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Failure to confirm previous identification of two putative museum specimens of the Atlantic sturgeon, *Acipenser sturio*, as the Adriatic sturgeon, *A. naccarii*

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Abstract MtDNA analyses were employed to examine the species identification of three preserved museum sturgeon specimens from the River Guadalquivir, two of which were originally described as *Acipenser sturio* L., 1758 by Hernando (1975; Doñana, Acta Vertd 2: 263–264). Later the same two specimens were identified as *A. naccarii* Bonaparte, 1836 by Garrido-Ramos et al. (1997; Mar Biol 129: 33–39) on the basis of morphometric and nuclear DNA analyses. Three different laboratories using independent techniques were unsuccessful in extracting authentic DNA from tissue samples from the three specimens and in obtaining any verifiable PCR product. We cannot confirm the previous molecular identification of two of these specimens by Garrido-Ramos et al. as *A. naccarii*. We suggest that these specimens are indeed *A. sturio*.

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Introduction

The European Atlantic sturgeon, *Acipenser sturio*, is considered by many to be the only sturgeon species native to the Iberian Peninsula (Almaça 1988; Doadrio et al. 1991; Elvira et al. 1991a, b; Elvira and Almodóvar 1993; Pereira 1995). Recent studies, however, have raised questions concerning which other *Acipenser* species are endemic to the region. Garrido-Ramos et al. (1997) concluded that the Adriatic sturgeon, *A. naccarii*, also existed historically in Spain. These authors conducted a morphometric study of museum sturgeon specimens. Additionally, they performed a molecular analysis of two specimens, Nos. EBD 8173 and EBD 8174 from the Guadalquivir River in the collection of the Biological Station of Doñana, Sevilla (Spain). Hernando (1975) had previously identified these specimens as *A. sturio*. Garrido-Ramos et al. (1997) claimed that the snout shape and position of barbels, as well as the presence of *A. naccarii* species-specific satellite DNA in tissue extracts, indicated that the two specimens should be considered *A. naccarii*, and not *A. sturio*. Since these particular morphological characters are especially affected by ontogenetic allometry, Elvira and Almodóvar (1999) conducted further analysis of the specimens. Based on a more detailed morphometric comparison of the same specimens with the other 28 in collections of 16 Spanish museums, Elvira and Almodóvar (1999) attributed these two specimens to *A. sturio*.

At present, the Siberian sturgeon, *Acipenser baerii*, is also caught periodically in the rivers of Spain (Elvira and Almodóvar 1999). These individuals are apparently

escapees from commercial sturgeon aquaculture. Individuals of *A. baerii* can be distinguished from the other *Acipenser* species by their fan-shaped gill rakers, a distinctive, diagnostic morphological character unique to *A. baerii* (Sokolov and Vasil'ev 1989). Unfortunately, it is difficult to recognize this character in young *A. baerii* (Prokes et al. 1997). It is also difficult to distinguish *A. baerii* from *A. naccarii* through a mtDNA comparison, but the two species can be readily distinguished from *A. sturio* using such methodology (Birstein et al. 1998).

In order to verify the results of Garrido-Ramos et al. (1997), we used mtDNA analyses to examine tissue samples from the two sturgeon specimens studied originally (EBD 8173 and EBD 8174) plus one more specimen (EBD 8401) from the same collection (Biological Station of Doñana, Sevilla, Spain), which also came from the Guadalquivir River.

Materials and methods

Materials

Small pieces of muscle tissue were obtained from each of the following three preserved sturgeon specimens from the collection of the Biological Station of Doñana, Sevilla: (1) EBD 8173; Guadalquivir River, Alcalá del Río, Sevilla, Spain; 12 April 1974; TL = 1755 mm; alcohol; (2) EBD 8174; Guadalquivir River, Alcalá del Río, Sevilla, Spain; 11 May 1975; TL = 1520 mm; stuffed; (3) EBD 8401; Guadalquivir River, Coria del Río, Sevilla, Spain; winter 1981; TL = 1610 mm; alcohol.

Molecular methods

Replicate muscle tissue samples were preserved in alcohol and mailed to the authors' three different laboratories in New York, Berlin, and Madrid, where each team used its own methods.

