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Preliminary results from characterization of the Iberian Peninsula sturgeon based on analysis of the mtDNA cytochrome *b*

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ABSTRACT

Historically, the Atlantic sturgeon *Acipenser sturio* L., 1758 was considered the only sturgeon species that inhabited rivers of the Iberian Peninsula. Nevertheless, in a recent paper, Garrido-Ramos *et al.* (1997) identified specimen EBD 8174 (museum collection of the Doñana Biological Station, Spain), from the Guadalquivir River, as the Adriatic sturgeon *Acipenser naccarii* Bonaparte, 1836, suggesting that both *A. sturio* and *A. naccarii* could be native to Spain. To test this theory, we compared partial mitochondrial cytochrome *b* (cyt *b*) gene sequences obtained from museum specimens of *A. sturio* that originated from the Iberian Peninsula, the Adriatic and the North Sea, as well as from live individuals of the Gironde River population in France. Specimens of *A. naccarii* and of the Siberian sturgeon *Acipenser baerii* Brandt, 1869 from fish farms were also included in the study. DNA from museum specimens was successfully amplified using the protocol of France and Kocher (1996) for DNA extraction from formalin-fixed and ethanol-preserved samples. Phylogenetic analysis was performed on partial cyt *b* gene sequences (over 402-bp and 245-bp). We identified specimen EBD 8174 as *A. sturio*, confirming the opinion that *A. sturio* is the only sturgeon species of the Iberian Peninsula. Further molecular analyses of museum specimens are needed for a description of the historical intraspecies genetic variation within *A. sturio*. This information is crucial for any future recovery plan for this species. Our comparison also showed interspecies sequence divergence ranging from 6.91 % (*A. sturio/A. naccarii*) to 7.43 % (*A. sturio/A. baerii*).

Key words: *Acipenser sturio, Acipenser naccarii, Acipenser baerii*, genetic variation, molecular analysis.

RESUMEN

***Resultados preliminares de la caracterización del esturión de la península Ibérica mediante el análisis del gen citocromo* b**

Históricamente, el esturión atlántico Acipenser sturio *L., 1758 ha sido considerado la única especie de esturión que vive en los ríos de la península Ibérica, siendo así demostrado por múltiples estudios morfológicos recientes. Sin embargo, un trabajo reciente identificó el ejemplar del río Guadalquivir EBD 8174 como* Acipenser naccarii *Bonaparte, 1836, sugiriendo que ambas especies de esturión podrían ser nativas de España. Para comprobar esta nueva teoría, se han analizado las secuencias del gen citocromo* b *mitocondrial en muestras de esturión atlántico preservadas en varios museos procedentes de la península Ibérica, el mar Adriático y el mar del Norte, así como material fresco del río Garona. Además, se han estudiado con fines comparativos varias muestras de* A. naccarii *y* Acipenser baerii *Brandt, 1869 procedentes de piscifactorías. Las muestras de museos fueron amplificadas con éxito usando un protocolo específico utilizado para material fijado en formol y preservado en alcohol. La muestra de esturión de España (EBD 8174) fue identificada como* A. sturio. *Este resulta-*

do confirma que A. sturio *es la única especie de esturión en la península Ibérica. De cualquier manera, antes de abordar los planes de recuperación, sería necesario realizar análisis moleculares complementarios para describir las posibles variaciones genéticas existentes entre poblaciones. La comparación de las secuencias de citocromo* b *de* A. sturio *con* A. naccarii *y* A. baerii *mostró valores de divergencia de 6,91 % y 7,43 % respectivamente.*

Palabras clave: Acipenser sturio, Acipenser naccarii, Acipenser baerii, *variación genetica, análisis molecular.*

INTRODUCTION

The Atlantic sturgeon *Acipenser sturio* L., 1758, is native to Western Europe and historically was present in many large Spanish rivers (Holčík *et al.*, 1989). Moreover, *A. sturio* was considered the Iberian Peninsula's only sturgeon (Almaça, 1988; Doadrio, Elvira and Bernat, 1991; Elvira, Almodóvar and Lobón-Cerviá, 1991a, b; Elvira and Almodóvar, 1993; Pereira, 1995). The *A. sturio* population in the Guadalquivir River was intensively exploited for caviar beginning in the 1930s, and its population dropped dramatically from the 1960s (Elvira, Almodóvar and Lobón-Cerviá, 1991a,b).

