



## Mitochondrial Cytochrome b Sequence Variation in Three Sturgeon Species (*A. stellatus* Pallas, 1771, *A. gueldenstaedtii* Brandt, 1833, *H. huso* Linnaeus, 1758) from the Black Sea Coasts of Turkey

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### Abstract

It has been assumed that sturgeons originated in the Tethys Sea, and the Black Sea is their most important diversification center. Hence Turkey has had a significant place throughout history. Although there are some genetic studies about sturgeon species that inhabit the Black Sea, these studies have not included samples from Turkey. In this study, the phylogenetic relationship, cladistic positions, and genetic variations were determined from the Cyt-b (1141bp) mtDNA of 3 sturgeon species (n = 28), collected from Turkish coast of the Black Sea between 2005 and 2007. Nineteen haplotypes, and haplotype diversity ranging from 81.8% to 96.4%, were detected from Cyt-b sequences. Studied A+T rates were found between 52.6 and 53.8% for all species. Ti/Tv rates were estimated for each species: *A. gueldenstaedtii* (9:1), *A. stellatus* (7:2), and *H. huso* (6:0). Nucleotide diversity and nucleotide differences calculated for three species varied from 0.167-0.378% and 1.89-4.2, respectively. Genetic distances were calculated (1.258-5.288%). Each species was separated by phylogenetic reconstruction with a high bootstrap value (100%). All *A. gueldenstaedtii* samples replaced in the Black Sea Lineage Group and were separated into 2 clades (A and B). Similarly, *A. stellatus* samples were separated into 2 clades, but *H. huso* samples were not separated.

**Keywords:** Sturgeon, *A. stellatus*, *A. gueldenstaedtii*, *H. huso* Turkey, Black Sea, mtDNA, Cyt-b, sequence analysis, phylogenetics.

### Karadeniz'in Türkiye Kıyılarında Dağılım Gösteren Üç Mersin Balığı Türünün (*Acipenser stellatus*, *Acipenser gueldenstaedtii*, *Huso huso*) Mitokondriyal Sitokrom b Sekans Varyasyonu

#### Özet

Mersin balıklarının Tethys Denizi kökenli olduğu ve farklılaşma merkezinin Karadeniz olduğu düşünüldüğünde Türkiye çok önemli bir yere sahiptir. Karadeniz'deki mersin balığı türleri hakkında yapılmış çalışmalar bulunmasına rağmen Türkiye kıyılarından örnekleme yapılmamıştır. 2005-2007 yılları arasında Türkiye kıyılarından örneklenen 3 türe ait (*Acipenser stellatus*, *A. gueldenstaedtii*, *H. huso*) 28 örnekte Cyt-b geni (1141bp) sekans verilerinden genetik mesafeler ve filogenetik dendogramlar oluşturulmuş ve kladistik analizler yapılmıştır. Cyt-b geninde 19 haplotip ve %81,8-96,4 arasında değişen haplotip çeşitliliği belirlenmiştir. A+T oranı tüm türler için %52,6 ile 53,8 arasında bulunmuştur. Ti/Tv oranı *A. gueldenstaedtii*'de (9:1), *A. stellatus*'ta (7:2) ve *Huso huso* için (6:0) olarak bulunmuştur. Nükleotid çeşitliliği ve nükleotid farklılığının sırasıyla %0,167-%0,378 ve 1,89-4,2 arasında değiştiği belirlenmiştir. Türler arası mesafe Kimura-2 parametresine göre hesaplanmıştır (%1,258-5,288). Filogenetik ağaçlar sonucunda %100'lük seç-bağla değeri ile türler ayrılmıştır. *A. gueldenstaedtii* örneklerinin hepsi Karadeniz soy grubu içerisinde yer aldığı ve 2 farklı gruba (A ve B) ayrıldığı belirlenmiştir. Benzer olarak *A. stellatus* örneklerinin de 2 gruba ayrıldığı tespit edilmiştir. Buna karşın, *H. huso* örneklerinde ayrılma görülmemiştir.

