

SHORT NOTE

MOLECULAR IDENTIFICATION OF *Acipenser sturio* SPECIMENS: A WARNING NOTE FOR RECOVERY PLANS

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Abstract

Partial sequences of the cytochrome b gene were generated for six sturgeons caught recently in the historic range of *Acipenser sturio*. Four samples studied were identified as *A. sturio*: two individuals from the Gironde River (France), and two individuals caught in the North and Baltic Seas. Two remaining samples were identified as from the clade of fishes which includes the Adriatic sturgeon *A. naccarii* and Siberian sturgeon *A. baerii*. Several DNA sequence differences were found between individuals of *A. sturio* from the Gironde River population and from the North and Baltic Seas. A careful genetic evaluation of each captured putative *A. sturio* individual which potentially can be used for breeding in restoration projects is recommended. © 1998 Elsevier Science Ltd. All rights reserved.

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INTRODUCTION

The European Atlantic or Baltic sturgeon *Acipenser sturio* is one of the most endangered sturgeon species (Rochard *et al.*, 1991; Birstein, 1993; Birstein *et al.*, 1997a). Historically, this anadromous species thrived in the Northeastern Atlantic Ocean, especially in the shallow parts of the North and Baltic seas and in the Mediterranean and Pontic regions (Holčík *et al.*, 1989). *Acipenser sturio* has been commercially important since ancient times. Bony remains of this species are known from the Neolithic settlements (3d-2d millennia B.C.) along the southern coast of the Baltic Sea (Tsepkin, 1984) and were found in the Roman Empire cities along the Rhine (Kinzelbach, 1987).

From the 1890s and until the early 1900s, *A. sturio* was so highly overexploited in Western Europe that only a few fish were caught annually in the North and Baltic Seas during the first part of our century (Holčík *et al.*, 1989). In France, the Gironde River population

of *A. sturio* was intensely exploited for caviar since the 1920s, and it decreased dramatically since the 1970s (Rochard *et al.*, 1990). Now this species is listed as endangered by many European countries (Lelek, 1987; Holčík *et al.*, 1989; Lepage and Rochard, 1995) and the IUCN, as well as being placed on the CITES list. *Acipenser sturio* was included in the 1996 IUCN Red List of Threatened Animals with the status Critically Endangered (IUCN, 1996, p. 70).

At present, only a population of about a few thousand Atlantic sturgeon exists in the Gironde River system in France (Rochard *et al.*, 1990; Castelnaud *et al.*, 1991; Williot *et al.*, 1997). In Spring 1995, natural reproduction of *A. sturio* was reported in this river for the first time since 1988 (Anon., 1995). Presumably, there is a small stock of *A. sturio* in the eastern part of the Black Sea (Pavlov *et al.*, 1985, 1994), but all attempts to find these fish in that area during the last 2 years have failed (Artyukhin, pers. comm.). During the last decade single Atlantic sturgeon individuals reappeared in the North Atlantic and Baltic Sea. In 1985 and 1989, two sturgeons were caught in the North Sea near the German coast (Debus, 1995), and another was captured near the Dutch coast in 1994 (Timmermanns and Melchers, 1994). In 1992, two juvenile *A. sturio* were reported from the tributary of the Rhine (Volz and De Groot, 1992). The same year another Atlantic sturgeon was captured in the Guadalquivir Estuary in Spain (Elvira and Almodovar, 1993). In May 1996, a big mature female was caught in the Estonian waters of the Baltic Sea (Paaver, 1996).

There is a great awareness concerning the endangered status of *A. sturio* in Europe. This awareness has resulted in an ongoing recovery project for *A. sturio* in France (Rochard *et al.*, 1990; Williot *et al.*, 1997). In May 1995, for the first time a sturgeon female was captured simultaneously with a male in the Gironde Estuary (Anon., 1995). These fish were artificially bred and juveniles obtained from this breeding were released into the Garonne and Dordogne rivers. There has been a discussion of the need of a similar recovery plan in Spain (Elvira *et al.*, 1991). In addition, on 1 July 1994,

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The Society to Save the Sturgeon *Acipenser sturio* was founded at the Senckenberg Institute in Frankfurt on Main, Germany (Elvira and Gessner, 1996). This Society's aims are to save and protect the last specimens of this species, to build up a reasonable broodstock and organize a restocking programme. The Society has also announced a reward of 10 000 DM for every Atlantic sturgeon captured alive (Hochleithner, 1995).

