Primer	Sequence
LS-19 F	CATCTTAGCCGTCTGGGTAC
LS 19 R	CAGGTCCCTAATACAATGGC
LS 34 F	TACATACCTTCTGCAACG
LS 34 R	GATCCCTTCTGTTATCAAC
LS 54 F	CATCTAGTCTTTGTTGATTACAG
LS 54 R	CAAAGGACTTTGAAACTAGG
LS 57 F	GCTTGGTTGCTAGTTTGC
LS 57 R	GTACAGTATGAGACCACAGGC
LS 68 F	TTATTGCATGGTGTAGCTAAAC
LS 68 R	AGCCCAACACAGACAATATC
Aox 23 F	CAGTGTGCTAGCTTCTCAATA
Aox 23 R	GTTAGCTTAACCATGAATTGTG
Aox 45 F	TTGTTCAATAGTTTCCAACGC
Aox 45 R	TGTGCTCCTGCTTTTACTGTC

PCR Multiplex

We used the same seven pairs of primers for microsatellite loci in the same amplification reaction. Amplifications were done in 25 µL final volume with 1X PCR Buffer, $MgCl_2$, 200 µM of each nucleotide, DNA template, 0.4 µL of each primer, 0.5 units of AmpliTaq Gold DNA Polymerase and nuclease free water. Reaction mixes were amplified in GeneAmp 9700 PCR System (Applied Biosystems) using the following program: a first denaturation step at 95˚C for 10 minutes, 40 cycles of denaturation at 95˚C for 30 seconds, annealing at 53˚C for 30 seconds, and extension at 72˚C for 60 seconds and a final extension step at 72˚C for 10 minutes. Amplified products were run on 3% agarose gel electrophoresis.

Results and discussions

Microsatellite multiplexing analysis is a powerful technique that can be successfully used in genetic studies. Recent studies have shown that the relevance of multiplex polymerization chain reaction allow the analysis of high allelic variation for different loci [5, 6]. In this respect we optimized a protocol for multiplex PCR amplification for seven microsatellite loci in *Huso huso.*

The PCR-multiplex requires extensive optimization of annealing conditions for maximal amplification efficiency of the different primer–template systems. Initially we have determined the optimal annealing temperature of each set of primers by temperature gradient PCR. The following electrophoresis patterns we have obtained for our seven microsatellite loci is shown in Figures 1-7.

In the next step the microsatellite loci were co-amplified in the same multiplex PCR reaction. We obtained fragments in range of 130 – 170 bp for LS-19, 130 – 170 bp for LS-34, 220 - 260 bp for LS-54, 170-210 bp for LS-57, 140 – 180 bp for LS-68, 130 – 170 bp for Aox23 and 120 – 160 bp for Aox45. The amplicons were analyzed by electrophoresis in agarose gel 3% resulting in the electrophoresis pattern shown in Figure 8.

Figure 1: Electrophoresis in agarose gel 2% for LS 19 locus. Lane 1: Molecular weight marker 50 bp; lanes 2 – 5: DNA fragments amplified by Gradient Temperature PCR - 2 – 54.5ºC; 3 – 52.9ºC; 4 – 51.7ºC; 5 – 51ºC; lane 6: negative control.

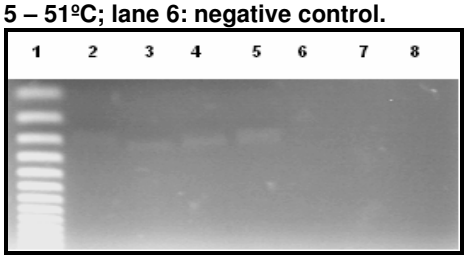


Figure 2: Electrophoresis in agarose gel 2% for Aox45 locus. Lane 1: Molecular weight marker 50 bp; lanes 2 – 5: DNA fragments amplified by Gradient Temperature PCR - 2 – 54.5ºC; 3 – 52.9ºC; 4 – 51.7ºC; 5 – 51ºC; lane 6: negative control.

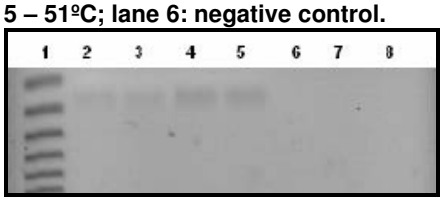


Figure 3: Electrophoresis in agarose gel 2% for Aox 23 locus. Lane 1: Molecular weight marker 50 bp; lanes 2 – 5: DNA fragments amplified by Gradient Temperature PCR - 2 – 54.5ºC; 3 – 52.9ºC; 4 – 51.7ºC; 5 – 51ºC; lane 6: negative control.