Contrary to the prevailing scientific opinion, Garrido-Ramos *et al.* (1997) recently suggested that two sturgeon species could be native to Spain: not only *A. sturio*, but also the Adriatic sturgeon *Acipenser naccarii* Bonaparte, 1836, which was formerly recognized as endemic to the Adriatic Sea basin only (Tortonese, 1989; Birstein and Bemis, 1997). In addition to a revaluation of morphological data, Garrido-Ramos *et al.* (1997) claimed that their conclusion was supported by a molecular identification of two specimens, EBD 8173 and EBD 8174, from the collection of the Doñana Biological Station (Spain), as *A. naccarii*. However, three laboratories in different countries could not confirm these results because the paper of Garrido-Ramos *et al.* (1997) did not contain a description of the molecular methods used, and, therefore, its results could not be reproduced (Doukakis *et al.*, 2000). Doukakis *et al.* (2000) suggested that Garrido-Ramos *et al.* (1997) might have worked not with the authentic extracted DNA, but with a contaminant. Furthermore, new morphological studies of these two specimens, and a comparison of them with many museum sturgeon specimens from rivers of the Iberian Peninsula, are in disagreement with the conclusions of Garrido-Ramos *et al.* (1997) (Elvira and Almodóvar, 1999, 2000; Rincón, 2000).

In the meantime, data concerning the molecular taxonomy of *A. sturio* are scarce (Wirgin, Stabile and Waldman, 1997; Birstein, Betts and DeSalle, 1998; Birstein and DeSalle, 1998; Ludwig and Kirschbaum, 1998). A molecular study of all available *A. sturio* specimens, including those kept in different museums, is crucial to any recovery programme for this species (Birstein and Doukakis, 2000; Ludwig *et al.*, 2000). Consequently, the goals of the present study were: (1) To elaborate a reliable method of DNA extraction from the museum (archival) specimens; (2) To identify and characterize by molecular methods the *A. sturio* specimens from the Iberian Peninsula and other European areas; (3) To determine the relationships between *A. sturio* and related sturgeon species.

MATERIALS AND METHODS

Sample collection

For DNA extraction, we used muscle tissue (from fresh, stuffed or ethanol-preserved specimens) from 7 specimens of *A. sturio*, 3 *A. baerii* and 3 *A. naccarii*, from different localities (table I). Fresh material was stored at –80 °C until DNA extraction.

DNA extraction

Homogenization after freezing of fresh tissue samples in liquid nitrogen was followed by a standard procedure of phenol-dicloromethane extraction and alcohol precipitation (Wirgin *et al.*, 1997). Tissues from stuffed samples were incubated in 1 ml of shaking TE9 buffer (500 mM Tris, 20 mM EDTA, 10 mM NaCl, pH 9.0; Shiozawa *et al.*, 1992) at room temperature for 24 hours, with one change of the buffer. The TE9 buffer was discarded and the tissue samples were then minced and incubated for 5 hours at 55 °C in TE9 buffer containing 50 µl of 20 % sodium dodecyl sulfate (SDS) and 12.5 µl of 30 mg/ml Proteinase K and incubated at 55 °C during 5 hours. Thereafter, another 12.5 µl of 20 mg/ml Proteinase K was added and the tissue

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

Sample	Species	Collection/Museum	Location of capture or farm	Preservation	Fragment of cyt *b*
S1	*A. sturio*	Doñana Biological Station, Seville EBD 8174, May 1975	Guadalquivir River, Alcalá del Río, Seville, Spain	Stuffed	155-bp
S2	*A. sturio*	Cemagref, Bourdeaux	Gironde River, France	Fresh	402-bp
S3	*A. sturio*	Cemagref, Bourdeaux	Gironde River, France	Fresh	402-bp
S4	*A. sturio*	Cemagref, Bourdeaux	Gironde River, France	Fresh	402-bp
S5	*A. sturio*	Cemagref, Bourdeaux	Gironde River, France	Fresh	402-bp
S6	*A. sturio*	Senckenberg Museum, Frankfurt SMF 2448	Adriatic Sea, Trieste, Italy	Ethanol	155-bp
S7	*A. sturio*	Senckenberg Museum, Frankfurt SMF 7637	Eider River, Nübbel, Germany	Ethanol	155-bp
B1	*A. baerii*	Doñana Biological Station, Seville EBD 8157, September 1995	Guadalquivir River, Coria del Río, Seville, Spain (released)	Fresh	402-bp
B2	*A. baerii*	Department of Animal Biology I Complutense University, Madrid, 1996	Duratón River, Spain (released)	Fresh	402-bp
B3	*A. baerii*	Department of Animal Biology I Complutense University, Madrid, 1996	Duratón River, Spain (released)	Fresh	402-bp
N1	*A. naccarii*	Aquatic Ecology Station, Seville	Sierra Nevada fish farm, Spain	Fresh	402-bp
N2	*A. naccarii*	Aquatic Ecology Station, Seville	Sierra Nevada fish farm, Spain	Fresh	402-bp
N3	*A. naccarii*	Aquatic Ecology Station, Seville	Sierra Nevada fish farm, Spain	Fresh	402-bp

samples were incubated again overnight at 55 °C. Standard phenol-dicloromethane 1:1 extractions were used to isolate DNA. DNA was precipitated in 1.6 ml of ice-cold 100 % EtOH and 0.1 vol. of 3 M sodium acetate (pH 6.8); incubated at –20 °C overnight; centrifuged at 16 000 g for 5 min at 4 °C; and washed in cold 70 % EtOH. Samples were air-dried and resuspended in 10-30 µl of ddH_2O.