New York, mtDNA: cytochrome b (cyt b) gene, NADH5 gene, control region

In order to identify the species origin of the museum samples, we attempted to amplify and sequence small regions of mtDNA molecule (portions of the cyt *b* and NADH5 genes, and of the control region) using the polymerase chain reaction (PCR) and sturgeon-specific amplification primers (see below). Then we compared the nucleotide sequences of the amplified regions to our database of extant sturgeon mtDNA [Birstein and DeSalle 1998, GenBank AF006123–AF006188 (cyt *b*); and unpublished]. The gene regions chosen contained species-specific nucleotides as characterized in previously sequenced voucher specimens.

DNA extraction was performed using established methods: the phenol/chloroform preparation (DeSalle et al. 1993) and DTAB/CTAB (Gustincich et al. 1991). The phenol/chloroform preparation included an overnight incubation of small pieces of tissue in the presence of 300 µl Liftons buffer (100 m*M* EDTA, 25 m*M* Tris, pH 7.5, 1% SDS) and 30 µl 20 mg ml^{-1} proteinase K. Then, 25 µl 5 *M* KAc (pH 4.8) and 350 µl phenol were added, the aqueous layer was extracted, and an additional purification step was performed using 350 µl chloroform. The extracted aqueous layer was then mixed with 1 ml 100% EtOH, and the DNA was allowed to precipitate at −20 °C overnight. After spinning, the DNA pellet was resuspended in a mixture of 200 µl RNAse-free water, 25 µl 7.5 *M* NH_4OAc, and 700 µl EtOH and placed at −20 °C for 20 min. The DNA was pelleted and air-dried before resuspension and use in the PCR. The DTAB/CTAB protocol consisted of grinding small pieces of tissue in 300 µl of 1 *M* Tris EDTA (pH 8.8), adding 600 µl DTAB [8% dodecyltrimethylammonium bromide, 1.5 *M* NaCl, 100 m*M* Tris (pH 8.8), 50 m*M* EDTA] and incubating at 68 °C for 15 min with occasional mixing. Two chloroform purification steps were then performed. The extracted aqueous layer was mixed with 900 µl water and 100 µl CTAB (5% hexadecyltrimethylammonium bromide, 0.4 *M* NaCl). After mixing and spinning, the supernatant was discarded and the DNA was resuspended in 300 µl of a 1.2 *M* NaCl solution; 750 µl of 100% EtOH was added to reprecipitate the DNA. After spinning for 10 min, the DNA pellet was washed with 300 µl 70% EtOH, spun, and air-dried before resuspension.

To test the extraction success, PCR amplification was initially attempted for a small region (approx. 200 bp) of the cyt *b* gene. The universal sturgeon primers B7-2 (5′-GCCTACGCCATTCT CCG-3′) and S2a (5′-CCTCCAATTCATGTGAGTACT-3′) were used in a PE 486 thermocycler under the following conditions: 1 min at 94 °C, 1 min at 46 °C, 1 min at 72 °C for 33 cycles. These amplification attempts were unsuccessful and assumed to be the result of an impure DNA preparation. We then divided the products obtained from each of the phenol/chloroform extractions into several separate aliquots and treated them with one of the following preparations according to the manufacturer's instructions: BIO 101 Gene Clean (BIO 101 Inc., La Jolla, California, USA), Wizard DNA Purification Systems (Promega, Madison, Wisconsin, USA), or DNAzol Genomic Isolation Kit (Molecular Research Center Inc., Cincinnati, Ohio, USA). The DTAB/CTAB extraction product was also treated but only with the BIO 101 Gene Clean procedure. Amplifications were then attempted for an additional cyt *b* fragment B1 (5′- CCATCCAACATCTCTGCTTGATGAAA-3′) (J. Groth, Dept. of Ornithology, AMNH), and B2 (5′-CAGAATGATATTT GGCCTCA-3′), plus fragments in the NADH5 gene region (ND5, 5′-AATAGTTTATCCAGTTGGTCTTAG-3′) (Bembo et al. 1995) and RND5-3 (5′-AAGCCCATGAGTGGTAGG-3′), and the control regions HF400 (5′-CAGTCCTGCTTTTGG GGTTTGAC-3′) and dlp1.5 (5′-GCACCCAAAGCTGARRTT-CTA-3′) (Baker et al. 1993).

