Genetic analysis of six sterlet (*Acipenser ruthenus*) populations - recommendations for the plan of restitution in the Dniester River

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Abstract

The aim of the present study was the genetic analysis of the Dniester population of sterlet *Acipenser ruthenus* and comparison of it to five other sterlet populations, in order to develop a population recovery plan. The genetic analysis of six sterlet populations from Eurasian rivers (Dniester, Dnieper, Danube, Volga, Kama and Ob) was carried out using microsatellite DNA markers. The genetic variation and genetic distance between studied populations were evaluated. Significant genetic differentiation was detected between the analyzed populations through the pairwise genetic differentiation index (Fst) test. The D_A measure of genetic distance between pairs of populations indicated that the shortest genetic distance (in relation to the Dniester population) was between the Dniester and the Dnieper populations (0.184). The results obtained represent a starting point of sterlet conservation program in Dniester River.

Keywords: *Acipenser ruthenus*, Aquaculture, Genetic analysis, Microsatellite DNA, Restitution

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Introduction

Almost all Acipenseriformes species are endangered and some acipenserid species are almost extinct (Birstein, 1993, Birstein *et al.*, 1997). The causes of the disappearance of sturgeon include biological traits of populations (late sexual maturation and long intervals among periods of spawning) and anthropogenic effects (environmental pollution destroying spawning habitats, human interventions preventing the migration of fish to their spawning grounds and overfishing).

The sterlet (*A. ruthenus*) is a relatively small sized species among the fish of the Acipenseridae family, characterized by rapid sexual maturation (Sokolov and Vasiliev, 1989). This is an important game and commercial fish owing to the features which have enabled breeders to obtain fast growing hybrids, for example the bester, a hybrid of the giant sturgeon, *Huso huso* (L.), female and a sterlet male (Chebanov and Billard, 2001). The sterlet is a representative of freshwater sturgeon which creates isolated river populations (Sokolov, Vasiliev 1989). It originally inhabited the rivers of Eurasia, being widely distributed in rivers flowing into the Caspian, Black, Baltic, White, Barents Seas and the Sea of Azov (Berg, 1948). Sterlet stocks have diminished especially due to the damming of rivers (Kotlyarevskaya, 1989) and water pollution (increasing petroleum residues, phenols, PCBs, etc.) causing mass mortality among spawners (Romanov and Altuf'ev 1993, Lepilina and Romanov 2005). In Ukraine, the sterlet is listed in the Red Data Book of Ukraine (Gringevsky, 1995, Fopp-Bayat *et al*., 2008), and occurs in the lower Danube and the Dniester Rivers, where its distribution has been limited since the construction of the Dubosary dam. This fish, at the beginning of the twentieth century, occurred in the Bug River following its probable migration from the Dniester (Wilkosz, 1904). The sterlet population in the Dniester River basin, whose members infiltrated the Bug River, is extremely endangered (Tretyak et al. 2008), while the sterlets in the Bug are completely extinct (Kolman *et al*., 2012). Because the sterlet populations in the Dniester River are drastically diminishing, artificially supported breeding in aquaculture conditions should be employed to either protect endangered populations or to reintroduce extinct populations of sterlet. Many species and populations remain extremely low in abundance and may not be capable of recovery without the aid of deliberate releases of hatchery produced sturgeons. Any risk of negative ecological, genetic or economic impacts of aquaculture can be reduced by a careful survey of genetic inventories of systems which are stocked (Fopp-Bayat *et al*., 2013).

The sterlet population in the Dniester River is extremely threatened. Hence a protection plan has been launched in cooperation between Poland and Ukraine, and a broodstock of spawners has been created in aquaculture conditions in Ishkhan Fish Farm in Ukraine. This fish farm is registered as a fish farm with breeding stocks of sterlet and confirmed by a certificate issued by the Ministry of Agrarian Policy of Ukraine number UA3516673271. The protection plan includes the formation of broodstock, under genetic control, of these fish in aquaculture conditions and production of material for

restoration of the Dniester River population. Such action will help in the future, increasing the number of fish in the existing population and restoring endangered populations.

