Analysis of ancient mitochondrial DNA of the Baltic Sea sturgeon (*Acipenser* sp.)

Dorota Fopp-Bayat[1], Slawomir Ciesielski[2], Miroslaw Luczynski[2]

[1]Department of Ichthyology, Faculty of Environmental Sciences and Fisheries, University of Warmia and Mazury in Olsztyn, 10-718 Olsztyn, Oczapowskiego 5, Poland, e-mail: foppik@uwm.edu.pl
[2]Department of Environmental Biotechnology, Faculty of Environmental Sciences and Fisheries, University of Warmia and Mazury in Olsztyn, 10-718 Olsztyn, Sloneczna 45G, Poland
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ABSTRACT

Genetic relatedness between Baltic Sea sturgeon (*Acipenser sturio* L.) specimens caught in different geographic areas is not clear. According to previous studies, fish captured in different locations within the historic area of *A. sturio* habitation are genetically different to each other. We have examined a fragment (191 base pairs) of mitochondrial *cytochrome b* gene of four specimens of *A. sturio* found in Poland: three fish were preserved in museums of natural history and the bone of one fish was from an archaeological site. DNA sequences of the three museum samples were identical, whereas the DNA sequence of the archaeological sample differed in the 918 position of the *cytochrome b* gene. All the analyzed DNA fragments were similar to those of Acipenser baeri and genetically distant to *Acipenser sturio* and *A. oxyrinchus*.

INTRODUCTION

The European Atlantic sturgeon, also called the Baltic Sea sturgeon (*Acipenser sturio* L.) is an endangered anadromous fish species (IUCN 2003). Historically, this species thrived in the northeastern Atlantic Ocean, in parts of the North Sea, in the Baltic Sea as well as in the Mediterranean and Pontic regions (Holčik 2000). It has been commercially important since ancient times.

At the beginning of the 20th century *A. sturio* occurred along the entire coastline of Europe, from the White Sea through the Norwegian, North, Baltic, Atlantic ocean, Mediterranean to the Black Sea (Holčik et al. 1989; Kolman and Zarkua 2002). In Poland, the Odra and the Vistula (and their tributaries) were the two major rivers where the sturgeons were common and which they ascended to spawn (Kulmatycki 1933). Damming of rivers and intensive fishery reduced the abundance of the Baltic Sea sturgeon to such an extent that in the 1920s the species was regarded as endangered within the Baltic Sea basin (Kulmatycki 1933). In the second half of the 20th century only single individuals were occasionally caught (Birstein and Doukakis 2000; Rudnicki 1966; Zelichowska 1964). In 1996 *A. sturio* was included in The Project of Migratory Fish Restoration in Poland (Sych et al. 1996). Currently, only a small population of *A. sturio* exists in the Gironde River system in France (Birstein and Doukakis 2000).

Genetic relatedness between *A. sturio* from different geographic locations is not clear. According to Birstein et al. (1998a), fish captured in different locations within the historic area of *A. sturio* occurrence genetically differ from each other. Based on *cytochrome b* (*cyt b*) gene analysis of *A. sturio* individuals obtained from different locations, Birstein at al. (1998a) showed its intraspecific genetic differentiation.

Restoration of an endangered species needs data to be gathered on its genetic characteristics. As the Baltic Sea sturgeon became very rare (if not extinct) in Europe, it was necessary to turn to museum specimens. In a variety of situations, studies of ancient DNA have helped in solving genetic and biological issues when live specimens were practically unavailable (for example, Ciesielski 2001; Ciesielski et al. 2002).

The research on ancient DNA is usually focused on the analysis of mitochondrial DNA (mtDNA) fragments. As each vertebrate cell contains numerous mtDNA molecules, some intact mtDNA often persist in samples of ancient tissues (Hagelberg et al. 1991). Among the mitochondrial genes the *cyt b* is probably the most extensively studied mtDNA segment in fish (for example, Birstein et al. 1998a; Kocher et al. 1989).

The objective of our study was to establish a technique for the investigation of archival samples of *A. sturio*. In this study the fragment (191 bp) of mitochondrial *cyt b* gene in three museum specimens of *A. sturio* and in one bone of *A. sturio* obtained from an archaeological site was amplified and sequenced. Comparison of our data to the respective DNA fragments obtained from GenBank showed that all the analyzed sequences were most similar to those of *A. baeri* and genetically distant to *A. sturio* and *A. oxyrinchus*.

