

# Molecular analysis of *Acipenser sturio* L., 1758 and *Acipenser oxyrinchus* Mitchill, 1815: A review

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## ABSTRACT

Molecular phylogeny based on the analysis of partial sequences of mitochondrial (mt) cytochrome *b* (cyt *b*), 12S, and 16S genes supports a close relationship between the European Atlantic sturgeon *Acipenser sturio* L., 1758, and the American Atlantic sturgeon, *Acipenser oxyrinchus* Mitchill, 1815. These sister species form a separate clade within the genus *Acipenser* and appear to be the most basal clade with respect to all other species of the genus. Intraspecifically, at least two different genetic forms have been found within *A. sturio* (in the Northern and Baltic seas), but no defined subspecies exist. For *A. oxyrinchus*, fixed differences in the control region and cyt *b* gene distinguish the two subspecies, the Atlantic sturgeon *A. oxyrinchus oxyrinchus* Mitchill, 1815 and the Gulf of Mexico sturgeon *A. oxyrinchus desotoi* Vladykov, 1955. Within *A. o. oxyrinchus*, a pronounced latitudinal cline in haplotype diversity exists from north to south along the eastern coast of North America. This greater genetic diversity in southern populations is most likely a result of population continuity through the Pleistocene. Monomorphism within the two northernmost, post-Pleistocene *A. o. oxyrinchus* populations indicates a probable founder effect. *A. o. desotoi* also displays a significant geographic structuring of populations in different river systems. Because of the lack of *A. sturio* individuals necessary for population studies and the close relationship between this species and *A. oxyrinchus*, the genetic characteristics and structure of *A. oxyrinchus* can be regarded as a model for *A. sturio*. The only opportunity for future detailed molecular study of *A. sturio* resides in using tissue samples from sturgeon specimens kept in various European museums.

**Key words:** Atlantic sturgeon, cytochrome *b*, genetic diversity, phylogeny.

## RESUMEN

**Análisis molecular de *Acipenser sturio* L., 1758 y *Acipenser oxyrinchus* Mitchill, 1815: una revisión**

La filogenia molecular basada en el análisis de secuencias parciales de los genes mitocondriales (mt) citocromo *b* (cyt *b*), 12S y 16S apoya la próxima relación entre el esturión atlántico europeo *Acipenser sturio* L., 1758 y el esturión atlántico americano *Acipenser oxyrinchus* Mitchill, 1815. Estas especies hermanas forman un clado separado dentro del género *Acipenser* y parecen constituir el clado más basal con relación a todas las otras especies del género. Intraespecíficamente se han encontrado, al menos, dos formas genéticas diferentes dentro de *A. sturio* (en los mares del Norte y Báltico) pero no existen subespecies definidas. Para *A. oxyrinchus* las diferencias fijadas en la región control y en el gen del cyt *b* distinguen las dos subespecies: el esturión atlántico *A. oxyrinchus oxyrinchus* Mitchill, 1815 y el esturión del golfo de México *A. oxyrinchus desotoi* Vladykov, 1955. Dentro de *A. o. oxyrinchus* existe un pronunciado cline latitudinal en

la diversidad de haplotipos desde el norte hasta el sur a lo largo de la costa este de América del Norte. Esta mayor diversidad genética en las poblaciones meridionales es, más probablemente, un resultado de la continuidad poblacional durante el Pleistoceno. El monomorfismo dentro de las dos poblaciones post-pleistocénicas más septentrionales de *A. o. oxyrinchus* indica un probable efecto fundador. *A. o. desotoi* siempre muestra una significativa estructuración geográfica de poblaciones en diferentes sistemas fluviales. Debido a la falta de individuos de *A. sturio* necesarios para los estudios poblacionales y a la próxima relación entre esta especie y *A. oxyrinchus*, las características y estructura genéticas de *A. oxyrinchus* pueden ser consideradas como modelo para *A. sturio*. La única oportunidad para un futuro estudio molecular detallado de *A. sturio* reside en el uso de muestras de tejidos de ejemplares de esturión almacenados en varios museos europeos.

**Palabras clave:** Esturión atlántico, citocromo b, diversidad genética, filogenia.

## INTRODUCTION

At present, the European Atlantic or Baltic sturgeon *Acipenser sturio* L., 1758 is one of the most endangered sturgeon species (Rochard, Castelnau and Lepage, 1990; Birstein, 1993, 1999a; Lepage and Rochard, 1995; Birstein, Bemis and Waldman, 1997; Anatsky, 1999). Historically, its range included the Northeastern Atlantic Ocean, especially the shallow parts of the North and Baltic Seas and coastal areas of the Mediterranean and Pontic regions (reviews in Holčík *et al.*, 1989; Kinzelbach, 1997). Currently, only a small population of *A. sturio* exists in the Gironde River system in France (Castelnau *et al.*, 1991; Williot *et al.*, 1997). In the spring of 1995, natural reproduction of this species was reported in this river basin for the first time since 1988 (Anon., 1995). Unfortunately, it is not known which river was used by sturgeon for spawning at that time. Analysis of the catch data of *A. sturio* provided by commercial fishermen over the period 1980-1994 indicates that juveniles from this population disperse over a very large area, from the Bay of Biscay to Scandinavia, as well as the British Isles (Rochard, Lepage and Meazé, 1997).