The starting point for this work was a genetic study of sterlet inhabiting the Dniestr River (small population) and other populations of sterlet of the Eurasian continent. Such studies based on genetic assays allow researchers to identify suitable fish material for the creation of broodstock. By knowing the genetic diversity of a newly created broodstock, breeders are able to carry out genetic monitoring during a restitution program and to trace genetic changes in natural populations and in aquaculture. The purpose of the present study was to perform genetic analysis of the Dniester population of sterlet and to compare it to five other sterlet populations in order to generate information needed for an optimal strategy underlying a conservation program of sterlet in the Dniester River.

Materials and Methods

Fin clips of the sterlet, *A. ruthenus* from six populations were sampled in 2007-2012. The samples were collected from 72 specimens of sterlet and preserved in 96% ethanol until DNA isolation. Sterlet populations from the following rivers were studied: the Dniester (seven samples), Dnieper (five samples), Danube (seven samples), Volga (17 samples), Kama (24 samples) and Ob (ten samples). Genomic DNA for microsatellite amplification was extracted from tissue samples using a Sherlock AX Kit (A&A Biotechnology, Poland).

Eleven microsatellite loci were tested (Afu-54, Afu-68 - May *et al*., 1997, AfuB-68 - Welsh and May, 2006, Aox-23, Aox-27 - King *et al*., 2001, Spl-101, Spl-106, Spl-163, Spl-168, Spl-173 - McQuown *et al.*, 2000, Atr-113 - Rodzen and May, 2002). Analysis of microsatellites was conducted based on six loci which were distinguished by good quality of the amplified product and the polymorphic band patterns: Afu-68, AfuB-68, Spl-163, Spl-101, Spl-106, Spl-173. PCR reaction mixture was prepared in a total volume of 25 µl with 40 ng DNA template, 1x PCR reaction buffer (50 mM KCl, pH 8.5; Triton X-100), 0.4 mM of each primer, 0.25 mM of each deoxynucleotide triphosphate (dNTP), 3.3 mM $MgCl_2$ and 0.6 unit Go Taq Flexi DNA Polymerase (Promega, Madison, WI, USA). Re-distilled water was used to bring the reaction mixture to the desired final volume. PCR reactions were conducted under the following reaction profile: one cycle at 94^{o}C for 5 min, 30 cycles at 94^{o}C for 30s, the locus-specific annealing temperature for 30s, 72^{o}C for 30s, and final extension at 72^{o}C for 10 min. Amplification was conducted in a Mastercycler gradient thermocycler (Eppendorf, Germany).

In order to enable genotyping of PCR products with an Applied Biosystem 3130 Genetic Analyser, forward primers were 5'-labeled with different fluorescent reporter dyes (Afu-68-FAM, AfuB-68-VIC, Spl-163-NED, Spl-168-PET, Spl-101-VIC, Spl-106-FAM). Fluorescent labeled primers as well PCR products were stored in black boxes to be protected against sunlight. The lengths of amplified DNA fragments were determined using an Applied Biosystems 3130 Genetic Analyser

sequencer against GeneScan 600 [LIZ] size standard (Applied Biosystems, California, USA). Individual microsatellite loci amplified using primers with different attached fluorescent dyes were arranged into sets and analyzed in a multiplex mode. Fragment size and allele were determined using the GeneMapper and the Genetic Analyser software (Applied Biosystems, California, USA) according to the manufacturer's recommendations. Genetic profiles containing lists of alleles detected within the examined loci were prepared for each specimen.

The allele frequencies, expected heterozygosity (H_e), observed heterozygosity (H_o) and the mean number of alleles per locus (N_a) for each population were computed using the microsatellite toolkit makro for MICROSOFT EXCEL^{TM} (Park, 2001). The number of effective alleles (N_e), number of private alleles (N_{pa}), the F_{ST} matrix and DA distance matrix of studied populations of sterlet, fixation index (F) and analysis of molecular variance (AMOVA) were calculated using GeneAlex (Peakall and Smouse, 2006). The Fstat software (Goudet, 2001) was used to calculate the allelic richness (A_R) per locus and population of sterlet. A phylogenetic tree was constructed using neighbor-joining clustering (NJ) based on genetic distance, with the aid of the MEGA 5.1 software (Tamura *et al*., 2011).