PCR amplification

The PCR 402-bp fragments of the cytochrome *b* (cyt *b*) gene were amplified using universal primers (L14841; 5´- AAAAAGCTTCCATCCAACATCT-CAGCATGATGAAA-3´, H15149; 5´-AAACTGCAG-CCCCTCAGAATGATATTTGTCCTCA-3´, Kocher *et al.*, 1989) synthesized by Boehringer Mannheim (Spain). Tissues may degrade due to the storage process, making DNA extraction from archival specimens extremely difficult (France and Kocher, 1996; Rosenbaum *et al.*, 1997; Wirgin *et al.*, 1997). Therefore, we designed a specific internal primer (L14993; 5´-ATGACTAATCCGAAATATTC-3´) to generate a 155 bp fragment in combination with H15149. This region was selected because of the high variability in it among *A. sturio*, *A. naccarii* and *A. baerii*.

Amplification was performed in 50 µl total volumes containing 1 unit of *Taq* I Polymerase (Biotools, Madrid, Spain), 5 µl of 10× reaction buffer (Biotools), 20 pmol of each primer, 2 mM $MgCl_2$, 1 µl of dNTP mix (12.5 mM stock; Biotools), and ddH_2O to volume. One microlitre of DNA was added to the PCR mix, and subjected to 35 cycles of amplification. DNA was amplified in a Perkin Elmer 9600 thermocycler using the following protocol: after a preliminary denaturation at 94 °C for 5 min, each cycle consisted of denaturation at 94 °C for 30 s, annealing at 44 °C for 30 s, and primer extension at 72 °C for 1 min. To improve the subsequent reamplification and sequencing, a final cycle was added, including an extension of 5 min at 72 °C. Negative controls (no DNA added) were run for each amplification. PCR products were electrophoretically separated in 1-1.5 % agarose gels with a molecular size ladder and stained in ethidium bromide solution to determine size and quality of fragments. Some PCR products from archived specimens were reamplified, in order to increase the quantity of DNA.

The amplification products were purified before sequencing, either with Qiaquick columns (Qiagen, Santa Clarita, CA, USA), or Biotools columns (Biotools, Madrid, Spain), following the manufacturer's instructions.

Sequencing of PCR products

After purification, sequencing was performed in both directions by dideoxy sequencing (Sanger, Nicklen and Coulson, 1977) with the primers we used for PCR amplification. Sequencing was performed in an ABI PRISM 377 automated sequencer (Applied Biosystems, Foster City, USA), using four-dye fluorescent technology.

The cyt *b* gene sequences

The following cyt *b* gene sequences from the related sturgeon species retrieved from GenBank were used for the phylogenetic study: *A. sturio* (accession no. AF006134), *A. baerii* (AF006123), *A. naccarii* (AF006150), *Acipenser sinensis* Gray, 1835 (AF006158), *Acipenser persicus* Borodin, 1897 (AF006156), *Acipenser oxyrinchus* Mitchill, 1815 (AF006154), *Acipenser gueldenstaedtii* Brandt & Ratzeberg, 1833 (AF006126), *Acipenser brevirostrum* LeSueur, 1818 (AF006124) and *Polyodon spathula* (Walbaum, 1792) (MTPSPCYTB).

Sequence analysis

Sequences were analysed on an ABI PRISM 377 automated sequencer and data were edited with a multiple sequence editor program (ABI 1992). The sequences are deposited in GenBank under accession numbers AF217206-AF217209.

All DNA sequences were aligned using Clustal X (Thompson, Higgins and Gibson, 1994). Sequence data obtained for the long (402 bp) and short (155 bp) cyt *b* segments were analysed for distance and character-based variation using PHYLIP (version 3.5; Felsenstein, 1993). Phylogenetic trees were generated according to the maximum parsimony, MP (Felsenstein, 1983), maximum likelihood, ML (Felsenstein, 1981), and the distance-based neighbour-joining, NJ (Saitou and Nei, 1987) algorithms. For the transition/transversion bias, pairwise distances between nucleotide sequences were computed according to Kimura's two-parameters model (Kimura, 1980). The statistical significance of branching orders was assessed by the bootstrap resampling technique (100 replicates). Trees were rooted using as an outgroup *P. spathula*, a close relative of the Acipenseridae (Bemis, Findeis and Grande, 1997). A majority-rule consensus tree was constructed using the CONSENSE program from the PHYLIP package. The Kishino-Hasegawa-Templeton test (Templeton, 1983; Kishino and Hasegawa, 1989) was used to determine branch lengths from distance and ML test analyses.