**Anahtar Kelimeler:** Mersin Balığı, *A. stellatus*, *A. gueldenstaedtii*, *Huso huso*, Türkiye, Karadeniz, mtDNA, Sitokrom b, sekans analizi, filogenetik

### Introduction

Throughout history, sturgeon fish has had great value in different aspects. The high cultural,

commercial, ecological, and zoological value of sturgeons has promoted an interest in sturgeon biology including their systematics and evolution (Bemis *et al.*, 1997). More information is needed

about the ecology, genetics, environmental physiology, toxicology, and behavior of sturgeons to understand the environmental impacts on different species. The *Acipenseriformes* order (sturgeons and paddlefish) includes 27 species in four currently recognized genera in a Holarctic distribution. This is an ancient assemblage with recognizable acipenserid fossils from the Upper Cretaceous and fossil relatives extending the origin of *Acipenseriformes* into the Lower Jurassic (Grande and Bemis, 1991; Findeis, 1997).

These species are mainly distributed in the water systems; such as rivers, lakes, and sea water (brackish water) of China, Central Asia, Lake Aral, the Caspian Sea, the Black Sea, and in North America (Grande and Bemis, 1991; Bemis et al., 1997). There are six sturgeon species inhabiting in the the Danube river basin, The Black Sea including the Turkish seas (Geldiay and Balik, 1996). However, due to anthropogenic impacts, Atlantic sturgeon (*Acipenser sturio*), Sterlet (*Acipenser ruthenus*) and Ship sturgeon (*Acipenser nudiventris*) have nowadays almost disappeared from the region, while population of Beluga (*Huso huso*), Russian sturgeon (*Acipenser gueldenstaedtii*) and stellate sturgeon (*Acipenser stellatus*) are experiencing severe decline (Williot et al., 2002).

Sturgeon fish are slow growing and mature very late in life that may take 20 years (depending on species) and, even then, do not spawn every year. Thus, they are particularly susceptible to exploitation and to many other risks, including pollution and habitat fragmentation. Fishermen easily catch adult sturgeon because of their large body and recognized spawning migration routes. Their dependence on shallow spawning areas and fast flowing rivers leads to the effects of exposure to environmental factors, as well (Bemis and Kynard, 1997). Today, almost all sturgeon species categorized by the International Union for Conservation of Nature (IUCN) as endangered, vulnerable, or least concern (Birstein et al., 1997b).

Meristic and morphological similarities among a number of species of sturgeons living within the same basin can be defined as a certain species of *Acipenser*, particularly during the juvenile period. For this reason, it is impossible to rely on literature for the type of comparisons that must be made (Bemis et al., 1997; Birstein et al., 1997b). Although many diverse characteristics and methods have been used to analyze the genetic structure in exploited and endangered species like sturgeons (for example, ecological approaches, tagging, parasite distribution, physiological and behavioral traits, morphometrics and meristics, calcified structures, cytogenetics, immunogenetics and blood pigments, and the use of molecular genetic techniques in fisheries), researches on the genetics of this species have increased dramatically over the last three decades, largely due to the increased availability of techniques and increased

awareness of the value of genetic data. Nowadays, there are many different types of DNA markers used in molecular genetics of sturgeon, to identify stock discrimination of commercially important species and to determine species and their origin including: allozyme (Chikhachev, 1983; Ivanenkov and Kamshilin 1991; Kuz'min, 1991; Pourkazemi, 1996; Kuz'min, 2002), DNA sequence data (DeSalle and Birstein, 1996; Birstein et al., 1998), PCR-Single Strand Confirmation Polymorphism (SSCP) (Rehbein, 1997; Ludwig et al., 2000), RAPD (Comincini et al., 1998; Gharaei et al., 2005), Species-specific PCR (DeSalle and Birstein, 1996; Birstein et al., 1998; Mogue et al., 2006), PCR-RFLPs (Wolf et al., 1999; Ludwig et al., 2002), AFLP (Congiu et al., 2001), Microsatellites (Jennekens et al., 2001; Zhu et al., 2002; Moghim et al., 2012), and SNP (Timoshkina et al., 2011).