MATERIALS AND METHODS

Sample collection

Samples were acquired from the collections of three Polish Natural History Museums: Wroclaw, Szczecin and Gdynia (Table 1). In the case of museum specimens, pieces of dried tissues were cut out from the dry-preserved fish. The fish specimens were stored in museums for decades and two of them (Gdynia and Szczecin) were whole preserved fish, morphologically identified as *A. sturio*. One sample, a 700-1000 year old bone of *A. sturio*, was obtained from an archaeological excavation site located in Gdansk (northern Poland).

Table 1. List of studied specimens of the Baltic Sea sturgeon

Sample No.	Place and time of the fish catch	Place of specimen storage	Catalogue Number of specimen	Total Length (TL, cm) of a specimen and analyzed tissue
1.	Odra River near Wroclaw, beginning of the XXth century	Museum of Natural History, Poland Wroclaw University	none	unknown/skin
2.	Vistula River near Chelmno, 1965	Faculty of Marine Fishery and Food Technology, Academy of Agriculture in Szczecin, Poland	MI1-006	280/gills
3.	Vistula River near Tczew, 1972	Oceanographic Museum and Maritime Aquarium, Sea Fisheries Institute, Gdynia, Poland	none	180/skin
4.	Unknown place, X–$XIII^{th}$ century	Archaeological site in Gdansk, Poland	none	unknown/a fragment of a bone

DNA extraction

In order to avoid contamination, the preparation of skin, gills and bone samples and the DNA extractions were performed in one laboratory and stored in a freezer located in another room. Preparation of buffers and PCR set-up was performed in yet another laboratory, which was UV illuminated for periods of time when it was not in use. The DNA extraction was replicated. Thus, the results for the ancient DNA samples were based on repeated analysis derived from two independently processed DNA extracts. For each PCR, reaction blank controls were conducted. Analysis of the 700-1000 year old *A. sturio* bone was conducted two years after the analysis of the three museum samples.

Approximately 80 mg of archival tissues were taken, and hard pieces of the skin were powdered with a fine sand paper. Samples were then transferred into a tube with 500 μl of a lysis buffer (100 mM Tris-HCl, 10 mM EDTA, pH 8.0, 0.5 mg of proteinase K, and 0.25 mg of dithiothreitol) and incubated at 55°C for 8 hours. The aqueous phase was extracted once with an equal volume of phenol, and once with chloroform-isoamyl alcohol solution (24:1). The DNA was precipitated with 0.6 ml of isopropanol for 15 min and then the sample was centrifuged to obtain a DNA pellet. The pellet was washed with ethanol, centrifuged again, and air dried. Finally, the DNA pellet was resuspended in 40 μl of sterile water. The procedure of DNA extraction from archival fish bone has been previously described by Ciesielski et al. (2002).

PCR amplification

The fragment of *cyt b* gene was amplified using primers B7-2 (5'-GCCTACGCCATTCTCCG-3') and s2A (5'-CCTCCAAT-TCATGTGAGTACT-3'), which spanned 191 bp region of the *cyt b* gene from 829 to 1019 nucleotide (Birstein et al. 1998b).

Double-stranded PCR amplifications were performed in 50 μl reaction volumes, containing 2 units of *Taq* DNA polymerase (Promega, Winsconsin, USA), 5 μl of reaction buffer (500 mM KCl, 100 mM Tris-HCl pH 8.5; 1% Triton X-100), 20 pmol of each primer (MWG-BIOTECH, Ebersberg, Germany), 2.5 mM $MgCl_2$, 500 μM of dATP, dCTP, dGTP, and dTTP, and 2 μl of DNA template. DNA was amplified using the Perkin Elmer 9600 thermal cycler (PE-Applied Biosystems, California, USA) beginning with preliminary denaturation at 95°C for 5 min. The amplification cycle consisted of 94°C for 30 s, 52°C for 30 s, and 72°C for 45 s for a total of 30 cycles, ending with a final elongation step at 72°C for 3 min. Initial PCR products of Gdynia and Szczecin samples were reamplified using the same PCR conditions.

DNA sequencing

The PCR product was purified from oligonucleotides, primers and dimers, using GenElute PCR DNA Purification Kit (SIGMA). Then sequencing was performed using a Perkin Elmer ABI 373 automated DNA sequencer and the DyeDeoxy Cycling Sequencing reaction (PE-Applied Biosystems, California, USA). All the amplified fragments were sequenced with both light- and heavy-stranded primers. The results of sequencing were verified using the BLAST program (Altschul et al. 1990). The obtained sequences were deposited in GenBank under the Accession Numbers: AF468655, AY442324, AY971519 and AY971520.