RESULTS

Characteristics of the cyt *b* gene partial sequences

The long amplified fragment (402-bp) corresponds to the 5´-end of the cyt *b* gene. The short am-

ATGGCAAACA	TCCGAAAAAC	ACACCCACTA	CTTAAAATTA	TTAATGGAGC	50
				1	
ATTCATTGAC	CTCCCCACAC	CCTCCAACAT	CTCCGTGTGA	TGAAA**Y**TTTG	100
2		3	4		
GCTCACTC**M**T	AGGCCTCTGC	CTTGT**S**ACAC	AAATC**Y**TAAC	AGGACTATTT	150
	5	6		7 8 9	
CTTGCAATAC	A**Y**TACACAGC	**Y**GACATTTCA	ACAGCCTTCT	CCTC**YR**T**Y**GC	200
	10				
CCACATCTGC	CGAGA**Y**GTAA	ATTACGGATG	ACTAATCCGA	AATATTCATG	250
	11	12 13			
CAAACGGGGC	CTC**Y**TTCTTC	TT**Y**AT**Y**TGCT	TGTACCTTCA	CGTAGCACGA	300
14 15	16 17			18 19 20	
GG**Y**AT**R**TACT	A**Y**GG**Y**TCATA	CCTCCAAAAA	GAAACCTGAA	A**Y**AT**Y**GG**R**GT	350
21 22	23		24	25 26	
RATCCT**Y**CTG	CT**Y**CTCACCA	TAATAACCGC	CTTCGTGGG**R**	TATGTA**Y**T**R**C	400
CC					402

Figure 1. Consensus sequence of a 402-bp cytochrome *b* fragment from *A. sturio*, *A. naccarii* and *A. baerii*. Numbers mark the variable positions among genotypes observed

Table II. Variable nucleotide positions of cytochrome *b* fragment sequences. Numbers refer to sequence position in figure 1

		Variable nucleotides																								
Genotypes	Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
STU	S1	?	?	?	?	?	?	?	?	?	?	T	C	T	C	A	C	C	C	C	G	G	T	C	G	T	A
STU	S2	C	C	G	T	T	C	C	G	C	T	T	C	T	C	A	C	C	C	C	G	G	T	C	G	T	A
STU	S3	C	C	G	T	T	C	C	G	C	T	T	C	T	C	A	C	C	C	C	G	G	T	C	G	T	A
STU	S4	C	C	G	T	T	C	C	G	C	T	T	C	T	C	A	C	C	C	C	G	G	T	C	G	T	A
STU	S5	C	C	G	T	T	C	C	G	C	T	T	C	T	C	A	C	C	C	C	G	G	T	C	G	T	A
STU	S6	?	?	?	?	?	?	?	?	?	?	T	C	T	C	A	C	C	C	C	G	G	T	C	G	T	A
STU	S7	?	?	?	?	?	?	?	?	?	?	T	C	T	C	A	C	C	C	C	G	G	T	C	G	T	A
BAE 1	B1	T	C	C	C	C	T	T	A	T	C	C	T	C	T	G	T	T	C	C	A	A	C	T	A	C	G
BAE 2	B2	T	A	C	C	C	T	T	A	T	C	C	T	C	T	G	T	T	C	C	A	A	C	T	A	C	G
BAE 1	B3	T	C	C	C	C	T	T	A	T	C	C	T	C	T	G	T	T	C	C	A	A	C	T	A	C	G
NAC	N1	T	C	C	C	C	T	T	G	T	C	C	T	C	T	A	T	T	T	T	A	A	C	T	A	C	G
NAC	N2	T	C	C	C	C	T	T	G	T	C	C	T	C	T	A	T	T	T	T	A	A	C	T	A	C	G
NAC	N3	T	C	C	C	C	T	T	G	T	C	C	T	C	T	A	T	T	T	T	A	A	C	T	A	C	G

plified fragment (155-bp) corresponds to positions from 247 to 402 of the cyt *b* gene of the white sturgeon, *Acipenser transmontanus* Richardson, 1836 (Brown *et al.*, 1989; GenBank accession no. X14944).

Twenty-six polymorphic sites were found on the 402-bp fragment among 10 specimens of *A. sturio*, *A. baerii*, and *A. naccarii* (figure 1). They showed a single base-pair change and the sequence variation was predominantly due to transitions (n = 24). No deletions or insertions were observed. When we compared the 155-bp fragment obtained from S1, S6, and S7 with the other samples, we found 16 changes due to transitions in the sequences (table II). Both regions studied had a low G content (average 16.2 %) and almost equal A, C, and T contents (average 26.7, 29.3, and 27.8 %, respectively). The base composition of this cyt *b* region in *Acipenser* was similar to that found in teleosts (representatives of the order Perciformes) (Cantatore *et al.*, 1994).

Sequence variation and diversity of the cyt *b* genotypes

Four cyt *b* genotypes were identified in *A. sturio* (STU), *A. naccarii* (NAC), and *A. baerii* (BAE-1 and BAE-2) (table II). The sequence from the *A. sturio* specimen EBD 8174 was identical to those from sturgeon representing the Gironde River, Adriatic, and North Sea populations. However, at position 126 we found G in all *A. sturio* sequences, whereas Birstein and DeSalle (1998) identified T in the sequence from a North Sea individual. We did not observe any variable site within this fragment in *A. naccarii*, and we found a substitution (position 109) in one *A. baerii*.