For identification of phylogenetic relationship among sturgeon species, mtDNA is preferred as it is the rapid rate of evolution, the maternal mode of inheritance and the relatively small size of mtDNA make the analysis of this molecule one of the methods of choice for many studies (Ferguson et al., 1995). Furthermore, the low effective size of mtDNA compared to the nuclear markers makes it more likely to provide a population-specific marker, resulting in generally greater genetic differentiation than nuclear markers (Ferguson et al., 1995). In the course of evolution, the variation ratio of the Cytb gene sequence is higher than that of other functional areas. This gene has also been one of the most frequently utilized segments of mtDNA because it is easy to align and it has been characterized in many vertebrates (Stepien and Kocher, 1997; Kocher et al., 1989; Zardoya and Doadrio, 1999; Orti et al., 1994; Brito et al., 1997). In the case of fish, the cytochrome b gene has been used to address many phylogenetic questions, from relationships among closely related species to deep phylogenetic questions such as the relationships among *Acipenser gueldenstaedtii*, *A. baerii* and *A. persicus*, so the Cytb gene is considered to be a good marker to research on genetic differentiation and phylogenetic relationship.

This study characterized the mtDNA variation in the 3' end of the Cyt-b gene in three sturgeon species found at the Turkish coast of the Black Sea. Genetic structure was assessed by evaluating the pattern of variation in mtDNA sequences within this selected geographic range.

This is the first molecular study based on an endangered sturgeon species from the Turkish coast of the Black Sea. Thus, it may shed light on the mode of diversification of Black Sea fauna of the area. Second, mutual monophyly at the Cyt-b level of Turkish and other Black Sea countries' sturgeons was preliminarily tested in this study. To this aim, 4 species of sturgeon from several downstream areas and seashores of the Turkish coast of Black Sea were included in the study.

## Materials and Methods

### Sample Collection and DNA Extraction

Between 2005-2007, on the Black Sea coast of Turkey, *A. gueldenstaedtii* (12), *A. baerii-like* (1) *A. stellatus* (7) and *H. huso* (8) species were sampled (Table 1). Sampling for sturgeon species was carried out at seven sites in the Black sea area ranging from Rize to Sakarya. Samples from the cultured Russian sturgeon whose eggs were transferred from Russia to Istanbul University were also considered. There is one *A. baerii-like* haplotype in the *A. gueldenstaedtii* samples. Although the situation of fish with this haplotype is controversial (Bristein *et al.*, 2000), it was evaluated in this study to make comparison with other studies. A non-destructive sampling method was used in this study by taking caudal fin tissue from the fish. Caudal fin tissues about 1–1.5 cm<sup>2</sup> were preserved immediately in 98% ethanol and stored at an ambient temperature in the field and -20°C in the laboratory.

Total genomic DNA was extracted from 15-20 mg of tissue by the DNA extraction kit (PROMEGA SV Wizard kit) using the following the manufacturer's protocol or standard proteinase K, phenol/chloroform extraction (Sambrook *et al.*, 1989). The DNA concentration and quality was assessed by a UV/visible spectrophotometer (BIO-RAD, The SmartSpec Plus) at 260/280 nm. Furthermore, the extracted DNA was examined on 1% agarose gels stained with EB and stored at -20°C until needed.

### PCR Amplification and Sequencing

Approximately 1.227 bp of mitochondrial segment, including the Cyt-b gene, were amplified with Cyt-f (5'-TGACTTGAAAACCACCGTTGTTA-3') (position 14.387 – 14.410 in *Acipenser stellatus* mitochondrion, complete genome) designed for brown trout (Bernatchez and Donznan, 1993) and Cyt-r (5'-CTTCGGTTTACAAGACCG-3') primers (position 15.597-15.614 in *Acipenser stellatus* mitochondrion, complete genome) (Ludwig *et al.*, 2000). Each 50µl reaction contained 200-250 ng DNA, each primer, PCR Master Mix (2X) (Promega) (Reaction buffer, pH 8.5), and ddH<sub>2</sub>O. The 2X PCR Master Mix (Promega), which is a premixed and