Until recently, another small stock of *A. sturio* was also observed in the eastern part of the Black Sea (Pavlov *et al.*, 1985, 1994). Sturgeon spawned in the lower part of the Rioni River in Georgia (Ninua, 1976). All attempts to catch *A. sturio* in that area in the 1990s have failed (E. Artuykhin and Z. Zarkua, pers. comm.).

During the last decade, several single *A. sturio* individuals were caught in the North Atlantic and the Baltic Seas. In 1985, 1989 and 1995, three sturgeon were caught in the German waters of the North Sea near the Helgoland Island (Debus, 1995, 1997; K. Busse, pers. comm.). In 1993, one *A. sturio* was cap-

tured in the North Sea near the Dutch coast (Timmermanns and Melchers, 1994) and in 1992, two juveniles were reported from a tributary of the Dutch part of the Rhine (Volz and De Groot, 1992). In May 1996, a large mature female was caught in the Estonian waters of the Baltic Sea (Paaever, 1996, 1997, 1999). Therefore, only single individuals of *A. sturio* are currently available for genetic research.

Historically, the North American Atlantic sturgeon, *Acipenser oxyrinchus* Mitchill, 1815, was considered to be the same species as *A. sturio*. Later, *A. oxyrinchus* was recognised as the separate subspecies *A. sturio oxyrinchus*, with the European Atlantic sturgeon known as *A. sturio sturio* (Smith, 1891; Vladykov and Greely, 1963). In 1963, Magnin and Beaulieu suggested elevation of these subspecies to species ranks, with the European form retaining the name *A. sturio*, and the American form recognised as *A. oxyrinchus* (Magnin and Beaulieu, 1963; Magnin, 1964). Since then, they are usually considered sister species (e.g., Birstein and Bemis, 1997; Choudhury and Dick, 1998), but recently Artyukhin and Vecsei (1999) lowered them to subspecies level again. However, this opinion contradicts current data on genetic differences between *A. sturio* and *A. oxyrinchus* (see below).

Two subspecies, the Atlantic sturgeon, *A. oxyrinchus oxyrinchus* Mitchill, 1815 and the Gulf of Mexico sturgeon, *A. oxyrinchus desotoi* Vladykov, 1955, were described within *A. oxyrinchus* (Vladykov, 1955; Vladykov and Greely, 1963). Currently, the Atlantic subspecies occurs from the Hamilton Inlet of Labrador in Canada to the St. Johns River in eastern Florida, while the Gulf sturgeon inhabits most river systems of the northern Gulf of Mexico from the mouth of the Mississippi River to the Suwannee River and coastal waters up to Florida Bay, Florida (Smith and Clugston, 1997).

In contrast to the European Atlantic sturgeon, different populations of *A. oxyrinchus* have been studied intensively and numerous genetic data for this species exist in the literature.

In the present paper, we review all of the genetic data known for both *A. sturio* and *A. oxyrinchus*.

## RESULTS AND DISCUSSION

### General genetic characteristics and the position of *A. sturio*-*A. oxyrinchus* within the Acipenseridae

The chromosome number in *A. sturio* is  $116 \pm 4$  (Fontana and Colombo, 1974; Tagliavini *et al.*, 1999). The *A. sturio* karyotype consists of 35 pairs of meta- and submetacentric large and medium-sized chromosomes, two pairs of small acrocentrics and about 20 pairs of microchromosomes (Fontana and Colombo, 1974; Tagliavini *et al.*, 1999). The DNA content in *A. sturio* is 3.6 pg/nuclei (Fontana, 1976), which is characteristic of sturgeons with 120 chromosomes (Birstein, Hanner and DeSalle, 1997). For *A. o. oxyrinchus* the DNA content was reported as 4.55 pg/nuclei (Blackledge and Bidwell, 1993), but the difference in the DNA content between these two species appears to be due to different methods of evaluation (microdensitometry for *A. sturio* and flow cytometry for *A. oxyrinchus*). For *A. oxyrinchus*, the number of chromosomes in *A. o. desotoi* cultured cardiac cells was 99-112 (Li *et al.*, 1985).

