

GENETIC CHARACTERIZATION OF ATLANTIC STURGEON STOCKING MATERIAL USED IN LITHUANIA TO RESTORE THE BALTIC SEA POPULATION

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Background. Recent genetic analyses of fish remains, obtained from archaeological sites, revealed that more than 2000 years ago the Baltic Sea was inhabited by sturgeons closely related to the Atlantic sturgeon, *Acipenser oxyrinchus* Mitchell, 1815. As some wildlife populations of the Atlantic sturgeon are still present in Canadian rivers, we decided to use those fish to restore the extinct Baltic population.

Materials and methods. Fin clips of 50 *A. oxyrinchus* were collected from fry representing two hatcheries. At the hatchery No. 1 sturgeons were reared from fertilized eggs received from the Acadian Sturgeon and Caviar Inc., Canada. At the hatchery No. 2, larvae provided by the Regional Research Institute for Agriculture and Fisheries (Germany) were reared for several months until they were released into rivers. The molecular data of 22 and 28 specimens from hatcheries No. 1 and No. 2, respectively, were compared with homological D-loop sequences and some microsatellite loci derived from two museum specimens of sturgeons from the Tadas Ivanauskas Museum of Zoology, Kaunas, Lithuania. Signs of possible introgressive hybridization between *Acipenser sturio* Linnaeus, 1758 and *A. oxyrinchus* in two museum specimens were checked by comparison of alleles at loci *AoxD161*, *AoxD188*, *AoxD297*, *AoxD242*, and *AoxC30*. A possible affinity of between sturgeons released in Lithuanian rivers in 2015 and samples representing hatcheries No. 1 and No. 2 was estimated based on multi-locus genotyping data using the Structure software, version 2.3.4.

Results. The same D-loop haplotype H1 characteristic for the museum specimens (MK637525 and MK637526) was also found as the only haplotype distributed among sturgeons reared in hatcheries and used for population re-establishment. A possible hybridisation between *A. sturio* and *A. oxyrinchus* was not confirmed for two museum specimens studied. The first results of genetic application undertaken in Lithuania also revealed the possibility to discriminate the individuals obtained from different hatcheries based on the set of 13 microsatellite loci *Aox45*, *AoxD54*, *AoxD161*, *AoxD297*, *AoxD188*, *AoxD234*, *AoxD242*, *AoxC45*, *AoxC30*, *AoxD241*, *AoxD64*, *AoxD186*, and *Ls-68*. The analysis of *A. oxyrinchus* specimens representing F1 generation of parental individuals reared in two separate hatcheries in Canada and Germany revealed significant differences in the allele composition and high probabilities for individuals released into the natural environment to be assigned to the hatchery of their origin.

Conclusion. The results of the molecular analysis will be useful for identification of age, pedigree, and for initiating genetic monitoring of the restored population of the Atlantic sturgeon in the Baltic Sea.

Keywords: *Acipenser oxyrinchus*, genetic diversity, D-loop, DNA microsatellites

INTRODUCTION

Sturgeons have a long lifespan, delayed maturation, and complex life cycle, involving anadromy (Sulak and Randall 2002, Williot et al. 2011) and these factors could have been beneficial while going through severe environmental changes in the past (Dudu et al. 2011). Nevertheless, today these features, along with extensive

anthropogenic activity, have a strong impact on declines of sturgeon populations in their natural habitats all over the world (Billard and Lecointre 2001, Bloesch et al. 2006). According to the IUCN Red List of Threatened Species, only two out of 25 species in the family Acipenseridae have the status of least concern. Whereas 16 species are critically endangered, two are considered

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endangered, three species are vulnerable, and two species are considered near threatened (Nelson et al. 2013).