ready-to-use solution, contains Taq DNA Polymerase (50 unit/ml), dNTPs (400 µM dATP, 400µM dGTP, 400µM dCTP, 400 µM dTTP), MgCl<sub>2</sub> (3mM) and reaction buffers. Samples were amplified in a PTC-200 gradient thermal cycler (MJ Research, Waltham, Massachusetts, USA) using the following protocol: after a preliminary denaturation at 94°C for 1 min, each of the 35 cycles consisted of denaturation at 94°C for 1 min, annealing at 47°C for 30 s, and primer extension at 72°C for 1.5 min. To improve the subsequent reamplification and sequencing, a final cycle was added; including an extension at 72°C for 5 min. Negative controls of DNA extraction and negative PCR control were subjected to amplification. The success of the PCR reaction was visualized using 1.5-2.0% agarose gel electrophoresis with a molecular-size ladder, and stained with ethidium bromide (EB) solution to determine the size and quality of fragments under UV light. DNA bands in gels were viewed under UV light and the images were recorded.

The PCR products were purified before sequencing using Wizard® SV Gel and the PCR Clean-Up System, following the manufacturer's instruction. The purified products were then sent to MACROGEN (Macrogen Inc., Seoul, Korea) for sequencing, using both forward and reverse strands.

Nucleotide sequences of the Cyt-b segment of mtDNA were established for 28 sturgeons. Differing variants of the sequenced regions were deposited in the GenBank/NCBI under accession numbers KC130090–KC130117. The following Cyt-b gene sequences from the related sturgeon species were retrieved from the GenBank and used in the present study (AF238688, AF238694, AF238669, AJ249692, AF238699, AJ563386, AF238679, AJ277594, AJ563395, AJ563394, AF238700, AF238705, AJ277596) *A. gueldenstaedtii*, (AF238620, AF238615) *A. persicus*, (AJ245833, AJ245834, AF238660) *A. naccarii* (AF238658, AF238653, AF238631, AF238640, 11102385) *A. baerii*, (AJ249693, AY846685, AY846696, AY846689) *A. stellatus*, (AJ245828) *A. brevistrum*, (AJ245832) *A. nudiventris*, (AJ249694) *A. ruthenus*, (AY510085) *A. dabryanus*, (AJ245830) *A. medirostris*, (AJ245839) *A. sturio*, (AJ245840) *H. huso*, (AJ252187) *H. dauricus*, (NC005834) *Psephurus gladius*, (NC004419) *Polyodon spathula*. For comparative analysis, 37 Cyt-

**Table 1.** Sampling location of sturgeon species used in these studies [number of sample (sampling no)]

Samling locations	Species				Total
	<i>A. gueldenstaedtii</i>	<i>A. baerii/like</i>	<i>A. stellatus</i>	<i>H. huso</i>	
Rize	-	-	-	1 (25)	1
Trabzon	-	-	3 (26-27-28)	3 (20-29-69)	6
Giresun	4 (40-44-53-54)	1 (46)	2 (48-60)	3 (18-56-59)	10
Samsun	5 (37-61-67-91-62)	-	2 (32-66)	1 (76)	8
Sakarya	1 (63)	-	-	-	1
Hatchery originated samples from Russia	2 (2-11)	-	-	-	2
Total	12	1	7	8	28

bp sequences from the GenBank were combined with the sequences of the present study and used to infer the phylogenetic relationship of sturgeons in the Turkish coast of the Black Sea. For the analysis, one representative of a closely related species, *Polyodon spathula*, was used as the outgroup.

The genetic structure was assessed by evaluating the pattern of variation in mtDNA sequences within this selected geographic range.