All of this evidence confirms that both *A. oxyrinchus* and *A. sturio* belong to the 120-chromosome group of sturgeons and, therefore, *A. sturio* belongs to the group of 120-chromosome sturgeon species; members of the other group of species have 240 chromosomes (for detailed discussion of the problem of ploidy in sturgeons, see Birstein, Hanner and DeSalle, 1997; Birstein and DeSalle, 1998; Vasil'ev, 1999). On the basis of karyotypic structure, a high number of nucleoli in nuclei, and a high level of duplicated loci, the 120-chromosome sturgeon species are usually considered tetraploids (Ohno *et al.*, 1969; Birstein, Hanner and DeSalle, 1997). The American paddlefish *Polyodon spathula* (Walbaum, 1792), a representative of the second acipenseriform family Polyodontidae, also has 120 chromosomes and similar karyotypic characteristics to those of the 120-chromosome sturgeons (Dingercus and Howell,

1976). In *P. spathula*, the duplicated loci were found for insulin, glucagon, glucagon-like peptide, and proopiomelanocortin (Nguen *et al.*, 1994; Danielson *et al.*, 1999). Since karyotypes of the shovelnose sturgeon *Scaphirhynchus platyrhynchus* (Rafinesque, 1820) (which belongs to presumably the oldest sturgeon subfamily Scaphirhynchinae – see Mayden and Kuhajda, 1996) and of *P. spathula* are similar, the tetraploid karyotype of 120 chromosomes is regarded as ancestral for acipenseriforms (reviewed in Birstein, Hanner and DeSalle, 1997; Vasil'ev, 1999). However, some authors describe the 120-chromosome sturgeon species, including *A. sturio*, as diploids (Fontana *et al.*, 1996; Tagliavini *et al.*, 1999).

A molecular phylogenetic study of all sturgeon species showed that *A. sturio* and *A. oxyrinchus* are clustered together in one clade, and that this clade is the sister-group to the other main clades of sturgeon species (Birstein and DeSalle, 1998). This conclusion was based on combined analysis of data for fragments of three mitochondrial (mt) genes, the 12S, 16S, and cytochrome *b* (cyt *b*) (150-bp, 350-bp, and 650-bp, respectively). The position of this clade in the phylogenetic tree supports the hypothesis that *A. sturio* (together with *A. oxyrinchus*) is probably a descendant of ancestral forms of *Acipenser* (Nesov and Kaznyshkin, 1983). Based on the fact that the main geological changes in the North Atlantic Ocean occurred during the Lower (135-95 My ago) and Upper (95-65 My ago) Cretaceous (Smith, Smith and Funnell, 1994), it is assumed that the *A. sturio*-*A. oxyrinchus* lineage originated during the Middle Cretaceous, ca. 90 My ago (Birstein and DeSalle, 1998).

### Molecular difference between *A. sturio* and *A. oxyrinchus*

Wirgin, Stabile and Waldman (1997) compared a partial sequence (203-bp) of the control region of the mtDNA of *A. sturio* (an individual from the Gironde River) to sequences from 159 individuals representing both *A. o. oxyrinchus* and *A. o. desotoi*. They found a minimum of 31 and maximum of 33 nucleotide changes between *A. sturio* and *A. oxyrinchus* individuals. There were also three sites of insertions/deletions recognised: a CA insertion in positions 35-35, and deletions of GC in positions 58-59 and A in position 119 of *A. oxyrinchus* relative

to *A. sturio*. The number of nucleotide changes in pairwise comparisons between the two subspecies of *A. oxyrinchus* ranged between 5 and 8, with no insertions and/or deletions, and is therefore much lower than between *A. sturio* and *A. oxyrinchus*. With the exclusion of insertions and deletions, nucleotide divergence between these two species was much higher (about 15 %) than between the two subspecies of *A. oxyrinchus* (maximum 3.5 %; Ong *et al.*, 1996). Also, we found 7 nucleotide changes in the 295-bp *cyt b* gene regions between *A. sturio* and *A. oxyrinchus* (figure 1; see also Birstein and DeSalle, 1998). We observed even more changes in a 642-bp fragment of the ND5 gene (table I).

Wirgin, Stabile and Waldman (1997, p. 387) concluded that "the level of [molecular] differentiation observed argues strongly for full species status of each of the western and eastern Atlantic sturgeons." Therefore, molecular data support the traditional recognition of *A. sturio* and *A. oxyrinchus* as separate species (Magnin and Beaulieu, 1963; Magnin, 1964) and not subspecies, as Artyukhin and Vecsei (1999) suggested recently.

#### Are there intraspecies forms within *A. sturio*?

A comparison of two fragments of the *cyt b* gene in *A. sturio* individuals from different locations showed considerable intraspecific genetic differentiation (Birstein, Betts and DeSalle, 1998). Four *A.*

*sturio* individuals were used for this analysis: two from the Gironde River in France, one caught in the North Sea near the Dutch coast (Timmermanns and Melchers, 1994), and one captured in the Baltic Sea in Estonian waters (Paaver, 1996, 1997). The sequences from two individuals from the Gironde River were practically identical (GR1 and GR 2) and differed in only a few changes from the sequence of the individual from the North Sea (GR1, GR 2, and NS, respectively, in figure 1 and table II). The partial *cyt b* sequence from the Baltic Sea individual (BS) differed slightly from that of the NS (6 changes) and GS1/ GS 2 (4 changes) (figure 1, table II). Ludwig *et al.* (2000) also described a difference in the distribution of microsatellite alleles in the museum specimens of *A. sturio* from the populations in the North and Baltic Seas. They found some alleles in the North Sea individuals only (alleles 121 and 124 of the microsatellite Afu-19; 147 of Afu-34; 120 of Afu-39 and 152 of Afu-68), while two alleles (130 of the microsatellite Afu-19 and 180 of Afu-180) were observed exclusively in the Baltic Sea specimens. All these data point to the possibility of intraspecies differentiation within *A. sturio*.