Table III. Genetic distances among 245-bp sequences analysed following Kimura's two-parameter model. Cells in bold characters show the genetic distance analysed in the 155-bp sequence

	STU	BAE-1	BAE-2	NAC	*Acipenser sinensis*	*Acipenser persicus*	*Acipenser naccarii*	*Acipenser gueldenstaedtii*	*Acipenser brevirostrum*	*Acipenser oxyrinchus*	*Acipenser sturio*	*Polyodon spathula*
STU	0.00											
BAE-1	6.91	0.00										
BAE-2	7.43	0.41	0.00									
NAC	6.91	1.65	2.09	0.00								
A. sinensis	10.80	9.78	10.35	10.75	0.00							
A. persicus	6.91	1.65	2.09	1.65	10.75	0.00						
A. naccarii	7.83	2.50	2.95	0.82	11.73	2.50	0.00					
A. gueldenstaedtii	6.91	0.00	0.41	1.65	9.78	1.65	2.50	0.00				
A. brevirostrum	6.91	0.82	1.25	2.50	10.75	2.50	3.35	0.82	0.00			
A. oxyrinchus	4.23	9.67	10.23	8.73	12.73	8.73	8.73	9.67	10.62	0.00		
A. sturio	0.41	6.89	7.40	6.89	10.75	6.89	7.80	6.89	6.89	4.22	0.00	
P. spathula	12.95	12.89	13.52	12.89	8.36	12.89	13.92	12.89	13.92	14.96	12.89	0.00

Pairwise estimates of sequence divergence in the 402-bp fragment of the cyt *b* gene among haplotypes of *A. sturio*, *A. baerii* and *A. naccarii* were 0.41-7.43 % (table III). Evidently, the American Atlantic sturgeon *A. oxyrinchus* is the closest relative of *A. sturio*: we observed a 4.2 % divergence between the cyt *b* sequences of these species. *P. spathula*, which we used as an outgroup, presented the highest sequence divergence with *A. sturio* (12.9 %). *A. baerii* and *A. naccarii* showed a similar divergence from *A. sturio* (6.9 and 7.4 %, respectively).

The 402-bp fragment of the cyt *b* gene sequence was aligned with those of the related sturgeon species retrieved from GenBank. A phylogenetic analysis of this sequence data was performed to determine the position of the samples. The nucleotide sequence alignment consensus for all taxa was 245 bp long. Figure 2 shows the consensus tree built using the NJ method with a 7.17 ts:tv weighting assumption; *P. spathula* was used as an outgroup. The MP and ML analyses resulted in a similar and congruent tree after collapsing poorly