All the sequences were aligned with the help of the Higgins and Sharp algorithm using the program CLUSTAL W (Thompson et al. 1994). The transition/transversion ratio was estimated with the program PAMP included in the package PAML (Yang 1995). The phylogenetic relationship between the selected sturgeon species was inferred by employing a maximum likelihood approach using DNAML software (Felsenstein 1995). The esti-

mated transition/transversion ratio and empirical base frequencies were taken into consideration. The tree was rooted using the homologous sequence of *Huso huso*.

RESULTS

In this study a 191 bp long fragment of the *cyt b* gene was amplified, and a 135 bp long part of it was used for considerations upon phylogenetic relationships among several species of Acipenseridae. The 135 bp fragment of *cyt b* gene, which corresponded to 853-987 nucleotide positions, has been published for all the considered species.

The DNA sequences determined for the 3 museum samples were identical, whereas the DNA sequence of the archaeological sample differed in the 918 position of the *cyt b* gene (Figure 1). The amino acid sequences were identical for all the studied samples.

The comparison of the studied *cyt b* fragment of four sturgeon specimens from Polish collections with those of *Acipenser baeri*, *A. oxyrinchus* and *A. sturio* showed close relatedness of studied specimens to *A. baeri* and their distant relatedness to other sturgeons considered in this study (Figure 2).

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         853
A.s.     CCGAACAAAC TAGGCGGAGT ACTGGCCCTT CTATTCTCCA TCCTAGTCCT AATATTGGTA
A.o.o.   .......... .......... .......... ........T. .T........ ..........
A.o.d.   .......... .......... .......... ........T. .T........ ..........
A.b.     ..A..T.... ....T..... T..A...... ........T. .....A.... ......A..G
Gdansk   ..A..T.... ....T..... T..A...... ........T. .....A.... ......A..G
Wroclaw  ..A..T.... ....T..... T..A...... ........T. .....A.... ......A..G
Gdynia   ..A..T.... ....T..... T..A...... ........T. .....A.... ......A..G
Szczecin ..A..T.... ....T..... T..A...... ........T. .....A.... ......A..G
         913
A.s.     CCAGTCCTCC ACACCTCCAA ACAACGGGGA AATACATTTC GGCCCCTCTC CCAAATCCTA
A.o.o.   .......... .......... ......A... .......... ....T..... ....G.....
A.o.d.   .......... .......... ......A... .......... ....T..... ....G.....
A.b.     .....A.... .......T.. ......A... ..C..G..C. .A.....T.. T.....T...
Gdansk   .....A.... .......T.. ......A... ..C..G..C. .A.....T.. T.....T...
Wroclaw  .......... .......T.. ......A... ..C..G..C. .A.....T.. T.....T...
Gdynia   .......... .......T.. ......A... ..C..G..C. .A.....T.. T.....T...
Szczecin .......... .......T.. ......A... ..C..G..C. .A.....T.. T.....T...
         973
A.s.     TTTTGAGCCC TAGTG
A.o.o.   .......... .....
A.o.d.   .......... .....
A.b.     ..C....... .G...
Gdansk   ..C...C... .G...
Wroclaw  ..C...C... .G...
Gdynia   ..C...C... .G...
Szczecin ..C...C... .G...
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Figure 1. Alignment of partial mitochondrial *cytochrome b* gene sequences from the four studied samples (Gdansk, Wroclaw, Gdynia, and Szczecin; Accession Numbers AY442324, AY971520, AY971520, AF468655, respectively). Sequences of A.s. (*A. sturio* AJ428497), A.o.o. (*A. oxyrinchus oxyrinchus*; AF006163), A.o.d. (*A. oxyrinchus desotoi*; AF006164), and A.b. (*A. baeri* AJ245825) were obtained from the GenBank.