Earlier, two forms differing in meristic characters were described within *A. sturio* (Marti, 1939; Ninua, 1976). Marti (1939) showed that the number of dorsal and lateral scutes in the Baltic Sea *A. sturio* is lower than in the Black Sea *A. sturio* (9.6 and 14.3, and 27.7 and 32.8, respectively). Later it was found

Table I. Nucleotide changes in a partial sequence (642-bp) of the ND5 gene of *A. sturio* from the Gironde River and *A. oxyrinchus desotoi*<sup>1</sup>. (1): Data from Doukakis, Birstein and DeSalle (unpublished). Tissue samples from the same individuals GR1 and AD as in figure 1 and table II were used. (2): Position numbers refer to the ND 5/6 primer of Bembo *et al.* (1995)

Species	Positions of nucleotides <sup>2</sup>									
	39	50	68	70	103	128	154	164	173	193
<i>A. sturio</i>	A	C	C	G	T	A	G	C	C	C
<i>A. oxyrinchus desotoi</i>	G	T	T	A	C	T	A	G	T	T
	196	216	238	259	319	337	346	352	382	385
<i>A. sturio</i>	T	T	T	C	T	C	A	G	C	A
<i>A. oxyrinchus desotoi</i>	C	C	C	T	C	T	G	A	T	G
	388	398	412	445	466	469	478	517	532	544
<i>A. sturio</i>	T	T	G	A	A	A	G	C	T	T
<i>A. oxyrinchus desotoi</i>	C	C	A	G	G	G	A	T	C	C
	550	571								
<i>A. sturio</i>	T	A								
<i>A. oxyrinchus desotoi</i>	C	G								

Cytochrome *b* — a part of the region 1

NSa CCGCCTTCCCATACATCGGCGACACACTAGTGCAATGAATCTGAGGCGGCTT  
 BSa .....G.....  
 GR1a .....  
 GR2a .....  
 ADa .....G....GT..G.....  
 AOa .....G....GT..G.....

Cytochrome *b* — a part of the region 2

NSb GAATCATACTTTCTCTTTGCCTACGCCATCCTCCGATCTATTCCGAACAACTAGGCGG  
 BSb ....G...T..C.....  
 GR1b ....G.....  
 GR2b ....G.....  
 ADb ....G...T..C.....  
 AOb ....G...T..C.....

NSb AGTACTGGCCCTTCTATTCTCCATCCTAGTCCTAATATTGGTACCAGTCCTCCACACCT  
 BSb .....T.....  
 GR1b .....  
 GR2b .....  
 ADb .....  
 AOb .....

NSb TCCAAACAACGGGGAAATACATTTTCGGCCCCTCTCCCAAATCCTATTTTGAGCCCTAGT  
 BSb .....A.....  
 GR1b .....A.....A.....GA....G..  
 GR2b .....A.....A.....GA....G..  
 ADb .....A.....  
 AOb .....A.....

Figure 1. Variable areas in the regions 1 (384-435 nucleotides) and 2 (811-986) of the cytochrome *b* gene in *A. sturio* and *A. oxyrinchus*. NS is the *A. sturio* specimen from the North Sea (Dutch coast): NSa corresponds to a partial sequence of the sequence submitted to GenBank under No. AF006145 (372-494 nucleotides); NSb is a partial sequence of a sequence under No. AF006176. BS is the *A. sturio* specimen from the Baltic Sea (Estonia): BSa and BSb regions correspond to NSa and NSb, respectively. GR1 and GR2 are two *A. sturio* specimens from the Gironde River (Cemagref, France). AD is a specimen of *A. oxyrinchus desotoi* (caught in the Pearl River, Louisiana, USA): ADa corresponds to NSa (accession No. AF006153) and ADb corresponds to NSb (accession No. AF006164). AO is a specimen of *A. oxyrinchus oxyrinchus* (caught in the St. Lawrence River, Quebec, Canada): AOa (accession No. AF006140) and AOb (accession No. AF006163) correspond to NSa and NSb, respectively. The description of specimens NS, BS, GR1, and GR2 was given in Birstein, Betts and DeSalle (1998), and of AD and AO, in Birstein and DeSalle (1998). Nucleotide positions correspond to positions in the cytochrome *b* gene sequence of the white sturgeon, *A. transmontanus* (Brown *et al.*, 1989; GenBank accession no. X14944)

that sturgeon from the Atlantic Ocean (Gironde River), the Mediterranean, and the Black Sea have similar numbers of scutes: for the dorsal scutes 12.7, 13.0, and 13.5, respectively; and the lateral scutes, 35.1, 33.4, and 32.0, respectively (Magnin, 1963; Ninua, 1976). Based on these data, Holčík *et al.* (1989, p. 371) suggested that the Baltic Sea population of *A. sturio* "is probably separated from the remaining ones and may be a distinct subspecies."