Atlantic sturgeon, *Acipenser oxyrinchus* Mitchell, 1815 (see Birstein and Doukakis 2000), once was abundant in the Baltic Sea. It is thought, that *A. oxyrinchus* migrants had entered the Baltic Sea at least 4000 years ago and became a dominating species by replacing *Acipenser sturio* Linnaeus, 1758 approximately 2000 years ago (Popović et al. 2014, Chassaing et al. 2016). Seemingly, European waters had been colonized by migrants originating from North America, as studies of mtDNA D-loop revealed Baltic population to have had similar haplotypes A and B as St. Lawrence, St. John, and Hudson rivers populations in North America (Wirgin et al. 2000, Popović et al. 2014). However, the Baltic Sea population of *A. oxyrinchus* is now extinct in the wild as a consequence of intense anthropogenic activity, in particular, overfishing (Ludwig et al. 2002, Dadswell 2015). The last recorded individual in the Baltic Sea was caught off the coasts of Estonia in 1996 (Paaver 1999, Panagiotopoulou et al. 2014), which is considered as the year of the final extinction of the *A. oxyrinchus* population in Europe.

There are several restitution programs launched in Europe with the aim to re-establish local populations of *A. oxyrinchus* by breeding hatchery individuals and releasing them into local rivers. Currently, such restitution measures for sturgeons in western Europe are being carried out in Italy, France, and Germany (Williot et al. 2002). There are similar efforts in the Danube catchment area (Bloesch et al. 2006), Turkey (Memiş et al. 2006), and Poland (Kolman et al. 2011, 2014). Recently, a special program aimed to re-establish an extinct population of *A. oxyrinchus* has been approved by the Ministry of Agriculture of the Republic of Lithuania and increasing numbers of sturgeons are released into inland water bodies of Lithuania starting from 2011 and this program will continue until 2020.

The first results of the application of molecular markers used for estimation of genetic background of *A. oxyrinchus* stock released in natural environment during few years period of implementation of the restitution program undertaken in Lithuania and the analysis of two samples collected from museum specimens, which represent the extinct population of Atlantic sturgeon, serves as the basis for evaluation of genetic diversity among captive-bred individuals, as well as a dataset for the future genetic monitoring of restored population.

MATERIAL AND METHODS

Fin clips of 50 *A. oxyrinchus* individuals were collected from fry representing two distinct hatcheries. At hatchery No. 1*, located in Rusnė (Lithuania), sturgeons were reared from fertilized eggs received from Acadian Sturgeon and Caviar Inc., Canada. At hatchery No. 2, located in Simnas (Lithuania), larvae provided by Regional Research Institute for Agriculture and Fisheries (Germany) were reared for several months until being released in the Neris

and Šventoji rivers. Tissue samples representing two different hatcheries consisted of 28 and 22 individuals, respectively. Samples were preserved in 70% ethanol and used for DNA analysis. Additionally, dry fin-clips of two museum specimens of sturgeons (No. 153 and No. PP-0146) were collected at the Tadas Ivanauskas Museum of Zoology in Kaunas, Lithuania. Both individuals were caught in 1960 in the Baltic Sea, near Nida (Lithuania), and previous investigators identified them as representatives of *A. sturio*. DNA from both museum specimens and fresh tissue samples were extracted using Aljanabi and Martinez (1997) method.

A molecular investigation of representatives of museum specimens as well as two hatcheries was carried out by sequencing 196 base pairs (bp) D-loop fragment (mtDNA) and performing a fragment analysis of 13 microsatellite DNA loci.

The 196 bp D-loop fragment was amplified using a pair of primers HETERO1 (5'-ACCCTTAACCTCCCAAAG-3') and HETERO2 (5'-CATTTAATGGTAGATGAAAC-3') (Popović et al. 2014). The PCR volume for each sample was 25 µL and consisted of 1 × PCR buffer with 50 mM KCl, 0.2 mM dNTP, 5 pmol of each primer, 2.5 mM MgCl₂, 0.75 U Taq DNA Polymerase LC (Thermo Fisher Scientific Baltics, Lithuania), and 0.2 µg template DNA. PCR conditions were as follows: initial denaturation for 5 min at 95°C, 40 cycles for 30 s at 95°C, 25 s at 52°C, 25 s at 72°C, and the final extension step for 5 min at 72°C. The obtained mtDNA D-loop sequences of museum specimens were deposited in GenBank (MK637525-MK637526).