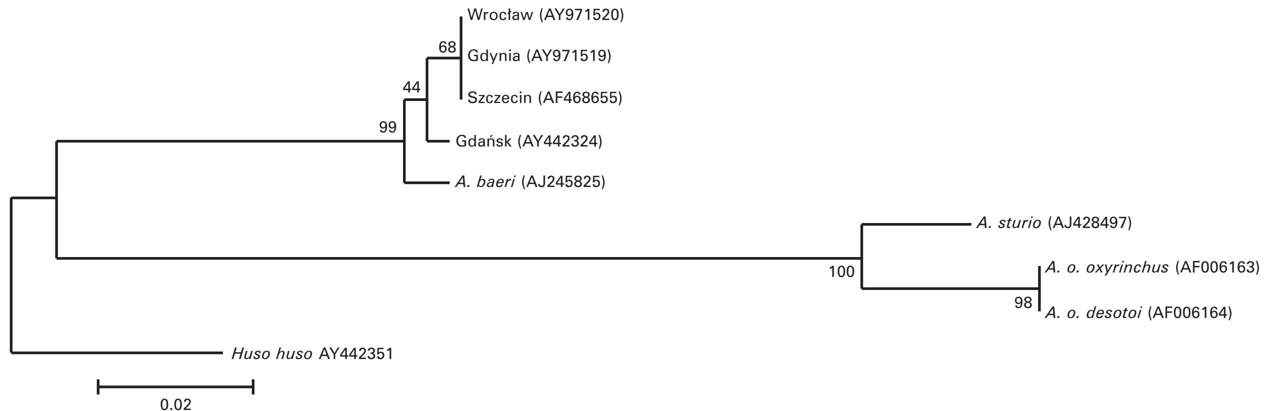

Figure 2. Neighbor-joining tree showing relationships between the analyzed samples and selected members of Acipenseridae family. The accession numbers are given in parentheses. The tree was rooted using homologous sequence of *Huso huso* and numbers adjacent to branch points are bootstrap percentages (n=1000 replicates). Bar = 0.02 shows estimated substitution per nucleotide.

DISCUSSION

DNA derived from archival tissues is often highly fragmented due to autolysis, bacterial degradation and other processes. This reduces the efficiency of PCR, so it is usually impossible to amplify DNA fragments longer than 200 bp (Pääbo 1989). In this study we were able to amplify 191 bp long fragment of the *cyt b* gene, and a 135 bp long part of it was used to study phylogenetic relationships among several Acipenseridae species. However, even using such short sequences, our analysis showed relationships similar to those reported by Birstein et al. (1998a), who based their conclusions on longer sequences of the *cyt b* gene.

Phylogenetic analysis showed that fish coming from Poland and identified morphologically as *A. sturio* belonged to the same clade together with *A. baeri* (Figure 2). *A. sturio*, *A. oxyrinchus oxyrinchus*, *A. oxyrinchus desotoi* were considerably different to those from Polish collections (Figure 1 and Figure 2). This may lead to the question about which sturgeon stock or population may be named *A. sturio* L.

There are stocks of sturgeon designated *A. sturio* in different geographical locations. Holčik (2000) suggests that several species have been confused under the name *A. sturio*, and he proposes a division of all sturgeons designated *A. sturio* into 2 groups. According to the author, the first group named *A. sturio* could encompass populations occurring in northern seas: the Baltic and North Sea. The other group would probably be composed of populations from the Mediterranean, Adriatic and Aegean Seas. Eastern populations, especially those inhabiting the eastern part of the Black Sea, may represent another species which awaits formal description (Holčik 2000).

A comparison of the 295 bp fragments of the *cyt b* of *A. sturio* individuals from different locations showed considerable intraspecific genetic differentiation (Birstein et al. 1998a). The partial *cyt b* gene sequence from the Baltic Sea individuals differed slightly from that of the North Sea (6 nucleotide changes) and from the Gironde River in France (4 nucleotide changes). Ludwig et al. (2000) also show differences in the distribution of microsatellite alleles examined in the museum specimens of *A. sturio* coming from the populations in the North and Baltic Seas. Some meristic characters, such as number of dorsal and lateral scutes, differ in *A. sturio* from the Black Sea to those from the Baltic Sea (Marti 1939), whereas *A. sturio* from the Atlantic Ocean, Mediterranean and Black Seas have a similar number of scutes (Ninua 1976). Based on these data, Holčik et al. (1989) suggest that the Baltic Sea population is probably different to the remaining ones and may constitute a distinct subspecies. Birstein et al. (1998a) support this view, suggesting the existence of intraspecific differentiation within *A. sturio*.

Among the four sturgeon individuals examined in this study, three fish were captured in the 20th century, whereas the fourth sample was obtained from an archaeological site and originated from fish that lived during the Middle Ages. Two tissue samples (museums in Szczecin and Gdynia) were taken from the whole specimens, which were morphologically identified as *A. sturio*. Surprisingly, all the individuals possessed the mitochondrial haplotype similar to *A. baeri* and different to those of *A. oxyrinchus* and *A. sturio* (Figure 1). This result would mean that the identification of the Baltic Sea sturgeon species remains an open issue.

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REFERENCES

Altschul, S.F., W. Gish, W. Miller, E.W. Myers, D.J. Lipman. 1990. Basic local alignment search tool. Journal of Molecular Evolution 42: 459-468.