Studies on the molecular difference between the Baltic and North Sea sturgeons, as well as the Gironde River individuals, support this hypothesis concerning the possible existence of at least two forms of *A. sturio*. Although more in-depth studies are needed to confirm this hypothesis, such studies may be impossible, since live Atlantic sturgeon are caught very rarely.

Table II. Nucleotide changes in a combined sequence (295-bp) of the cytochrome *b* gene of *A. sturio* from the Gironde River (two specimens, GR1 and 2), North Sea (NR), and Baltic Sea (BS)<sup>1</sup>. (<sup>1</sup>): Data from Birstein, Betts and DeSalle (1998). Two regions of the cyt *b* gene cover nucleotides (372-494) and (811-1018) (see figure 1). (<sup>2</sup>): Numbers correspond to positions in the sequence of the cytochrome *b* gene of the white sturgeon, *A. transmontanus* (Brown *et al.*, 1989)

Fish sample	Positions of nucleotides <sup>2</sup>									
	478	815	819	822	892	939	955	979	980	985
NS	A	C	C	T	C	G	G	A	G	A
BS	G	G	T	C	T	A	G	A	G	A
GR1	A	G	C	T	C	A	A	G	A	G
GR2	A	G	C	T	C	G	A	G	A	G

### Was *A. sturio* the only native sturgeon species in the Guadalquivir River in Spain?

In 1997, a group of Spanish authors suggested that two specimens from the collection of the Doñana Biological Station in Seville (EBD 8173 and 8174) were not *A. sturio*, but were rather the Adriatic (Italian) sturgeon, *Acipenser naccarii* Bonaparte, 1836 (Garrido-Ramos *et al.*, 1997). This theory completely contradicted the commonly accepted opinion that *A. sturio* was the Iberian Peninsula's only native sturgeon (Almaça, 1988; Elvira, Almodóvar and Lobón-Cerviá, 1991a, b; Elvira and Almodóvar, 1993). *A. naccarii* is an endangered species which lives in the Adriatic Sea and spawns mainly in the Po River basin in Italy (Tortonese, 1989; Rossi *et al.*, 1991). Recently, a second population of this species was found in Albanian waters of the Adriatic (Ludwig and Kirschbaum, 1998). This species is closely related to the Russian *Acipenser gueldenstaedtii* Brandt & Ratzeberg, 1833 and Siberian *Acipenser baerii* Brandt, 1869 sturgeons (Birstein, 1999b; Birstein, Doukakis and DeSalle, 2000), but not to *A. sturio*.

In addition to completing a morphological study, Garrido-Ramos *et al.* (1997) presented the results of a molecular study of one of the two specimens under discussion. Using a hypothesized "species-specific" nuclear satellite DNA (stDNA) Hind III from identified tissue samples of vouched specimens of *A. naccarii*, the authors claimed that they were able to hybridize this stDNA to the DNA extracted from the questioned *A. sturio* specimen. The presence of the "species-specific" satellite in the museum specimen and in extant *A. naccarii*, and the absence of this stDNA in extant *A. sturio*, led Garrido-Ramos *et al.* (1997) to conclude that "the museum specimen EBD 8173 captured in the Guadalquivir River corresponds to *A. naccarii*."

Unfortunately, the authors did not describe their method of DNA extraction and, therefore, their experiments could not be reproduced.

Later the same authors showed that this HindIII stDNA is not "species-specific", since it is present in at least two other sturgeon species, *A. baerii* and *Huso huso* (L., 1758) (Ruiz-Rejón *et al.*, 2000). Previously a similar or a closely-related family of stDNA was also found in the Russian sturgeon *A. gueldenstaedtii*, sterlet *Acipenser ruthenus* L., 1758, and stellate sturgeon *Acipenser stellatus* Pallas, 1771 (Mikhailova *et al.*, 1995). Evidently, additional proof is needed to support the conclusion of Garrido-Ramos *et al.* (1997) and Ruiz-Rejón *et al.* (2000) that *A. sturio* does not contain the HindIII or a closely related stDNA. Also, the possibility that in the case of the specimen EBD 8173, Garrido-Ramos *et al.* (1997) extracted an authentic and not a contaminant DNA, should be eliminated.

Three laboratories in different countries (Molecular Laboratory, American Museum of Natural History, New York; Institute of Freshwater Ecology and Inland Fisheries, Berlin; Department of Biodiversity and Evolutionary Biology, National Museum of Natural Sciences, Madrid) tried to obtain additional molecular information on the two specimens under study. Sequencing of the following mitochondrial genes was employed for comparison of *A. sturio* with *A. naccarii* and for testing the questioned specimens: cyt *b*, NADH 5, control region, and 12S rRNA. It had been shown previously that the 389-bp fragment of the 12S gene in the two species differed by 7 nucleotide changes (table III) and that *A. sturio* and *A. naccarii* could, therefore, easily be discriminated using this gene (Ludwig and Kirschbaum, 1998).