The 13 microsatellite DNA loci (*Aox45*, *AoxD54*, *AoxD161*, *AoxD297*, *AoxD188*, *AoxD234*, *AoxD242*, *AoxC45*, *AoxC30*, *AoxD241*, *AoxD64*, *AoxD186*, *Ls-68*) were selected and amplified in accordance with the methodology provided by Panagiotopoulou et al. (2014). These loci were amplified using PCR reaction mixture of 25 µL containing 0.25 µg of DNA, 5 pmol of each primer (the 5' ends of forward primers were fluorescently labelled), 0.2 mM of dNTPs, 3 mM of MgCl₂, 1 × buffer with KCl, and 0.625 U of Taq Polymerase (Thermo Fisher Scientific Baltics, Lithuania). The PCR conditions were as follows: initial denaturation for 15 min at 95°C, 35 cycles for 30 s at 94°C, 30 s at 61°C, or 63°C, 30 s at 72°C and the final extension step for 30 min at 60°C. Both DNA sequencing and DNA fragment analysis were performed using Genetic Analyzer 3500 (Applied Biosystems) at the Laboratory of Molecular Ecology (Nature Research Centre, Vilnius, Lithuania).

Species-specific mtDNA D-loop sequences, representing the most frequent haplotypes of *A. oxyrinchus* and *A. sturio* accessed through GenBank and DNA sequencing data obtained during the presently reported study were aligned using CLC 6.8.1 software**. The Neighbor-Joining dendrogram (Saitou and Nei 1987) was constructed using the same CLC 6.8.1 software. Genetic diversity parameters such as: total number of alleles per locus (N_T), number of effective alleles (N_E),

* Hatchery numbers were assigned solely for the purpose of this study.

** <http://www.clcbio.com>.

observed heterozygosity (H_o), and expected heterozygosity (H_e) were calculated using GenAlEx software version 6.5 (Paekal and Smouse 2012).

Signs of possible introgressive *hybridization between A. sturio* and *A. oxyrinchus* in two museum specimens were checked by comparing alleles at loci *AoxD161*, *AoxD188*, *AoxD297*, *AoxD242*, and *AoxC30*, as the allele range sizes of Atlantic and European sturgeons did not overlap at these loci.

The genetic structure of the population of *A. oxyrinchus* released into inland water bodies of Lithuania in 2015 was assessed and possibility to assign each studied fish to one of the samples representing different hatchery was estimated based on multi-locus genotyping data using Structure software, version 2.3.4 (Pritchard et al. 2000).

RESULTS AND DISCUSSION

Despite the relatively old age of the museum specimens (No. PP-1146 and No. 153) a sufficient amount of good quality DNA was extracted from dry fin-clips and the targeted fragments of mtDNA control region were amplified successfully. Newly obtained 180 bp sequences of the two museum sturgeons were aligned with the homologous sequences of both *A. sturio* (GenBank accession numbers FN256383, AJ428274, FN256380) and *A. oxyrinchus* (GenBank accession numbers AF162716.1, AF162721.1, AF162729.1, AF162722.1, AF162726.1) and phylogenetic relations of the above-mentioned sequences inferred by NJ reconstruction revealed the two museum specimens of sturgeons to be attributed to haplotype A of *A. oxyrinchus* (Fig. 1).

A comparison of the mtDNA D-loop sequences of both museum specimens and additionally obtained homologous sequences of captive-bred sturgeons released into Lithuanian inland waters in 2015 revealed that all the studied individuals of sturgeons represented the same haplotype A (GenBank access number AF162716.1) known as the most frequent haplotype H1, detected among representatives of extinct population of *A. oxyrinchus* (see Popović et al. 2014).