### Statistical Analysis

Genetic differences among *sturgeons* and proximal matrix relationships were tested using the statistical packages BIOEDIT (Hall, 1999), DnaSP 4.0 (Rozas and Rozas, 1999), MEGA 3.0 (Kumar et al., 2004), Arlequin (Schneider et al., 2000), and PAUP (Swofford, 2003). Nucleotide sequences from all specimens were compared and aligned among themselves and in respect to other sequences present in databases using the ClustalW computer software package (EMBL, Heidelberg, Germany). Minimal visual adjustments were made to the completed alignments using MEGA 3.0 (Kumar et al., 2004) and the BioEdit Sequence Alignment Editor (Hall, 1999). The protein sequence was predicted from the Cyt-b gene sequences using the BioEdit Sequence Alignment Editor (Hall, 1999). Haplotype numbers and haplotype diversity (h), nucleotide diversity ( $\pi$ ) and nucleotide differences (Tajima, 1983) were computed using DnaSP 4.0 (Rozas and Rozas, 1999) software.

All matrices were analyzed with two approaches: Bayesian inference and parsimony. Bayesian analyses were performed in MrBayes 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). For each single-gene dataset, the best-performing evolutionary model was identified under two different model selection criteria: Akaike information criterion (AIC) (Akaike, 1974) and the Bayesian information criterion (BIC) (Schwartz, 1978). These calculations were made in the Modeltest 3.7 (Posada and Crandall, 1998) and MrModeltest v.2.2 (Nylander, 2004) software using the graphical interface MrMTgui (1.0) (Nuin, 2008) and PAUP\* ver. 4.0b10 for Windows (Swofford, 2003; Nylander, 2004). Phylogenetic trees were constructed using maximum parsimony (MP; minimum heuristic search factor of 2), neighbor-joining (NJ) and the unweighted pair group method (UPGMA), by MEGA version 3 (Kumar et al., 2004) based on Kimura two-parameter distances (Kimura, 1980). The robustness of the inferred UPGMA, MP and ML trees were tested by bootstrapping with 1000 pseudo replications. These trees were determined by applying the 50% majority rule (strict consensus) for bootstrap replicates. In order to investigate the genetic differentiation among the three sturgeon species, an analysis of the pairwise Kimura-2 parameter distances between species was computed using the Arlequin 3.1

software (Schneider et al., 2000).

## Results

### Alignment and Base Composition of the Cyt-b Gene

The 1141 bp region of the 3' end of the mtDNA Cyt-b gene was aligned for 28 specimens; 19 haplotypes were identified with 100 segregating sites including 7 single variable sites and 93 parsimony informative sites (Figure 1). Haplotypes indicated minimal homoplasy because no more than two different nucleotides were identified at each segregating site. Haplotypes were not shared between species and were found to be specific to the species. While five haplotypes (H1, H2, H3, H4, H5) were observed in *A. gueldenstaedtii* (n = 12), only one haplotype (H6) was shared with one specimen of *A. baerii*, similar to the one from Giresun. Six (H7, H8, H9, H10, H11 and H12) and 7 haplotypes (H13, H14, H15, H16, H17, H18 and H19) were found in *A. stellatus* (n=7) and *H. huso* (n=8), respectively. Frequency distribution of haplotypes within and between species is given in Figure 1. H2 was the most frequent haplotype (33.3%) identified in *A. gueldenstaedtii* and shared by 4 specimens.

For all the sequences of three sturgeon species, the average nucleotide frequencies of thymine (T), cytosine (C), adenine (A), and guanine (G) were 26.6%, 31.9%, 26.5%, and 15.2%, respectively, and varied between 14.9 and 32.0%. The content of A+T (53.1%) was higher than that of G+C (46.9%) (Table 2). At the third position, strong compositional biases against G (only 7.5%) were observed. At all levels of sequence divergence, the ratio of unambiguous transitions to transversions in Cyt-b were estimated for each species; *A. gueldenstaedtii* (9:1), *A. stellatus* (7:2), and *H. huso* (6:0) and Ti's outnumbered Tv's, even in comparisons between the ingroup and the outgroup (Table 2). Meanwhile, the ratio of transitions/transversions (R) was 7.3. There was no significant difference in base compositional bias between ingroup and outgroup species. A total of twenty-five intraspecific variable sites were found among the 1141 bp of the Cyt-b gene in the 28 cases examined. Nucleotide diversity ( $\pi$ ) and nucleotide differences (k) calculated for three species varied from 0.167% to 0.378% and from 1.89 to 4.2, respectively. Most of the mutation events were transitions (88%, including 63.6% CT and 36.4% AG). Transversions were mostly AT (66.7%) and AC (33.3%).