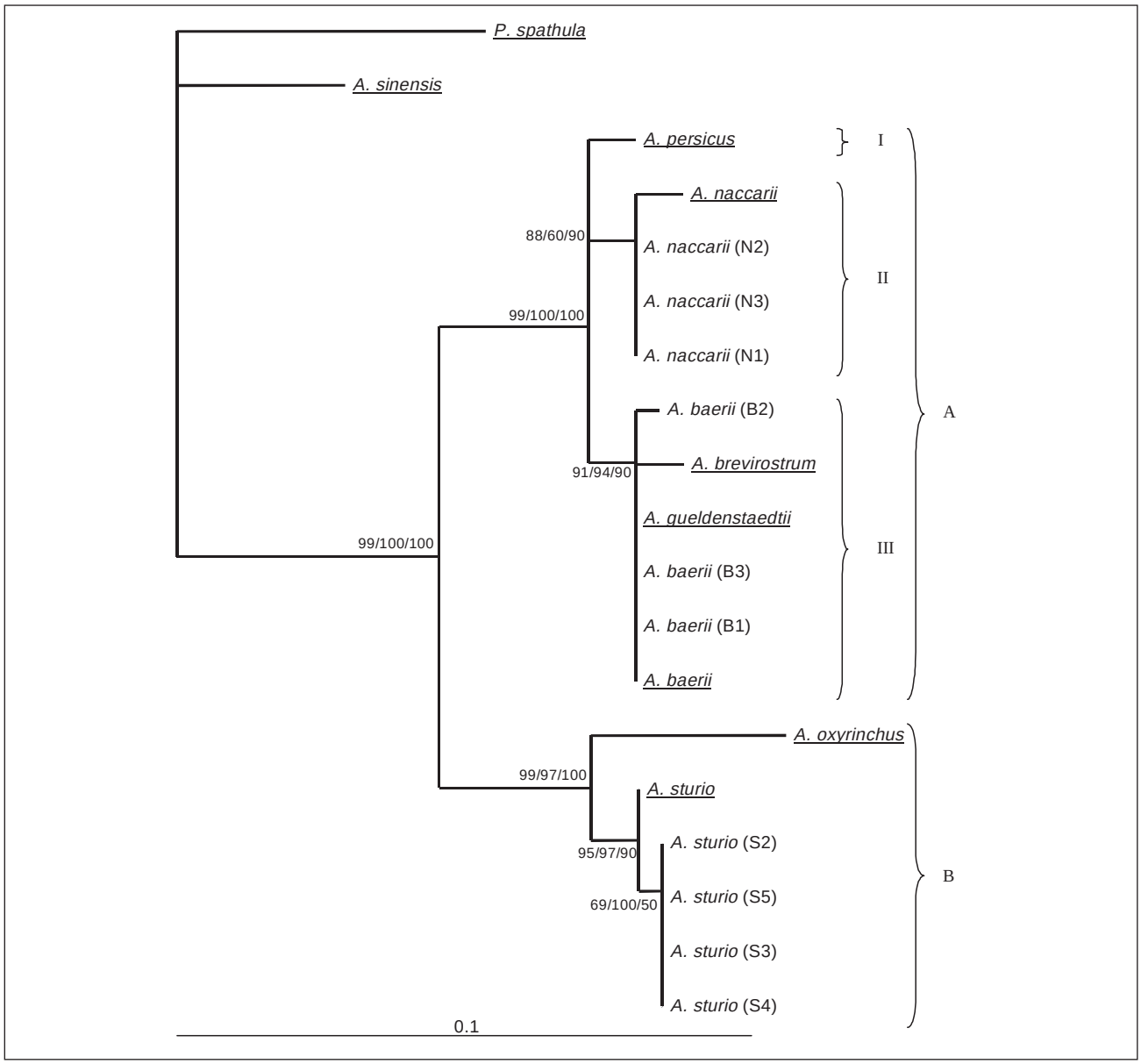

Figure 2. Estimated phylogenetic tree of 245-bp cytochrome *b* sequences. The percentages of bootstrap replicates supporting the clades are indicated at the branch points, based on (from left to right) neighbour-joining, maximum likelihood and equally-weighted parsimony methods. Bootstrap values < 50 % are not shown. Branch lengths are proportional to the estimated mean number of substitutions per site. GenBank species names are underlined