Berlin, mtDNA: 12S rRNA gene

Our previous analysis of the 400-bp region of the 12S rRNA gene allowed us to distinguish between *Acipenser sturio* from the Gironde River basin and *A. naccarii* from the Buna River, Albania (Ludwig and Kirschbaum 1998). We found seven diagnostic substitutions including three different restriction sites in this region. We used the same approach in the present study.

From each sample 25 µg tissue was washed in 0.68% NaCl over 24 h at 4 °C, then homogenized and transferred to 180 µl lysis buffer (QIAGEN Inc., Hilden, Germany) for 48 h. DNA was then purified following the standard protocol using the QIAamp Tissue Kit (QIAGEN Inc.). A part of the mitochondrial 12S rRNA gene was amplified with 5 U AmpliTaq DNA Polymerase (Perkin-Elmer Cetus) under the following conditions: 30 s at 94 °C, 1 min at 45 °C, and 2 min at 72 °C (using the primers of Kocher et al. 1989: L1091 (5′-AAAAGCTTCAAACTGGGAT-TAGATACCCCACTAT-3′) and H1478 (5′-TGACTGCA-GAGGGTGACGGGCGGTGTGT-3′). However, amplification resulted in a faint fuzzy band of about 400 bp when the DNA was visualized on the agarose gel.

The PCR product was purified with QIAquick Gel Extraction Kit (QIAGEN Inc.) and directly sequenced (Ludwig and Kirschbaum 1998). Additionally, the purified PCR products were cloned before sequencing to minimize the possibility of artifacts and amplification of nuclear pseudogenes.

We cloned the PCR products using TOPO TA Cloning Kit following the manufacture's instructions (Invitrogen Corp., San Diego, California, USA). Ten clones of each sample were amplified in 50-µl reaction volumes containing 2.5 U AmpliTaq DNA Polymerase (Perkin-Elmer Cetus), 0.2 m*M* degenerate primers (Kocher et al. 1989), and 25 ng DNA from each sample. The reaction was amplified in a Perkin-Elmer thermocycler 2400 programmed for 30 cycles each for 10 s at 94 °C, 10 s at 50 °C, and 2 min at 72 °C and

sequenced in an ABI 310 sequencer (ABI, Weiterstadt, Germany) as described by Ludwig and Kirschbaum (1998).

Madrid, mtDNA: cyt b gene

Several methods of DNA extraction and purification were tested. The usual protocol followed the Sambrook et al. (1989) phenol/chloroform method after grinding the sample to a fine powder in the presence of liquid nitrogen. We also tried extractions with buffers containing proteinase K and incubation overnight at 50 °C. No traces of DNA were observed after electrophoresis of the extracts on agarose gel. Aliquots of the extracts were then used for the PCR reaction with the primer H15149 (5′AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3′) (Kocher et al. 1989) and the reverse primer (5′-CGAACGTTGATATGAAAAACCATCGTTG-3′). No evidence of PCR amplification was obtained and we concluded that the DNA was degraded into small fragments. After electrophoresis of the main portion of the total extract, the 400 bp region (according to the size of control DNA fragments) was cut from the gel and DNA was extracted from it using BIO 101 Gene Clean (BIO 101 Inc.). This purified DNA was then employed both for the usual and booster PCR amplification (Ruano et al. 1989).

Results

New York, mtDNA: cyt *b* gene, control region, NADH5 gene

On no occasion did we obtain a DNA isolation which yielded a reliable, verifiable PCR product. PCR products were often faint with reactions requiring large amounts of template. Several positive PCR reactions were also accompanied by negative control products. When any of these products were sequenced, the results were ambiguous. The analysis of sequences from different genes from the same individual often gave a different species' identification. The results of a search for the cyt *b* sequence using the BLAST program (NCBI) suggested that the DNA was from either *Acipenser fulvescens* or from a *Scaphirhynchus* species (EBD 8401) or from *A. stellatus*, *A. brevirostrum*, or *A. transmontanus* (EBD 8174). The cyt *b* sequences obtained did not exactly match any of the sequences in our database and were not comparable to *A. sturio*, *A. naccarii*, or *A. baerii*. For the NADH5 region, no Acipenseriformes sequences have been submitted to GenBank, but a search using the BLAST program confirmed the sequence most closely resembled a *Cyprinus* or *Oncorhynchus* species. The NADH5 sequences were similar to the Acipenseriformes in our database but did not match any sequence. We had no success in amplifying any genes for specimen EBD 8173. Finally, our amplification and sequencing results were not reproducible. We concluded that these results indicated either slight contamination of PCR primers or reagents, surface contamination of the muscle samples due to prior handling, or an artifact of alcohol storage.