### Genetic Distances

Pairwise genetic distances among and within three sturgeon species and the outgroup, *Polyodon spathula*, were estimated by MEGA (Kumar et al., 2004) using the K2P method with gamma correction



and *A.baerii*-like *A. stellatus*, and *H. huso* appearing as monophyletic clades. Bootstrap support ranges from 50 to 100% under all tree-building methods. Bootstrap proportions are given for each method of analysis and shown for each node.

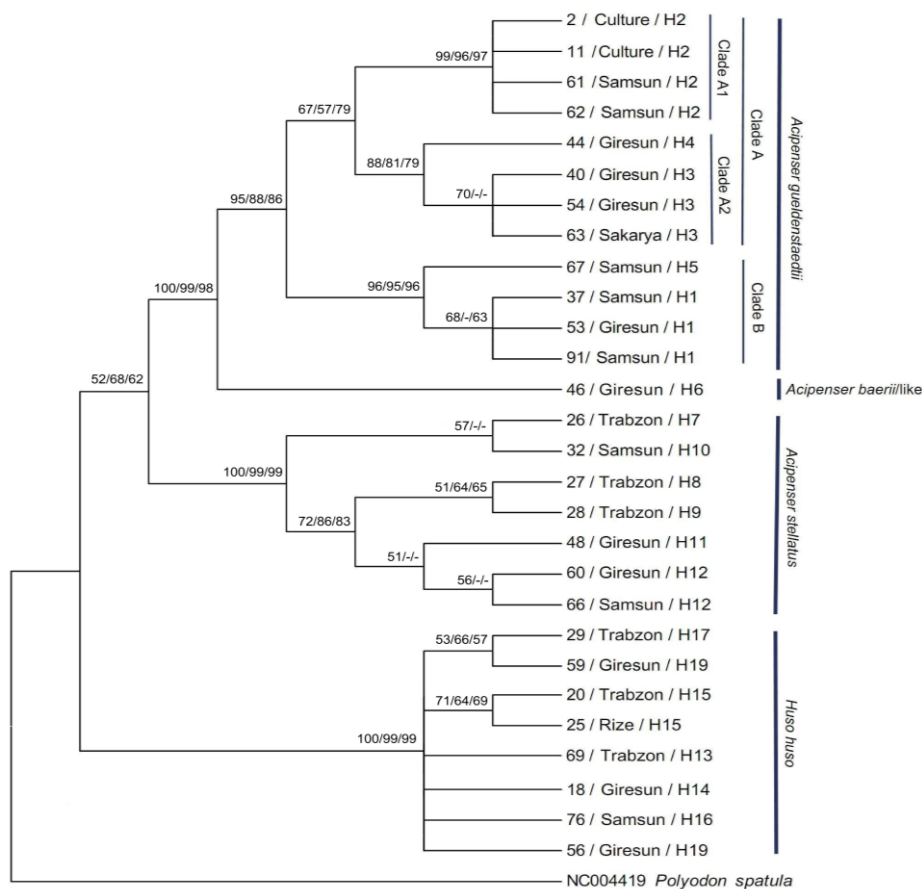
The results showed that the Black Sea sturgeons from the Turkish coast were grouped into two assemblages: *Acipenser* and *Huso*. The *Acipenser* group is represented by the species *A. gueldenstaedtii*, *A.baerii*-like and *A. stellatus*, and forms a separate branch in the UPGMA, MP and ML trees.