Birstein, V. J., J. Betts, R. DeSalle. 1998a. Molecular identification of *Acipenser sturio* specimens: a warning note for recovery plans. Biological Conservation 84: 97-101.

Birstein, V.J., P. Doukakis, B. Sorkin, R. DeSalle. 1998b. Population aggregation analysis of three caviar-producing species of sturgeons and implications for the species identification of black caviar. Conservation Biology 12: 766-775.

Birstein, V.J., P. Doukakis. 2000. Molecular analysis of *Acipenser sturio* L., 1758 and *Acipenser oxyrinchus* Mitchill, 1815: A review. Boletin Instituto Español de Oceanografia 16: 61-73.

Ciesielski, S. 2001. Examination of ancient DNA for inferring phylogenetic relationship among fishes. PhD thesis, University of Warmia and Mazury in Olsztyn, 84 p. (in Polish with English summary).

Ciesielski, S., P. Brzuzan, M. Luczynski. 2002. Recovery and analysis of mitochondrial DNA from ancient bones of common bream, *Abramis brama* L. Ancient Biomolecules 4: 43-46.

Felsenstein, J. 1995. PHYLIP (Phylogeny Inferrence Package, Version 3.57c. Department of Genetics, University of Washington, Seattle, WA, 1995).

Hagelberg, E., L.S. Bell, T. Allen, S.J. Jones, J.B. Clegg. 1991. Ancient bone DNA: techniques and applications. Philosophical Transactions of the Royal Society of London, Series B, Biological Sciences 333: 399-407.

Holčik, J. 2000. Major problems concerning the conservation and recovery of the Atlantic sturgeon *Acipenser sturio* L., 1758. Boletin Instituto Español de Oceanografia 16: 139-148.

Holčik, J., R. Kinzelbach, L.I. Sokolov, V.P Vasilev. 1989. *Acipenser sturio* Linnaeus, 1758. In: The Freshwater Fishes of Europe. General Introduction to Fishes. Acipenseriformes (ed. J. Holčik), pp. 367-394. AULA Verlag. Wiesbaden, Germany, 1 II.

IUCN. 2003. IUCN Red List of Threatened Animals. www.redlist.org

Kocher, T.D., W.K. Thomas, A. Meyer, S.V. Edwards, S.F. Pääbo, F.X. Villablanca, A.C. Wilson. 1989. Dynamics of mtDNA evolution in animals: Amplification and sequencing with conserved primers. Proceedings of the National Academy of Sciences of the United States of America 86: 6196-6200.

Kolman, R., Z. Zarkua. 2002. Environmental conditions of common sturgeon (*Acipenser sturio* L.) spawning in River Rioni (Georgia). Electronic Journal of Polish Agricultural Universities, Fisheries, 5, 2 (2002).

http://www.ejpau.media.pl/series/volume5/issue2/fisheries/art-01.html

Kulmatycki, W. 1933. On sturgeon conservation in Polish rivers. Ochrona Przyrody 12: 1-21 (*in Polish*).

Ludwig, A.N., I. Jenneckens, L. Debus, A. Ludwig, J. Becker, F. Kirschbaum. 2000. Genetic analyses of archival specimens of the Atlantic sturgeon *Acipenser sturio* L., 1758. Boletin Instituto Español de Oceanografia 16: 181-190.

Marti, V. Yu. 1939. The biology and harvest of *Acipenser sturio* in the Black Sea. Zoologicheskij Zhurnal 18: 435-442 (in Russian).

Ninua, N.Sh. 1976. Atlantic sturgeon in the Rioni River. Izdatelistvo Metsniereba, Tbilisi, Georgia 22 p. (*in Russian*).

Pääbo, S. 1989. Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification. Proceedings of the National Academy of Sciences of the United States of America 86: 1939-1943.

Rudnicki, A. 1966. The sturgeon: protection, poaching. Gospodarka Rybna 1: 21-22 (*in Polish*).

Sych, R., R. Bartel, K. Bieniarz, J. Mastynski. 1996. Project of migratory fish restoration in Poland. MS, Inland Fisheries Institute in Olsztyn, Poland, 40 p. (*in Polish*).

Thompson, J. D., D.G. Higgins, T.J. Gibson. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673-4680.

Yang, Z. 1995. Phylogenetic analysis by maximum likelihood (PAML) (Version 1.0. Institute of Molecular Evolutionary Genetics, Pennsylvania State University, U.S.A.).

Zelichowska, J. 1964. The sturgeon in the Vistula River near Torun. Gospodarka Rybna 8: 6-7 (*in Polish*).