None of the three laboratories could extract authentic DNA from the specimens in question, even though different DNA extraction methods were

Table III. Nucleotide changes in a partial sequence of the 12S ribosomal gene of *A. sturio* and *A. naccarii*<sup>1</sup>. (1): Data from Ludwig and Kirschbaum (1998). (2): Position numbers refer to the primer L1091 (Kocher *et al.*, 1989)

Species	Positions of nucleotides <sup>2</sup>						
	38	203	298	309	325	383	388
<i>A. sturio</i>	C	C	C	T	A	G	C
<i>A. naccarii</i>	G	T	T	C	G	A	G

used in each laboratory (Doukakis *et al.*, 2000). Moreover, Almodóvar, Machordom and Suárez (2000) later succeeded in obtaining and sequencing a 152-bp fragment of the *cyt b* gene from the specimen EBD 8174. These authors demonstrated the similarity of this sequence and those from two other archival museum specimens of *A. sturio* to the ones already published, and generated by themselves the *cyt b* gene sequences from the recently collected *A. sturio* individuals. Further detailed morphological studies of the two specimens under discussion also failed to support the Garrido-Ramos *et al.* (1997) conclusion (Elvira and Almodóvar, 1999; Rincón, 2000). Therefore, until further proof using molecular methods is provided, these two specimens should be considered *A. sturio*. However, Garrido-Ramos and his colleagues still insist that the specimen EBD 8173 is *A. naccarii* (Ruiz-Rejón *et al.*, 2000) and not *A. sturio*.

Currently, there is no convincing evidence that *A. naccarii* historically lived in Spanish waters, and future conservation efforts should respect this finding. Also, although several Siberian sturgeon, *A. baerii*, have been caught in the rivers of Spain, these individuals were typical escapes from fish farms (Birstein, Betts and DeSalle, 1998; Elvira and Almodóvar, 1999). Therefore, *A. baerii* should not be considered endemic to this system, either.

#### ***A. oxyrinchus* as a model for possible genetic traits in *A. sturio***

##### *General structure of the mtDNA*

In contrast to the length variation observed in the species of the Pacific coast (white sturgeon, *Acipenser transmontanus* Richardson, 1836, and green sturgeon *Acipenser medirostris* Ayres, 1854), no length variation in mtDNA molecules was observed among many specimens of *A. oxyrinchus* (specifically *A. o. oxyrinchus*) (Brown *et al.*, 1996; Waldman *et al.*, 1996; Ludwig *et al.*, 2000). Brown *et al.* (1996) discovered only one individual with longer

mtDNA. This was a case of heteroplasmy, i.e. a difference in the length of the D-loop region due to variation in number of repeated segments (see below): this specimen of *A. o. oxyrinchus* had three repeats in the D-loop instead of one. However, Miracle and Campton (1995) observed heteroplasmy in 18.5 % (31) of the individuals out of 168 *A. o. desotoi* studied. The heteroplasmic individuals displayed 1-4 copies of tandemly repeated segments. These data need more detailed confirmation. Ludwig *et al.* (2000) found only one individual of *A. sturio* with three repeats in the D-loop among 38 museum and 28 live specimens studied.

The general organization of the control region in the mtDNA of *A. oxyrinchus* is similar to that in other sturgeon species such as *A. transmontanus* (Buroker *et al.*, 1990). There are, however, some specific organizational details which differ in *A. oxyrinchus*. Within an approximately 1-1.1 kb segment between the 3' end of the *cyt b* gene and the 5' end of the conserved sequence block (CSB) of *A. oxyrinchus*, the following elements exist: the genes for tRNA<sup>Thr</sup> and tRNA<sup>Pro</sup>, an RTX segment (which has limited homology to the repeated sequence, RS), a 3'-flanking segment with 95 % sequence homology to the repeated sequence RS, an RS, a 5'-flanking segment with 60-70 % sequence homology to the RS, and then a region of a unique sequence of the control region (Brown *et al.*, 1996). Brown *et al.* (1996) determined the RS length in *A. oxyrinchus* as 80 bp, while Ludwig and Jenneckens (2000) considered it to be 79 bp in *A. o. oxyrinchus* and 80 bp in *A. sturio*. The RTX segment, which is characteristic of the *A. oxyrinchus* mtDNA, has not been found in the mtDNA of any other sturgeon species studied so far (Brown *et al.*, 1996).