Results of the phylogenetic trees for each species shown in Figure 2 indicated the presence of a clear grouping within the Black Sea sturgeon species

(*A. gueldenstaedtii*) supported by high bootstrap values (60 - 96%). Group I could be clearly divided into two clusters. The first included the hatchery-originated individuals (2 and 11) grouped within the individuals from the Samsun samples (61 and 62), and the second contained the other three (40, 44, 54) from Giresun grouped with one of the Sakarya samples (63). Group II contains four cases (37, 53, 67 and 91), three of which (37, 67 and 91) were from Samsun, and one of which (53) was from Giresun. A sample number 46 completely separated from *A. gueldenstaedtii* samples in dendograms were found closer to the *A.baerii*-like sample according to the results of the BLASTn, in which a nucleotide

**Table 3.** Kimura 2-parameter pairwise distances (gamma corrected) based on cytochrome-*b* sequence data (outgroup was included). Italic values are intra- species distances ( $\alpha$ : 0.05)

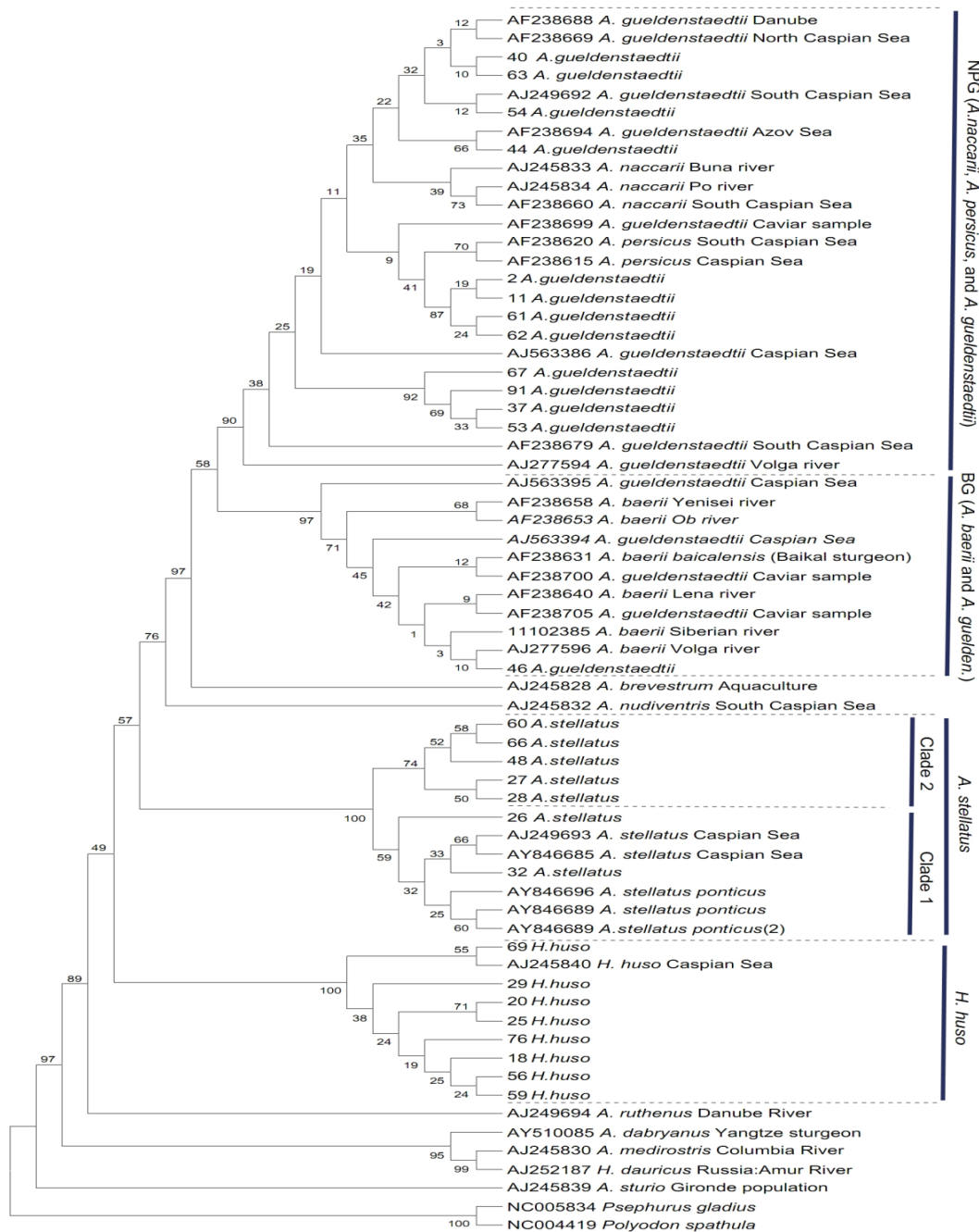
Species	A	B	C	D	E
<i>A. gueldenstaedtii</i> (A)	<b>0.0042</b>				
<i>A. stellatus</i> (B)	0.1722	<b>0.0032</b>			
<i>H. huso</i> (C)	0.1782	0.2793	<b>0.0017</b>		
<i>A.baerii</i> /like (D)	0.0163	0.1700	0.1259	-	
<i>P. spatula</i> (E)	4.3372	4.8568	4.6423	4.3696	-