Berlin, mtDNA: 12S rRNA

Sequences of cloned PCR fragments differed widely and were not comparable to the 12S rRNA sequences of *Acipenser sturio* (EMBL GenBank Y12663) or *A. naccarii* (EMBL GenBank Y12664). Summarizing the results of sequencing analysis, PCR products of about 400 bp length were amplified, but species identification was not possible. The differences among the cloned sequences in each sample strongly indicated that samples were contaminated with non-authentic DNA.

Madrid, mtDNA: cyt *b*

In our first experiment we amplified a 402 bp fragment. A comparison of the sequence from this fragment with the *Acipenser* sequences in GenBank showed no similarity to the cyt *b* gene sequence of any sturgeon species. We could not obtain an amplified product in our attempts to repeat the first result even under various PCR cycling conditions. We concluded that in the first experiment we worked with a contaminant, and not with authentic DNA extracted from the specimen.

Discussion and conclusions

Overall, the lack of success in obtaining repeatable DNA extractions and amplifications in three separate laboratories indicates that the preservation or storage of the tissue sample has made it extremely difficult, if not impossible, to extract DNA from these specimens. The presence of artifacts and contaminants is one of the great risks of using museum specimens for DNA analysis and can be the result of sampling methods and/or storage conditions of specimens. Also, there are numerous examples of pseudogenes in the nuclear DNA that are derived from the mtDNA, including copies of the cyt *b* genes and rRNA-segments (van der Kuyl et al. 1995; Zhang and Hewitt 1996), which can create a potential problem for species identification.

In our study, we could not replicate the extraction methods used by Garrido-Ramos et al. (1997) since the methods were not described. The reference cited by the authors for handling museum specimens (Pääbo 1989) did not provide any specific methodology. Therefore, we tested several commonly used DNA isolation protocols as detailed above. We did not use methods for formalin-preserved specimens (France and Kocher 1996; Vachot and Monnerot 1996; Jiang et al. 1997; Wirgin et al. 1997) since the museum specimens examined were preserved in alcohol or dried. Nevertheless, future studies of these samples should probably include such methodology.

Using different methods and approaches, we failed to extract DNA from the same museum sturgeon specimens studied by Garrido-Ramos et al. (1997). We feel that our results combined with those of Elvira and Almodóvar (1999) indicate that the main conclusion of Garrido-Ramos et al. that some museum specimens of *Acipenser sturio* in Spanish collections are in fact

A. naccarii needs confirmation. Future research should include proving the actual species-specificity of the *A. naccarii* satellite DNA since the original study examined only *A. sturio* and *A. naccarii* and not other *Acipenser* species. Since we were unable to confirm that specimens EBD 8173 and EBD 8174 are *A. naccarii* as postulated by Garrido-Ramos et al. (1997), we suggest that the possibility that these specimens could be *A. sturio* should not be ignored (Elvira at al. 1991a; Elvira and Almodóvar 1999). These results have a bearing on sturgeon restoration plans in Spain.

We suggest that the current plan offered by Ruiz-Rejón et al. (1998) to release *Acipenser naccarii* from aquacultured stock in Italy into the Guadalquivir River should be evaluated extremely carefully. Sympatric populations of *A. naccarii* and *A. sturio* have only been described from the Po River basin, Italy (Rossi et al. 1992). Very few data have been published on the genetic difference between *A. sturio* and *A. naccarii* (Birstein and DeSalle 1998; Ludwig and Kirschbaum 1998), although, theoretically these two species can hybridize (Holčík et al. 1989). Also, very little is known about intraspecific genetic variation in *A. sturio* (Kinzelbach 1997), but molecular data point to the possibility of at least two distinctive genetic forms within the species (Birstein et al. 1998). It is unclear whether Garrido-Ramos et al. (1997) examined both of these forms. Conservation measures for the endangered *A. sturio* are urgently needed, but for sustainable conservation of *A. sturio* and *A. naccarii* comparative molecular studies that include other museum specimens are a prerequisite. The integrity of *A. sturio* populations should be strictly protected, and premature stocking of other sturgeon species in Spain could jeopardize the potential rehabilitation of this species.

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