##### *Discrimination between *A. o. oxyrinchus* and *A. oxyrinchus desotoi**

Ong *et al.* (1996) used direct sequencing of a variable 203-bp region within the control region for

comparison of *A. o. oxyrinchus* and *A. o. desotoi*. This 203-bp segment corresponds to positions from 3 to 237 of the control region of *A. transmontanus* (Buroker *et al.*, 1990). Three fixed differences at positions 104, 199, and 200 were found among 15 polymorphic sites within this region between the two subspecies of *A. o. oxyrinchus* (Ong *et al.*, 1996). The other polymorphic sites were scattered throughout the repeated sequences beginning at position 87 and ending at position 231. These data confirm the difference between the two subspecies. It has been hypothesised that the phylogenetic separation of the two subspecies might have been initiated by population separation in the late Pliocene or Pleistocene (Bowen and Avise, 1990; Avise, 1992). This disjunction could have occurred with changes in the size of the Florida Peninsula associated with the sea level and climatic changes during this period.

The evolutionary history of *A. sturio* was probably similar. During the last glaciation, the Baltic Sea was covered with ice (Forsström and Punkari, 1997; Klimanov, 1997), so the Baltic Sea form of *A. sturio* must have originated during the post-glacial time, i.e. during the last 11 000 years. The colonization or recolonization of *A. sturio* in the Baltic Sea occurred from the southwestern part of the North Sea, which was not covered with ice.

However, a post-glacial founder effect is not the only possible explanation of the facts. Ludwig *et al.* (2000) considered another potential scenario: the decrease in population size in *A. sturio* and *A. o. oxyrinchus* caused by overfishing could result in a loss of genetic variability during the 19th-20th centuries. If so, the decrease in population size could lead to a lineage sorting effect.

#### *Stock distribution of A. oxyrinchus along the North American East coast*

The data on the structure of the *A. oxyrinchus* stocks may give an idea of possible structure and history of the former *A. sturio* populations. RFLP analysis of mtDNA using four diagnostic restriction enzymes (*Bgl* I, *Msp* I, *EcoR* V, *Hinf* I, and *Hinc* II) (Waldman, Hart and Wirgin, 1996; Waldman *et al.*, 1996) and sequencing of the 203-bp fragment of the control region mentioned above (Wirgin *et al.*, 2000) was used to characterise the stock structure of *A. o. oxyrinchus* along the Atlantic coast. Using sturgeon samples from 11 river systems, chi-square

analysis showed that populations could be grouped into three main highly differentiated stocks (with four subgroups in the southeastern stock):

- (1) Canadian: St. Lawrence and St. John Rivers;
- (2) Hudson River;
- (3) Southeastern:
  - (a) Albermarle Sound;
  - (b) Cape Fear River;
  - (c) Edisto River;
  - (d) Savannah, Ogeechee, and Altamaha Rivers.

A profound latitudinal cline in the number of composite mtDNA haplotypes and in haplotypic diversity was observed, which increased from north to south (Wirgin *et al.*, 2000). The number of haplotypes in each population ranged from one in each of the two most northern populations to 17 in the Savannah River. Haplotypic diversity (Nei and Tajima, 1981) among populations ranged from complete monomorphism (0.0, St. John River) in the two Canadian populations to considerable polymorphism in southeastern populations (0.90, the Savannah River). Previously, Brown *et al.* (1996) also observed a very low level of mtDNA sequence diversity in Atlantic sturgeon from the St. Lawrence River. These results, combined with the monomorphism of the two Canadian populations, suggest a strong post-glacial founder effect (but see above). Three haplotypes unique to northern populations were probably the result of nucleotide substitutions that occurred during post-glacial times, i.e. within the last 10 000 years. The greater genetic diversity in the southern populations is most likely due to continuity of these populations through the Pleistocene: all river systems with populations of sturgeons characterised by a high level of haplotypic diversity are in an area which was not glaciated (Swift *et al.*, 1986). Possibly, the second factor of high genetic diversity was faster mutation rates in these sturgeon because of their shorter generation times (Wirgin *et al.*, 2000). Twenty-five (64%) of the 39 composite mtDNA haplotypes found were unique to sturgeon from a particular population.

A similar analysis of RFLP data (*Bgl* I, *Msp* I, *EcoR* V, and *Hinf* I) and sequencing of a 203-bp variable fragment of the control region (the same as in Ong *et al.*, 1996) allowed Stabile *et al.* (1996) to identify 5 regional or river-specific stocks of *A. o. desotoi* from west to east, as follows:



- (1) Lake Ponchartrain and Pearl River (Louisiana);
- (2) Pasacagoula River (Mississippi);
- (3) Escambia and Yellow rivers (Florida);
- (4) Choctawhatchee River (Florida);
- (5) Apalachicola, Ochlockonee, and Suwannee rivers (Florida).

These data point to a strong reproductive isolation of *A. o. desotoi* stocks, a point which is reinforced by the low gene flow estimates between populations (details in Stabile *et al.*, 1996 and Wirgin, Stabile and Waldman, 1997). Haplotypic diversity (the RFLP data) ranged from 0.09 between the western and Choctawhatchee River stocks to 0.66 between the western and Escambia River-Yellow River stocks. Stabile *et al.* (1996) discussed two possible explanations for these results. First, these data indicate the possibility of strong homing fidelity of individuals within *A. o. desotoi*. Second, the homing fidelity may be reinforced by metabolic, temperature-dependent constraints: the sturgeon return to the same rivers from the Gulf of Mexico to summer near cold water springs in the cool water refuges (Clugston, Foster and Carr, 1995; Foster and Clugston, 1997).