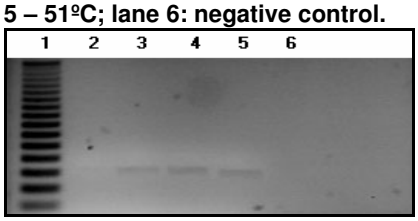


Figure 4: **Electrophoresis in agarose gel 2% for LS 34 locus. Lane 1: Molecular weight marker 50 bp; lanes 2 – 5: DNA fragments amplified by Gradient Temperature PCR - 2 – 54.5ºC; 3 – 52.9ºC; 4 – 51.7ºC; 5 – 51ºC; lane 6: negative control.**

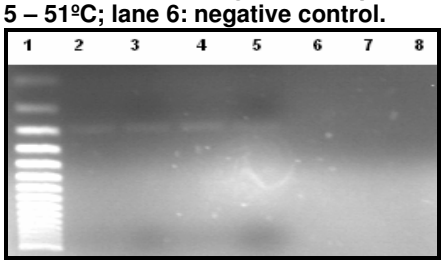


Figure 5: **Electrophoresis in agarose gel 2% for LS 57 locus. Lane 1: Molecular weight marker 50 bp; lanes 2 – 5: DNA fragments amplified by Gradient Temperature PCR - 2 – 54.5ºC; 3 – 52.9ºC; 4 – 51.7ºC; 5 – 51ºC; lane 6: negative control.**

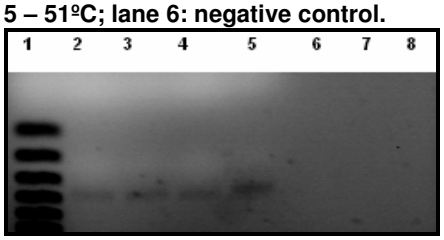


Figure 6: **Electrophoresis in agarose gel 2% for LS 68 locus. Lane 1: Molecular weight marker 50 bp; lanes 2 – 5: DNA fragments amplified by Gradient Temperature PCR - 2 – 54.5ºC; 3 – 52.9ºC; 4 – 51.7ºC; 5 – 51ºC; lane 6: negative control.**

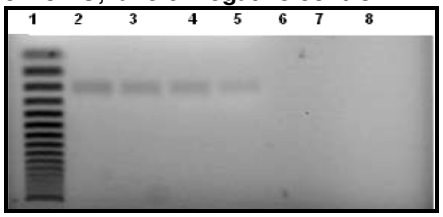


Figure 7: **Electrophoresis in agarose gel 2% for LS 54 locus. Lane 1: Molecular weight marker 50 bp; lanes 2 – 5: DNA fragments amplified by Gradient Temperature PCR - 2 – 54.5ºC; 3 – 52.9ºC; 4 – 51.7ºC; 5 – 51ºC; lane 6: negative control.**

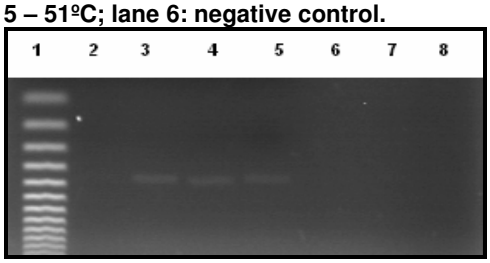
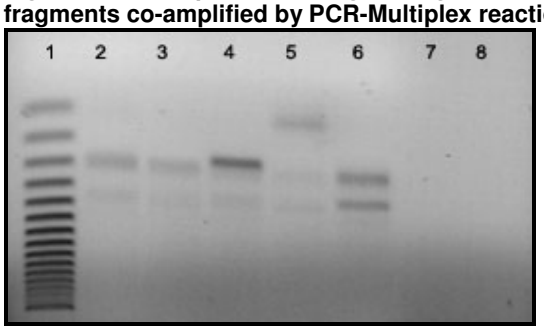


Figure 8: **Electrophoresis in agarose gel 3%: Lane 1: Molecular weight marker 50 bp; lanes 2 – 6 DNA fragments co-amplified by PCR-Multiplex reaction; lane 7: negative control.**



Conclusions

Our results demonstrate a correct amplification of all seven microsatellites tested. For further analysis via the genotyping technique we have decided to label the primers with four different fluorescent dyes. This technique could represent a very good method for individual identification and genetic distance evaluation within sturgeon stocks. It also could allow the evaluation of intra-specific genetic diversity and provide the ability to characterize the genetic variations in sturgeon populations.

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