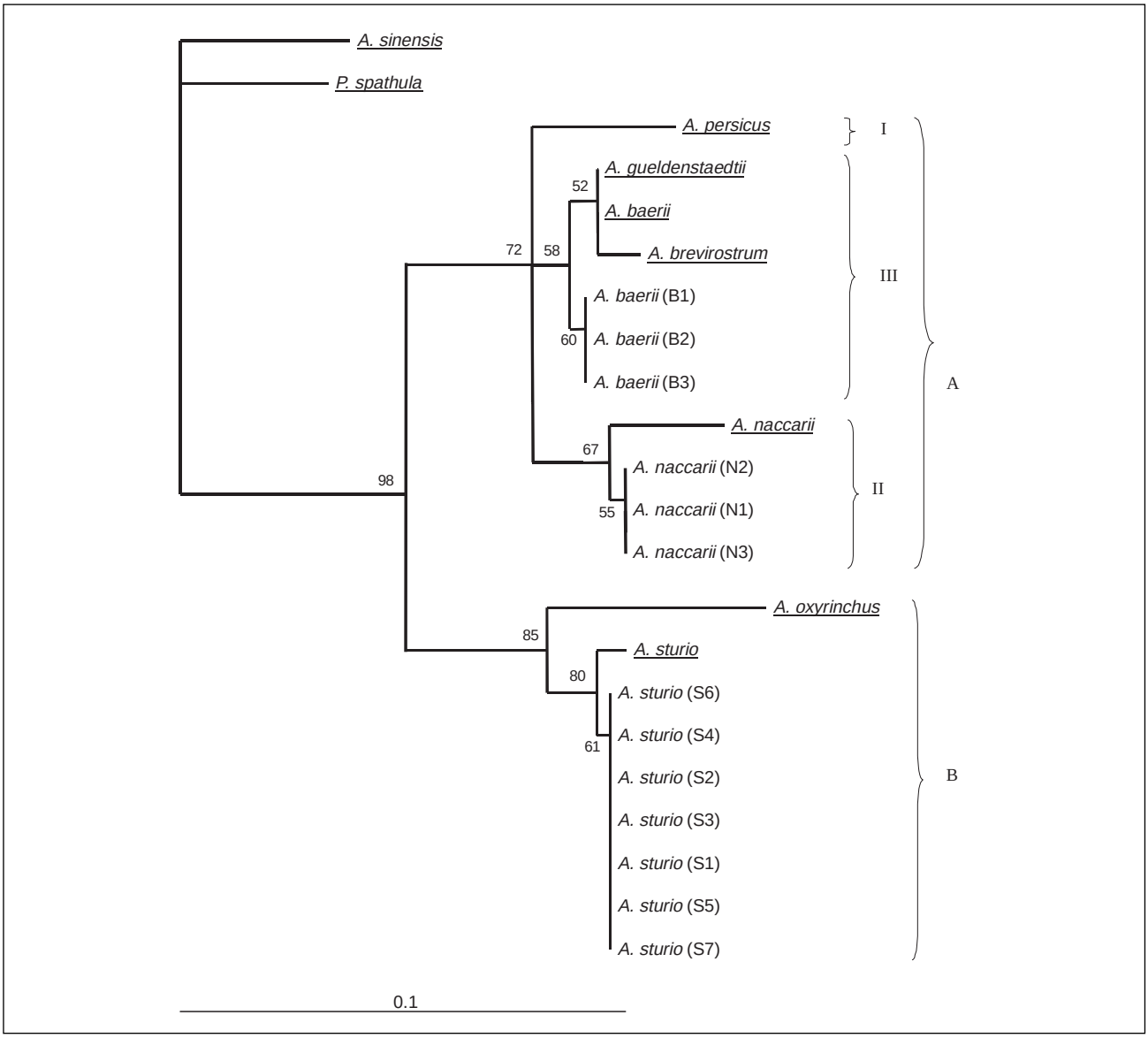

Figure 3. Estimated phylogenetic tree of 105-bp cytochrome *b* sequences according to the neighbour-joining method Kimura's distance. Bootstrap supports higher than 50 % are shown. Branch lengths are proportional to the estimated mean number of substitutions per site. GenBank species names are underlined

supported bootstrap values. The position of *A. sinensis* was not clearly resolved. Two clusters, A and B, can be clearly identified. Cluster A contained *A. persicus* (I), a clade of the *A. naccarii* specimens (II), and a clade which included *A. brevirostrum, A. gueldenstaedtii* and the *A. baerii* specimens (III). *A. persicus* was a sister species to (II) and (III). The clade (II) included all *A. naccarii* specimens, archival and alive. Its monophyly was well-supported (88 % for the NJ tree, 60 % ML tree, and 90 % for the MP tree). The *A. baerii* specimens analysed in the present paper, and the *A. baerii*, *A. brevirostrum*, and *A. gueldenstaedtii* specimens studied by Birstein and DeSalle (1998) (data from GenBank) also formed a well-supported clade. The second cluster, B, contained *A. oxyrinchus* and *A. sturio*.

The second phylogenetic tree was built using the 155-bp cyt *b* gene fragments obtained from *A. sturio* museum specimens originating from the Iberian Peninsula, Gironde River, North Sea, and the Adriatic (figure 3). The same overall pattern was observed for this short sequence as with the previous 245-bp segment. Sequences from all *A. sturio* samples, including that retrieved from GenBank, were clustered together, forming a monophyletic branch.

DISCUSSION

DNA from archival specimens

Currently, museum specimens are the only available material for *A. sturio* from many populations or from a geographical range of a population that has almost disappeared. Recent advances in molecular techniques offer a potential for DNA extraction from a diverse range of materials previously considered intractable (France and Kocher, 1996; Habelberg, Sykes and Hedges, 1989; Rosenbaum *et al.*, 1997; Pääbo, 1989; Wirgin *et al.*, 1997; see DeSalle and Bonwich, 1996, for a review). In our study the best yield in PCR products from archival samples was when we followed the protocol of France and Kocher (1996). However, only a short 155-bp fragment of the cyt *b* gene was successfully amplified. The tree based on the 105-bp sequences showed that a short sequence is suitable for discerning among the species studied: the nodes had good support bootstrap values (figure 3). We agree with Ludwig *et al.* (2000) that since *A. sturio* has almost disappeared in the wild, molecular analyses of specimens representing different populations in museum collections are crucial for any recovery plan for this species.

Phylogenetic implications

Clusters A and B in our trees (figures 2 and 3) generally support the phylogenetic relationships presented in Birstein and DeSalle (1998) for a longer region (650-bp) of the cyt *b* gene and for combined data for this region, plus a 350-bp fragment of the 16S rDNA and a 150-bp fragment of the 12S rDNA (figures 1 and 3 in that paper). The different position of *A. sinensis* in our tree, compared to the trees in Birstein and DeSalle (1998), was evidently caused by the high divergence of this cyt *b* gene fragment in *A. sinensis* from the other *Acipenser* species included in our study.

The relationships of species within clades of cluster A are basically congruent with the earlier phylogenetic studies. The position of the North American shortnose sturgeon of the Atlantic coast, *A. brevirostrum*, in our tree point to a close relatedness to the Russian sturgeon *A. gueldenstaedtii* inhabiting the Ponto-Caspian basin (Vlasenko *et al.*, 1989), and with the Siberian sturgeon *A. baerii*, living in Siberian rivers and Lake Baikal (Ruban, 1997). Morphologically *A. gueldenstaedtii* is similar to *A. persicus* (Vlasenko *et al.*, 1989) and *A. naccarii* (Tortonese, 1989), and it is considered to be related to *A. baerii* (Sokolov and Vasil'ev, 1989). Our results showed that *A. gueldenstaedtii* appears to be more closely related to *A. baerii* and *A. brevirostrum* than to *A. naccarii* and *A. persicus*. *A. persicus* is a sister species to the *A. baerii*, *A. gueldenstaedtii* and *A. brevirostrum* cluster, and to the *A. naccarii* clade. Conversely, Tagliavini *et al.* (1999) postulated a close relationship between *A. naccarii* and *A. gueldenstaedtii* and other clusters which includes *A. brevirostrum* and *A. baerii*.