### Molecular data and conservation projects

Since *A. sturio* is internationally listed as critically endangered (Anon., 1996), there is a serious concern in the European scientific community regarding this species. The Gironde River population of *A. sturio* has been studied for 20 years and a project for its restoration has been elaborated by French experts (Elie, 1997). A search for sturgeons from the Gironde (France) and Rioni (Georgia) Rivers, in an effort to create an artificial aquaculture of *A. sturio* in northern Germany, was recently suggested (Debus, 1995). In 1994, the Society to Save the Sturgeon *Acipenser sturio* was organised in Europe (Elvira and Gessner, 1996). The importance of genetic and molecular data illustrated in this paper stresses the necessity to base all practical efforts on the results of genetic research. Individuals caught in the historic area of *A. sturio*, especially young sturgeon, can be easily misidentified because of their morphological similarity to the Russian sturgeon, *A. gueldenstaedtii*, Siberian sturgeon, *A. baerii*, or Italian sturgeon, *A. naccarii*. The confusion with the last species is extremely im-

portant in the Adriatic Sea, where both *A. sturio* and *A. naccarii* historically spawned in the same rivers and local fishermen did not distinguish between them (Holčík *et al.*, 1989; Tortonese, 1989).

Currently, the problem of discrimination between *A. sturio* and *A. naccarii* is especially relevant due to the discussion of a project to introduce *A. naccarii* from an aquaculture stock developed in Italy into the Guadalquivir River in Spain (Ruiz-Rejón, Hernando and Domezain, 1998; Domezain, 1999). The implementation of this project should be postponed until convincing genetic data can prove that *A. naccarii* in fact historically existed in Spain. A species introduction must not be based on the poorly supported opinion of only one laboratory that one of many scores of sturgeon specimens kept in museum collections of Spain was not *A. sturio*, but *A. naccarii*.

The IUCN Red List classifies *A. o. oxyrinchus* as near threatened and *A. o. desotoi* as vulnerable (Anon., 1996). Recently, the US Fish and Wildlife Service and the US National Marine Fisheries Services were petitioned to list and protect both subspecies under the Endangered Species Act (Waldman and Wirgin, 1998). In 1994 and 1996, several thousand *A. o. oxyrinchus* from an artificially cultured Hudson River broodstock were released into the Hudson River and Chesapeake Bay (Secor, 1996; Waldman and Wirgin, 1998; Secor and Waldman, 1999). In the latter case, within 8 months the released fish had spread through the Chesapeake Bay from Baltimore Harbor to the lower James River in Virginia and even to neighbouring North Carolina (St. Pierre, 1999). However, Waldman and Wirgin (1998) recommended avoiding interstock transfer of *A. oxyrinchus* in future stocking to prevent disintegration of locally adapted gene pools. Another concern is the possibility of inbreeding depression of *A. oxyrinchus* due to a low number of artificially bred individuals used in hatchery-based stocking projects (St. Pierre, 1996, 1999).

### Concluding remarks: The need to study museum specimens

Genetic data on the current structure of *A. oxyrinchus* stocks enabled Wirgin, Stabile and Waldman (1997) and Wirgin *et al.* (2000) to reconstruct the history of this species. Also, genetic experiments on *A. oxyrinchus* demonstrated that ge-

netic data are crucial to any conservation project. The lack of experimental material is the limiting factor of genetic study of *A. sturio*, since only several live *A. sturio* were caught during the last decade; the only solution is to study museum specimens. Since new DNA extraction for formalin-preserved museum specimens has recently been developed (France and Kocher, 1996; Vachot and Monnerot, 1996; Jiang, Wu and Lin, 1997; Wirgin *et al.*, 1997), such a study on *A. sturio* is more realistic than ever. The first results using microsatellite DNA extracted from museum specimens of *A. sturio* from the North and Baltic Seas showed a high genetic similarity between these populations in the past, and a possible decline in genetic variability in both populations from 1823 to 1992 (Ludwig *et al.*, 2000).

Future molecular experiments should be performed in conjunction with morphological study of the same specimens. This will allow researchers to describe intraspecific forms within *A. sturio*, if they in fact existed. All of the main European museum collections holding *A. sturio* specimens should be involved in this work and it should be coordinated by a group of experts.

The evolutionary history of *A. sturio* is practically unknown. Below are some of the main outstanding questions:

- (1) Was the Gironde River basin a refugium from which colonization of other populations in the North and Baltic seas occurred?
- (2) How were the populations of the Gironde River, Iberian Peninsula, Mediterranean and Adriatic Sea genetically interrelated?
- (3) Finally, did the Black Sea population originate from the sturgeons of the Adriatic-Marmora Seas after the Black Sea became connected with the Mediterranean approximately 8 000 years ago (Ryan *et al.*, 1997), or is this population older?

Only results of a detailed genetic research can yield answers to these questions.

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