Results

Among ten microsatellite markers (Afu-54, Afu-68, AfuB-68, Aox-23, Aox-27, Spl-101, Spl-106, Spl-163, Spl-168, Spl-173, Atr-113), just six (Afu-68, AfuB-68, Spl-163, Spl-101, Spl-106, Spl-173) were selected for analysis. All the six selected markers were polymorphic and displayed successful amplification in all the sterlet populations. Table 1 contains data on the expected and observed heterozygosity, mean number of alleles, number of private alleles, number of effective alleles and fixation index. The average expected and observed heterozygosity ranged from 0.75 (Kama population) to 0.86 (Dniester population) and 0.65 (Volga population) to 0.78 (Danube population) respectively. The highest mean number of alleles (11.3) was observed in the Kama population, but the lowest (4.83) occurred in the Dnieper population of sterlet (Table 1). Private alleles were observed in all the populations (Table 1). The highest number of private alleles (3.00) was found in the Volga, while the Dnieper population had the lowest number of private alleles (0.33) (Table 1).

Table 1: Expected heterozygosity (H_e), observed heterozygosity (H_o), the mean number of alleles (N_a), number of private alleles (N_{pa}), Number of effective alleles (N_e) and Fixation Index (F) in studied population of sterlet *Acipenser ruthenus* based on microsatellite DNA analysis; SD – standard deviation.

Population	Dniester	Danube	Dnieper	Ob	Kama	Volga
Sample size	9	7	5	10	24	17
Loci typed	6	6	6	6	6	6
H_e	0.8595	0.7747	0.7630	0.7939	0.7484	0.8119
H_e SD	0.0288	0.0541	0.0561	0.0807	0.0718	0.0190
H_o	0.6852	0.7857	0.7000	0.7000	0.7847	0.6471
H_o SD	0.0632	0.0633	0.0837	0.0592	0.0343	0.0473
N_a	8.17	5.50	4.83	8.00	11.33	8.83
N_a SD	2.48	1.52	1.83	3.03	2.42	1.94
N_{pa}	2.833	0.667	0.333	2.500	2.667	3.000
N_e	5.876	4.079	3.753	5.311	5.027	4.901
F	0.164	-0.075	-0.046	0.037	-0.054	0.190

The allelic richness (A_R) observed at each locus ranged from 1.98 in the Ob population at *Spl-173* locus to 7.87 in the Dniester population at locus *Spl-106* (Table 2). F_{ST} values for each pair of the populations varied from 0.051 (the Kama-Danube) to 0.107 (the Ob-Dnieper) (Table 3). The DA distance of each population pair is shown in Table 3. The genetic variability at microsatellite loci was quantified by calculating the gene diversity (Nei, 1987) per population and locus (Table 4). The Ob population of sterlet showed the lowest gene diversity (0.389) at locus *Spl-173*, while the Dniester population presented the highest gene diversity (0.944) at locus *Spl-106* (Table 4).

Table 2: Allelic Richness (A_R) per locus and population of sterlet *Acipenser ruthenus* based on microsatellite DNA analysis.

Locus	Dniester	Danube	Dnieper	Ob	Kama	Volga	All
Afu-68	4.598	5.549	4.000	6.598	5.754	5.422	6.417
AfuB-68	5.961	5.789	3.000	6.966	5.863	4.511	7.024
Spl-163	6.922	4.845	7.000	6.241	6.652	5.482	6.563
Spl-101	5.843	2.933	5.000	5.768	4.045	5.566	6.299
Spl-106	7.873	5.945	7.000	5.745	5.785	6.180	7.266
Spl-173	5.118	4.077	3.000	1.984	3.497	4.036	4.608

Table 3: The F_{ST} matrix (normal) and D_A distance matrix (bold) of all studied populations of sterlet *Acipenser ruthenus*.

	Dniester	Danube	Dnieper	Ob	Kama	Volga
Dniester	0.000	**0.453**	**0.184**	**0.439**	**0.429**	**0.671**
Danube	0.072	0.000	**0.541**	**0.530**	**0.210**	**0.524**
Dnieper	0.063	0.104	0.000	**0.698**	**0.230**	**0.752**
Ob	0.064	0.082	0.107	0.000	**0.353**	**0.822**
Kama	0.063	0.051	0.066	0.061	0.000	**0.560**
Volga	0.069	0.077	0.101	0.088	0.072	0.000

The AMOVA revealed the variation among populations equal 9.0%, and among and within individuals reaching 8% and 83%, respectively (Table 5). The Neighbor-Joining tree (NJ) constructed according to the D_A genetic distance between each pair of populations demonstrated genetic differences (based on microsatellite DNA analysis) among the six populations of sterlet (Figure 1). The narrowest distance was observed between the Dniester and Dnieper populations, while the widest one was observed between the Kama and Volga populations (Figure 1). The NJ tree revealed two different clusters (Figure 1). The first cluster consisted of the Dniester, Dnieper, Ob and Kama populations and the second one was composed of the Danube and Volga populations.