Although Birstein and DeSalle (1998) used a much longer region of the cyt *b* gene (650 bp), their data showed that *A. gueldenstaedtii, A. persicus, A. baerii, A. naccarii,* and *A. brevirostrum* form an unresolved clade (figure 1 in their paper). They succeeded in placing *A. brevirostrum* outside this clade only when they used combined data for three genes, which contained a 1 150-bp-long sequence. In this analysis, *A. brevirostrum* was basal to the Ponto-Caspian and some Asian sturgeons (figure 3 in Birstein and DeSalle, 1998). A more detailed study of the relationships among Eurasian species of the same clade included a longer region of the cyt *b* gene (850-bp), a partial sequence of the ND5 gene (643-bp), and a fragment of the control region of mitochondrial DNA (725-bp), or approximately 2.3 kb bp (Birstein, Doukakis and DeSalle, in press). The traditionally recognised *A. gueldenstaedtii* in fact appeared to consist of two genetic forms, a "typical" and a cryptic one. The "typical" form is phylogenetically affiliated with *A. persicus* and *A. naccarii*. These data show that *A. persicus* is not a separate species; rather, it is conspecific with *A. gueldenstaedtii*. *A. naccarii* was diagnosed by only one G in position 164 of the control region. The cryptic form of *A. gueldenstaedtii* is very close genetically to *A. baerii*: it differed from *A. baerii* only by C-T transition at position 432 of the control sequence.

This example of complex species relationships found among a group of Eurasian sturgeons illustrates that reliable phylogenetic data can be obtained only if long DNA sequences are included in a study. Such results as those published by Tagliavini *et al.* (1999) can be considered only preliminary: these authors used very short sequences in their research and studied a small number of

sturgeon species. The latter leads to an inadequate picture of species relationships due to the problem of underdiagnosis of phylogenetic lineages (Davis and Nixon, 1992). In general, the low genetic variability shown by acipenseriforms (Birstein, Hanner and DeSalle, 1997) makes a study of phylogenetic relationships within this order complicated.

A. sturio/*A. oxyrinchus* cluster and *A. sturio* intraspecies variability

The grouping of *A. sturio* with *A. oxyrinchus* (figures 2 and 3) is congruent with previous molecular phylogenetic results, and supports the hypothesis that these species might be direct descendents of ancestral forms of *Acipenser* (Birstein and DeSalle, 1998). The present molecular study has not detected genetic difference among specimens of *A. sturio* from the Iberian Peninsula, Gironde River, North Sea, and Adriatic. Likewise, Ludwig and Kirschbaum (1998) did not find variable sites in a 400-bp fragment of the 12S ribosomal DNA when they compared *A. sturio* specimens from the North Sea and Gironde River. However, Birstein and DeSalle (1998) obtained six nucleotide changes in a 600-bp fragment of cyt *b* in two specimens of *A. sturio*, suggesting intra-species forms of *A. sturio* (see Birstein and Doukakis, 2000, for further discussion).

The specimen EBD 8174

Garrido-Ramos *et al.* (1997) found a HindIII satellite (st) DNA in the genomes of two specimens of *A. naccarii* from a fish farm that was absent in two specimens of *A. sturio* from the Gironde River. The HindIII stDNA family was then considered a species-specific nuclear DNA marker (see Ruiz-Rejón *et al.*, 2000). Garrido-Ramos *et al.* (1997) attributed specimen EBD 8174 to *A. naccarii*, based on the presence of the HindIII sequences in the DNA extracted from this specimen. The present study enabled us to positively identify specimen EBD 8174 as *A. sturio*. Our conclusion supports the results of previous morphometric and meristic studies of this specimen (Elvira and Almodóvar, 1999, 2000; Rincón, 2000).

CONCLUSIONS

Our preliminary results did not show genetic differences between *A. sturio* individuals from the Iberian Peninsula, Gironde River, Adriatic, and North Sea. We identified specimen EBD 8174 from the Guadalquivir River as *A. sturio*. The comparison of the cyt *b* gene sequences among *A. sturio*, *A. naccarii*, and *A. baerii* showed 22-24 fixed nucleotide changes among species in that region. The analysis of our data set, together with that retrieved from GenBank, supports the hypothesis of Birstein and DeSalle (1998) that *A. oxyrinchus* is the only sturgeon species closely related to *A. sturio*. Since *A. sturio* has almost disappeared in the wild, the molecular analyses of specimens representing different populations of this species in museum collections are specially important in terms of recovery plans for this species.

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