Evaluation of introgressive *hybridization between A. sturio* and *A. oxyrinchus*, described earlier (Popović et al. 2014), has become possible after comparing allele sizes

(bp) at loci *AoxC30*, *AoxD297*, *AoxD242*, *AoxD188*, and *AoxD161* discriminating a wild population of *A. oxyrinchus* from *A. sturio* and a successful identification of alleles at three out of five latter microsatellite loci for two museum specimens (Table 1). Due to the fact that allele sizes at loci *AoxD188*, *AoxD242*, and *AoxD297* fall into the range characteristic of *A. oxyrinchus* signals of possible hybridisation between *A. sturio* and *A. oxyrinchus* after examining two studied museum specimens No. PP-1146 and No. 153 could not be confirmed.

In order to evaluate the genetic diversity and the genetic structure of the sturgeon population which consists of sturgeons released into a natural habitat, two groups of young sturgeons obtained from hatcheries No. 1 and No. 2 were genotyped. Private alleles characteristic of each hatchery were identified at seven loci out of 13 microsatellite loci examined (Table 2). A smaller number of alleles detected at the majority of loci examined, as well as the number of private alleles, indicate that the population of sturgeons representing hatchery No. 1 was founded by a reduced number of parental individuals in comparison to those of the hatchery No. 2.

A comparison of genetic diversity parameters (the total number of alleles per locus (N_t), the number of effective alleles (N_e), observed heterozygosity (H_o), and expected heterozygosity (H_e) confirmed the diversity between the two studied samples representing hatcheries No. 1 and No. 2 but also it revealed the higher than expected heterozygosity characteristic of representatives of the hatchery No. 1 in the majority of studied loci except for *AoxD161* and *AoxC30* and for the representatives of the hatchery No. 2 at locus *AoxD54* (Table 3). The majority of loci under study exhibited more than two alleles (N_a) in the two studied stocks of *A. oxyrinchus* and the genetic diversity between those groups of fish were identified on the basis of many private alleles. Subsequent restocking, based on different stocking material, can increase these indicators on a global scale and will have a positive impact on the population of *A. oxyrinchus* in the Baltic Sea basin.

A similar genetic analysis of *A. oxyrinchus* restocking material derived from Canada was conducted during the restitution program in Poland where the restocking material was released in the local Polish rivers of the

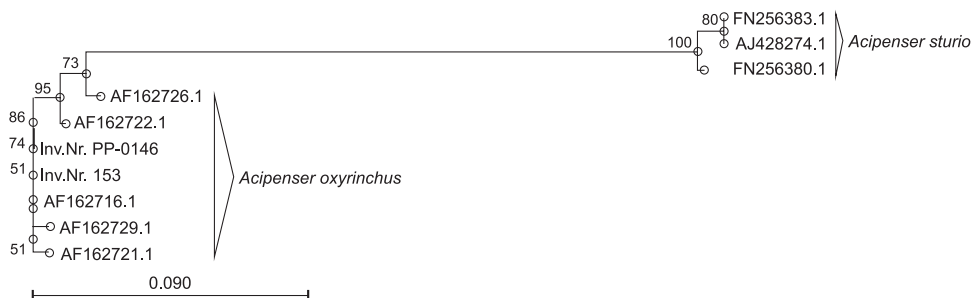


Fig. 1. Phylogenetic relations of mtDNA D-loop sequences of two museum sturgeon specimens No. PP-1146 and No. 153 with homologous sequences of *Acipenser sturio* (GenBank access numbers FN256380.1, FN256383.1, AJ428274.1) and *A. oxyrinchus* (GenBank access numbers AF162726.1, AF162722.1, AF162716, AF162729.1, AF162721.1) inferred using the Neighbor-Joining method; numbers above each branch show bootstrap values (1000 replicates)

Baltic Sea basin, e.g., Drwęca and Drawa rivers (Kolman et al. 2014). The participation of different countries and the multistage restitution program can positively affect the genetic condition of the sturgeon population in the Baltic Sea.