Table 4: Gene diversity (Nei, 1987) per locus and population of sterlet *Acipenser ruthenus*.

Locus	Dniester	Danube	Dnieper	Ob	Kama	Volga
Afu-68	0.826	0.881	0.650	0.894	0.850	0.825
AfuB-68	0.910	0.845	0.650	0.922	0.854	0.772
Spl-163	0.910	0.833	0.950	0.872	0.896	0.818
Spl-101	0.868	0.643	0.850	0.883	0.533	0.849
Spl-106	0.944	0.857	0.925	0.833	0.839	0.871
Spl-173	0.764	0.583	0.600	0.389	0.514	0.767

Table 5: AMOVA analysis of sterlet populations based on microsatellite DNA variation.

Source of variation	Degree of freedom	Sum of squares	Variance component	Percentage of variation
Among populations	5	39.053	0,232	9%
Among individuals	66	169.801	0.203	8%
Within individuals	72	156.000	2.167	83%
Total	143	364.854	2.602	100%

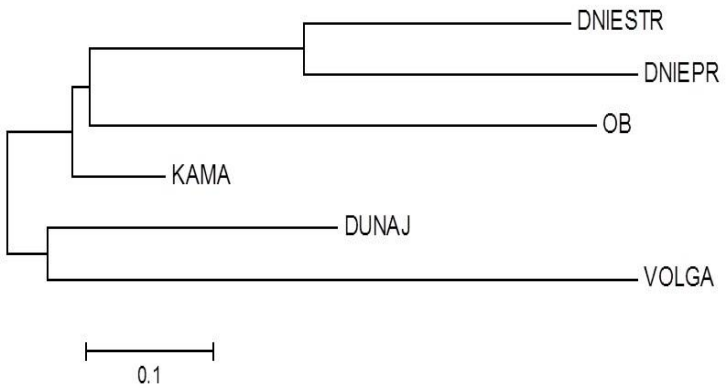

Figure 1: Neighbor-Joining tree (1000 bootstrap replications) of sterlet populations based on D_A distance values.

Discussion

The research provided particularly useful information for the effective conservation of the Dniester River population of sterlet and generated data that are output indicators of the gene pool in the newly formed reproductive broodstock. This study revealed high genetic diversity within the six populations of sterlet. It is very important to continue investigations which will provide us with more information about the genetic diversity in the analyzed population so as to plan an appropriate conservation strategy of sterlet resources in the Dniester River. Despite the recent decrease in the size of the sterlet population in the Dniester River, the population has not lost the rare private alleles. Therefore, adequate management is very important during the restitution of this unique population of sterlet.

The results of the phylogenetic analysis, compared with the other populations, showed the population that is genetically closest to the Dniester sterlet population, which may be useful during the restitution programme. If the size of the sterlet population in the river Dniester is insufficient, the broodstock can be refilled based on sterlets from the closest population to the restored population, in this case it is the Dnieper River population (genetic distance–0.184). Another vital finding is the most distant sterlet population (in relation to the population of the Dniester), which is the one in the Volga River (genetic distance–0.671). This information will be essential to justify the need of adaptive management for adequate protection of the genetic diversity of these fish and to develop management plans which will reflect genetic considerations. Effective conservation of the genetic diversity of the sterlet in the Dniester River heavily depends on the recognition of genetic diversity patterns of sterlet broodstocks in the conservation region. It is particularly important to sustain the genetic diversity of sterlet on a fish farm by regular monitoring of the genetic diversity of broodstock and progeny. Knowledge of the genotypes of tagged spawners enables controlled breeding based on genetic profiles of spawners so as to maintain the

genetic variation on an optimal level, thus preventing the inbreeding of the stock (Kaczmarczyk and Fopp-Bayat, 2013). It is then possible to produce stocking material with optimum genetic parameters, shaping and maintaining the genetic diversity of the stocked population over time. It is very important to monitor constantly the genetic condition of the broodstock. When the parameters of genetic diversity reach critical values, the broodstock should be amended with spawning individuals characterized by high genetic variation. In such a case, selection of spawning pairs based on genetic profiles is essential (Kaczmarczyk and Fopp-Bayat, 2013).