The present communication reports the results of our comparison and identification by molecular methods of several sturgeons caught in Europe.

MATERIALS AND METHODS

Samples

The location of sturgeon sources is given in Table 1. All tissue samples were fixed in 96% alcohol. Specimen Nos 1–3 were unambiguously identified as *A. sturio*. The species identification of sturgeon samples Nos 4–6 was uncertain, but all three individuals were caught in the historic range of *A. sturio*.

DNA isolation and manipulation

DNA was isolated according to the methods in Birstein *et al.* (1997b,c) and Birstein and DeSalle (1997). Tissue samples were treated overnight in 1 mg/ml final concentration of Proteinase K, 1% SDS and homogenization buffer. Phenol extraction was performed with an equal volume of phenol followed by an extraction with an equal volume of chloroform. The aqueous phase was ethanol precipitated twice and resuspended in ultrapure water for PCR.

PCR was accomplished for a fragment of the cytochrome (cyt) *b* gene. The reactions generated a 1.1 kb fragment using the universal primers situated with the 3' end at positions 105 and 1016 (Irwin *et al.*, 1989).

Sequences were generated with several internal primers (see Birstein and DeSalle, 1997) using an AB1373 automated sequencer. Sequences were manipulated with Sequencher software. The sequences are deposited in GenBank under access numbers AF006123–AF006188.

Species identification and phylogenetic analysis

For the identification of specimens as *A. sturio* we relied on a phylogenetic analysis of DNA sequences. We used the Birstein and DeSalle (1997) cyt *b* data base for all living species of *Acipenser*. Phylogenetic analysis was performed with PAUP program, version 3.1 (Swofford, 1993). Robustness of nodes critical to inferences concerning the placement of samples 4, 5 and 6 were assessed by the Bremer (1988, 1995) index and parsimony jackknife value (Farris *et al.*, 1996).

RESULTS AND DISCUSSION

Species identification

We previously reported the results of a phylogenetic analysis of over 600 bases of the cyt *b* gene of sample Nos 1 and 3 in Birstein and DeSalle (1997). We obtained 300 bases of the cyt *b* gene sequence for three of the remaining samples (Nos 2, 4 and 6) and 150 bases of the cyt *b* gene for sample No. 5 (Fig. 1). Owing to the robustness of the overall phylogenetic hypothesis with respect to the placement of *A. sturio*, sequences from these small regions were adequate to diagnose whether or not specimens 2, 4, 5 and 6 were indeed *A. sturio*. For these short regions of the cyt *b* gene samples Nos 1 and 2 were identical and differed by a few changes from the sequence for No. 3. Sample No. 6 was slightly different from samples 1, 2 and 3. Sequences for samples 4 and 5, on the other hand, were quite different from Nos 1, 2, 3 and 6. We performed a phylogenetic analysis of these

Table 1. List of sturgeon tissue samples studied and cytochrome *b* regions sequenced

No.	Location of sturgeon catch or maintenance	Sturgeon length (Tl) and weight (w)	Sample received	Name of collector	Region of cyt <i>b</i> sequenced ^a
1.	Gironde River Estuary, France (obtained in 1994) ^b	Tl = 45 cm	A fragment of muscles in 96% alcohol	P. Williot	1,2,3
2.	Gironde River Estuary, France (obtained in 1995)	Tl = 46 cm, w = 0.9 kg	A fragment of muscles in 96% alcohol	P. Williot	1,2
3.	North Sea, near the Dutch coast (captured in February 1994) ^b	Tl = 94 cm, w = 2.1 kg	A fragment of muscles in 96% alcohol	L. Debus	1,2,3
4.	Guadalquivir River near Seville, Spain (captured in Sept 1995)	Tl = 60 cm	A fragment of muscles in 96% alcohol	B. Elvira	1,2
5.	Hatchery of Propa-Gen International, Hungary (initially caught in Albania in 1995)	—	A fragment of fins in 96% alcohol	T. Gulyas	1
6.	Baltic Sea, Estonian waters (captured in May 1996)	Tl = 290 cm; w = 135 kg	A fragment of muscles in 96% alcohol	T. Paaver	1,2

^aRegion 1 corresponds to bases 436 through 552, region 2 corresponds to bases 810 through 1020, and region 3 corresponds to bases 105 through 342 from the cytochrome *b* gene of *A. transmontanus*, as represented in Brown *et al.* (1989).