Although formation of parental generation is dependent on a small number of spawning fish collected from the wild population (in case of sturgeon farm located in Canada) or it directly depends on a limited number of *A. oxyrinchus* individuals reared artificially (farms situated in Germany and Poland) it does not lead to the production of F1 generation with deficiency of heterozygotes (Table 3). Due to this phenomenon, good starting conditions and the possible formation of a highly variable and viable re-

established population of *A. oxyrinchus* in the Baltic Sea Region could be predicted.

Although the number of alleles for artificially reared *A. oxyrinchus* population examined in Poland (Panagiotopoulou et al. 2014) was considerably higher in comparison to the gene pool determined at the same loci studied in Lithuania the genetic diversity of the re-established population is expected to increase over time. Subsequently, a higher genetic diversity of the re-established population could be reached by adding new genetically unrelated female and male individuals participating in fertilization of eggs in hatcheries.

The process of restoration of the extinct population of *A. oxyrinchus* in the Baltic Sea region should be

Table 1

Allele sizes (bp) at loci *AoxC30*, *AoxD297*, *AoxD242*, *AoxD188*, *AoxD161* discriminating wild population of *Acipenser oxyrinchus* from *A. sturio* (according Popović et al. 2014) and alleles characteristic for hatcheries No. 1 and No. 2 and identified in museum specimens No. PP-1146 and No. 153

Locus	Allele size				
	<i>A. oxyrinchus</i>	<i>A. sturio</i>	hatcheries No. 1 and No. 2	No. PP-1146	No. 153.
<i>AoxD161</i>	126–158	162–170	114–147	—	—
<i>AoxD297</i>	185–353	169–173	183–223	202	195
<i>AoxD242</i>	163–215	243–315	138–192	165	—
<i>AoxC30</i>	269–341	257	265–301	—	—
<i>AoxD188</i>	262–354	242–250	283–342	305	338

Table 2

A comparison of the number and size of different alleles detected at seven microsatellite loci in two samples of young sturgeons representing hatcheries No. 1 and No. 2

Hatchery	Locus	Allele size							
		<i>Aox45</i>	<i>AoxD54</i>	<i>AoxD161</i>	<i>AoxD297</i>	<i>AoxD188</i>	<i>AoxD234</i>	<i>AoxD242</i>	
No. 1	Size [bp]	127	176	116	195	283	317	188	
		130	204	122	203	306	252	192	
		142		124	211	342	268	194	
				126	223		288	198	
				147	303		368		
			149	311					
		N_T	3	2	6	6	3	5	4
		N_p	0	0	4	3	1	2	1
	No. 2	Size [bp]	109	176	126	195	306	217	162
			127	196	130	199	310	221	166
130			200	138	211	338	225	170	
136			204	147	223	342	233	192	
142			216	151	300		237	194	
		224				268	198		
						276			
						284			
						288			
						300			
	N_T	5	6	5	5	4	10	6	
	N_p	2	4	3	2	2	7	3	

Size = allele size, N_T = total number of alleles per locus, N_p = number of private alleles (private alleles are shown in bold).

controlled by evaluating the population genetic structure of *A. oxyrinchus* released into inland water bodies. Informative and promising results could be obtained with the help of such genetic tool as the program Structure (Pritchard et al. 2000). Testing of two stocks of sturgeons representing two hatcheries No. 1 and No. 2 revealed the possibility to assign each studied fish to the sample that covers individuals belonging to the same spawning pool (Fig. 2).

The results of the presently reported molecular analyses will be useful for the identification of age of fish released into the natural environment in case it is captured and genotyped in the future and the obtained molecular data will be compared with the accumulated genetic data of young sturgeons representing different hatcheries in different years. The age of captured sturgeons will be identified by attributing individual genotypes to one of the samples representing the released sturgeons that had been reared in different hatcheries during the period of the implementation of the program of the restoration of *A. oxyrinchus* in the Baltic Sea.

CONCLUSIONS

Two museum specimens No. PP-1146 and No. 153, representing the extinct Baltic population of sturgeons were characterized by the same haplotype of D-loop fragment that was identified in the representatives of *A. oxyrinchus* participating in the process of population re-establishment in Lithuania.