The use of genetic markers in aquaculture provides valuable information about the genetic structure of a population and its genetic condition in captivity. In aquaculture, the gene pool of fish stocks is sometimes characterized by reduced diversity (decreased by drift over time). Genetic studies of fish show that small founding populations may quickly lose rare alleles (Allendorf, 1986). Usually, it is a consequence of wrong breeding methods, for example using only a few individuals with distinct characteristics chosen for a specific purpose, e.g. production of thousands of offspring (Wedekind, 2002). This method has an inherent risk of causing the bottleneck effect and inbreeding. If genetically depleted individuals from hatcheries are released into the wild, they may adversely affect wild fish (depletion of gene diversity, increased competition, contamination with pathogens or parasites) (Aho *et al*., 2006). Therefore, it is very important to maintain an optimal, effective population size per generation. Pante *et al*. (2001) suggested the size of an effective population between 25 and 94 individuals, for example broodstock in the breeding programmes of the Finnish Game and Fisheries Research Institute comprises at least 50 individuals of *Oncorhynchus mykiss*. In such studies, multi-locus molecular markers (e.g. microsatellite DNAs) can be used for monitoring the retention of diversity. The breeding system (e.g. small broodstock, sex-ratio biases, harem formation, inbreeding, high reproductive variance among individuals, etc.) and fluctuations in the population size over time can reduce the effective population size (Ne) and result in the loss of allelic diversity in a fish stock. Proper identification and analysis of recent dramatic changes in the population size (e.g. population bottleneck) seem to be an important aspect of any population monitoring programmes (Lacy, 1989).

Studies on the population genetics of sterlets were conducted by Reinartz *et al*. (2011) and comprised fish from the Danube, Volga and Kuban Rivers. They were an essential prerequisite for a conservation programme of the sterlet in the Danube River near the border between Germany and Austria. In the cited research the authors observed no relevant genetic differences among sterlets from different sampling points on the Danube River (Reinartz *et al*. 2011). Recently the new disomic single-locus DNA microsatellite markers were developed for Persian sturgeon (*A. persicus*) of the Caspian Sea (Moghim *et al*., 2013). The new developed microsatellite markers are necessary for effective management of Persian sturgeon and could be used to characterize the

natural populations of this species (Moghim *et al*., 2013). Moreover sometimes restitution is conducted based on genetically uncontrolled material from artificially reproduced stock. These fish are often raised in aquaculture conditions, thus increasing the danger of introducing non-autochthonous species or even hybrids. The genetic contamination of native acipenserid and the loss of biodiversity of broodstock have been indicated by Reinartz *et al*. (2011). Non-native sterlets (from the Volga River) or hybrids (the Volga-Danube) were discovered in the Danube River. During this study, inter-species hybrids between Siberian sturgeon and sterlet were also identified. Most Danube sterlets (approximately 77%) were pure native fish (Reinartz *et al*., 2011). To avoid the risk of negative introgression of native sterlets or hybridizations with exotic species, only native sterlet genotypes should be used for supportive stocking (Reinartz *et al*., 2011). In this case, genetic monitoring is very important for the conservation of natural populations and restocking plans.

In conclusion, the planned restitution programme of sterlet in the Dniester River must be executed based on the autochthonic reproducers originating from the Dniester drainage and should include instruments for protecting biodiversity, namely monitoring genetic diversity and controlling inbreeding in the broodstock. The advantages of aquaculture fish rearing and supportive release of produced fish will be the key points in the management of the sterlet population in the Dniester River. Successful stocking and protection of the sterlet population should be related to the successful hatchery breeding (with genetic monitoring of spawners) based on effective population sizes, which will be used to found new generations of sterlet. Additionally, the hatchery breeding should be conducted using pairs of spawners with identified genotypes to control the genetic diversity of progeny.

This preliminary research is the key point for the sterlet restitution plan in the Dniester River. The analyses described herein may be applied to genetic monitoring of the sterlet population during its restitution in the Dniester River.

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