^bSequences for these species are reported in Birstein and DeSalle (1997).

A. Cytochrome *b* - region 1

Sample #1	TTACTAACCTCCTCTCCGCCTTCCCATACATCGGCGACACACTAGTGCAATGAATCTGAGGCG
Sample #2
Sample #3
Sample #4	?????????????????.....T..G.....A.....
Sample #5	?????????????????.....T..G.....A.....T.
Sample #6	?????????????????.....G.....
Sample #1	GCTTTTCAGTGGACAACGCCACCCTCACCCGATTCTTCGCCTTTCACCTTCTTTTACCATTTG
Sample #2
Sample #3
Sample #4A.....T.....T.....C.....C.
Sample #5A.....T.....T.....C.....G.....G.
Sample #6

B. Cytochrome *b* - region 2

Sample #1	GAATGATACTTTCTCTTTGCCTACGCCATCCTCCGATCTATTCCGAACAACTAGGCGGAGTACT
Sample #2
Sample #3C.....
Sample #4C.....T.....C..C..A..T.....T.....T..
Sample #6T..C.....
Sample #1	GGCCCTTCTATTCTCCATCCTAGTCCTAATATTGGTACCAGTCCTCCACACCTCCAAACAACGAG
Sample #2G.
Sample #3G.
Sample #4	A.....T.....A.....A..G.....A.....T.....
Sample #6T.....
Sample #1	GAAATACATTTTCGACCCCTCTCCCAAATCCTATTTTGGACCCCTGGTGGCGACATACTAGTACTCA
Sample #2
Sample #3G.....AG.....A.....
Sample #4C..G..C.....T..T.....T.....C..AG.....
Sample #6G.....AG.....A.....

Fig. 1. Cytochrome *b* sequences for three identified samples (Nos 1–3) and three putative samples (Nos 4–6) of *Acipenser sturio*. See Table 1 for exact location of regions 1 and 2 as well as for the collection information for the six samples. Dots indicate identity with the sequence in the top row.

cyt *b* sequences to determine the placement of Nos 4, 5 and 6 (Fig. 2). Sample 2 was coded as missing for the cyt *b* 2 region in this analysis.

Figure 2 shows that sample No. 6 is part of a monophyletic clade together with the three *A. sturio* examined (Nos 1, 2 and 3) and with two subspecies of the American Atlantic sturgeon, *A. o. oxyrinchus* and *A. o. desotoi*. Specimen No. 5 is the sister taxon to the Adriatic sturgeon, *A. naccarii*, and specimen No. 4 is part of a group of closely related species including *A. baerii*. Taking into consideration that *A. baerii* is widely used for aquaculture throughout Western Europe including Spain (Williot *et al.*, 1993), our guess is that sample No. 4 might be an *A. baerii* individual which escaped from a fish farm. For a definitive species identification we would need to generate a longer sequence of the cyt *b* gene from this sample.

The phylogenetic tree in Fig. 2 was constructed using sequences from relatively short fragments of the cyt *b*

genes of all sturgeon species. But even with the short sequences, it gives relationships similar to those based on the combined data of 1100 nucleotides that we examined previously (Birstein and DeSalle, 1997). The region of the cyt *b* gene we sequenced, however, could be used for the elaboration of the molecular identification test similar to that which we described previously for the three commercial species of sturgeons, *Acipenser gueldenstaedtii*, *A. stellatus* and *Huso huso* (DeSalle and Birstein, 1996; Birstein *et al.*, 1997b).