A comparison of the genetic diversity parameters and the genetic structure based on the 13 microsatellite DNA loci analysis, revealed significant differences in allele composition of representatives of hatcheries No. 1 and No. 2 and high observed heterozygosity of both studied sturgeon samples.

A microsatellite data analysis carried out with the Structure software revealed the possibility to discriminate representatives of hatcheries No. 1 and No. 2 by assignment each individual to one of two respective samples.

The results of the molecular analysis will be used for the future genetic monitoring of the population of *A. oxyrinchus* undergoing the process of re-establishment.

Table 3

Comparison of genetic diversity parameters of representatives of hatchery No. 1 and hatchery No. 2 at 13 microsatellite loci

Locus	Hatchery No. 1					Hatchery No. 2				
	N	N_T	N_E	H_O	H_E	N	N_T	N_E	H_O	H_E
Aox45	28	3	2.95	0.82	0.66	22	5	3.24	0.77	0.69
AoxD54	28	2	1.77	0.64	0.44	22	6	1.94	0.36	0.48
AoxD161	28	6	2.37	0.43	0.58	22	5	3.80	0.91	0.74
AoxD297	27	6	4.43	0.93	0.77	22	5	4.57	0.86	0.78
AoxD188	27	3	2.45	0.89	0.59	22	4	2.06	0.55	0.51
AoxD234	28	5	4.03	1.00	0.75	22	10	6.05	1.00	0.83
AoxD242	26	4	2.80	0.81	0.64	22	6	2.86	0.86	0.65
AoxC45	28	1	1.00	0.00	0.00	22	1	1.00	0.00	0.00
AoxC30	28	2	1.77	0.29	0.44	22	2	1.15	0.14	0.13
AoxD241	28	3	2.67	1.00	0.63	22	7	5.69	1.00	0.82
AoxD64	26	3	2.73	1.00	0.63	19	7	5.69	0.95	0.82
AoxD186	27	4	3.85	0.93	0.74	21	6	3.69	0.81	0.73
Ls68	28	2	1.60	0.50	0.38	22	5	3.57	0.86	0.72

N = number of individuals, N_T = total number of alleles per locus, number of effective alleles (N_E), H_O = observed heterozygosity, H_E = expected heterozygosity in two samples of young sturgeons representing hatcheries No. 1 and No. 2.

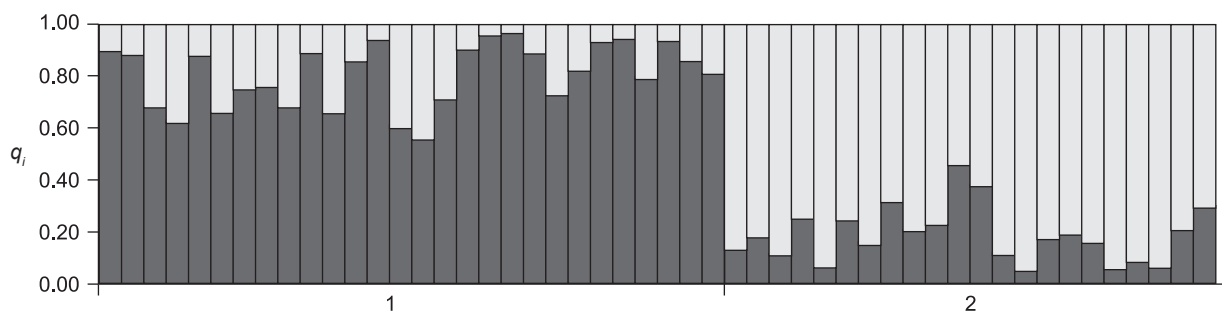


Fig. 2. Assignment of individual fish to one of the studied samples of *Acipenser oxyrinchus* representing hatcheries No. 1 (1) or No. 2 (2) estimated based on multi-locus genotyping data using Structure software, version 2.3.4 (Pritchard et al. 2000); q_i is individual proportion of membership to each of two clusters indicated in light or dark grey, respectively

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