Intraspecies difference

A comparison of two sturgeons from the Gironde River (Nos 1 and 2) with those captured in the North and Baltic Seas (Nos 3 and 6, respectively) showed considerable genetic differences among these fish. These differences are much higher than those between two subspecies of the American Atlantic sturgeon. Fixed differences in the nucleotide sequence of the control

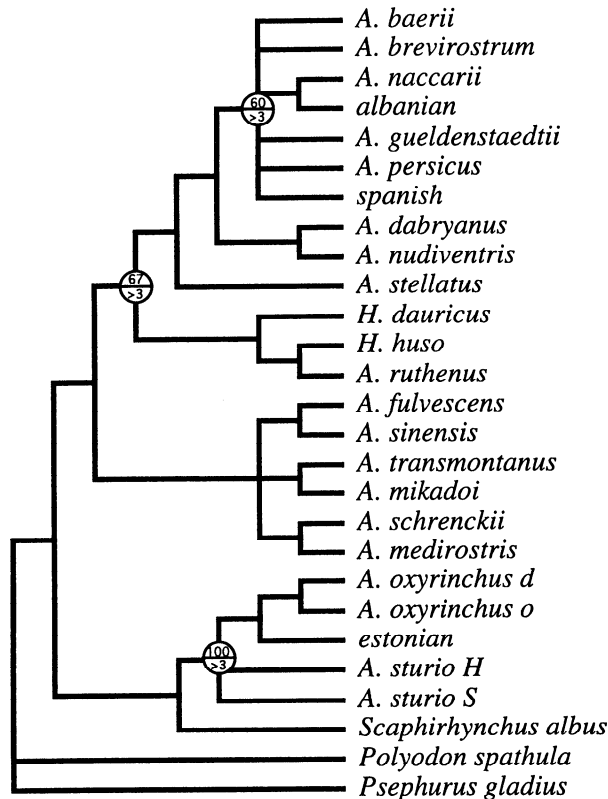


Fig. 2. Phylogenetic tree for 300 bp region of the *cyt b* gene. The four new samples described in this study plus two samples described in Birstein and DeSalle (1997), as well as sequences for all living sturgeon species for this 300 bp region were used to generate the tree. Two extant paddlefish species (*Polyodon spathula* and *Psephurus gladius*) were used as outgroups. *Albanian*, *spanish*, and *estonian* indicate sample Nos 5, 4 and 6, respectively; *A. oxyrinchus d* and *A. oxyrinchus o* are *A. oxyrinchus desotoi* and *A. o. oxyrinchus*, respectively; *A. sturio H* corresponds to sample No. 3, and *A. sturio S* corresponds to sample No. 1 (and 2). Only a 150 base region of the gene was obtained for sample No. 5 (see text). Bremer indices are indicated in the circles below the lines, and parsimony jackknife numbers are shown above the lines. Both sample Nos 4 and 5 are part of a clade, quite distant from *A. sturio*.

region of these subspecies was shown by Ong *et al.* (1996) and Wirgin *et al.* (1997).

Unfortunately, other data on the comparison of *A. sturio* from different populations are lacking, and only meristic characters have been compared (a review in Holčík *et al.*, 1989). Marti (1939) showed that the number of dorsal and lateral scutes of *A. sturio* from the Black and Baltic Seas differs. Later it was found that specimens from the Atlantic Ocean, Mediterranean and Black Seas have similar numbers of scutes (Magnin, 1963; Ninua, 1976). Based on these data, Holčík *et al.* (1989) suggested that the Baltic Sea population 'is probably separated from the remaining ones and may be a distinct subspecies'. Our results support these previous studies suggesting a difference between individuals from the Baltic Sea population of *A. sturio* and those from the Atlantic Ocean.

CONSERVATION ASPECTS

Our data suggest that the recovery plans for *A. sturio* should take into consideration two potential problems. First, sturgeons caught in the historic area of *A. sturio* can be easily misidentified because of the general morphological similarity of this species to *A. gueldenstaedtii*, *A. baerii* or *A. naccarii*. The confusion with the last species might be acute in the Adriatic Sea, where both species, *A. sturio* and *A. naccarii*, used the same rivers for spawning in the past and local fishermen did not discriminate between these species (Holčík *et al.*, 1989; Tortonese, 1989). Second, we provide data that show that different populations of *A. sturio* are genetically divergent. To establish this, we took the unique opportunity to study specimens representing both the Gironde River and North-Baltic Sea populations. We urge colleagues involved in work on the restoration of *A. sturio* populations in Europe to perform molecular identification of sturgeon individuals caught in different geographic localities. Also, we would like to stress that fish farmers have a responsibility to ensure that sturgeon farmed in western Europe (mostly *A. baerii*, but also *A. ruthenus* from the Danube River and *A. transmontanus* from North America) should not be allowed to escape into the wild and should never ever be released.

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