**Figure 2.** Phylogenetic tree of the genus *Acipenser* and *Huso* based on mtDNA cytochrome *b* gene sequences. Numbers on branches represent the bootstrap values obtained for 1000 replications corresponding to the UPGMA (the unweighted pair group method), MP (maximum parsimony) and ML (maximum likelihood) methods. Support values under 50% are not represented and given order of UPGMA/MP/ML. Identification numbers of individuals are followed by sampling sites and shared haplotypes.

sequence is compared to the contents of a nucleotide sequence database. The sequences of homologous mtDNA regions were used, sequenced by other authors, and deposited in the GenBank/NCBI under accession numbers given in the methods section. Since all sequences were used in the comparative analysis, Group II samples were separated from GenBank samples belonging to the Black Sea and the adjacent area, but Group I together and two subgroups were included in the GenBank samples (Figure 3). The strong phylogenetic split into two groups

corresponds to the main geographic areas. Group II seemed to be almost exclusive to the Turkish Black Sea waters while Group I might be entirely exclusive to other areas in the Black Sea. All trees (UPGMA, MP and ML trees) revealed that the *A. stellatus* was composed of two clusters. One of them was supported (>50% bootstrap) and contained representatives of cases 26 and 32. The second was a larger assemblage that consists of five individuals (27, 28, 48, 60 and 66). When analyzing the trees for *H. huso*, such a separation was not seen within the species. Samples



**Figure 3.** UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram based on Kimura two-parameter distances (Kimura, 1980), showing relationship between related sturgeon species retrieved from GenBank and three Black sea species used in the present study, using Cytochrome b sequences. The robustness of the inferred UPGMA tree was tested by bootstrapping with 1000 pseudoreplications.

(20 and 25; 29 and 59) were closer to each other but were not separated from the others.

## Discussion

### Characteristics of the Cyt-b Sequences

There are phylogenetic and population studies regarding the Eurasia species in the Black Sea, such as *Acipenser gueldenstaedtii*, *A. stellatus* and the *H. huso* species together with other sturgeon species (Ludwig et al., 2000; Bristein et al., 1998a, b; Doukakis, 2000; Jennecknes et al., 2000; Stabile et al., 1996; Brown et al., 1992). On the other hand, there have been no studies conducted thus far regarding the sturgeon species in Turkey based on either molecular or morphometric analysis. This study compared samples obtained from the Black Sea coast of Turkey both within the samples and with other studies by means of a sequence analysis of the mtDNA Cyt-b gene.

Approximately 1227 base pair (bp) fragments of the mitochondrial Cyt-b were amplified and then sequenced from 28 samples belonging to 4 species: *A. gueldenstaedtii* (12), *A. baerii*-like (1), *A. stellatus* (7) and *H. huso* (8). Complete sequences of 1141 bp Cyt-b were obtained after correction and alignment. Among the 28 sequences of the 4 sturgeon species, 19 distinct haplotypes were detected: 5 in *A. gueldenstaedtii*; 1 in *A. baerii*-like; 6 in *A. stellatus*; and 7 in *H. huso*. In general, one dominant haplotype (H2) was shared by 4 specimens in *A. gueldenstaedtii*. All haplotypes were unique to their own species.

Haplotype diversity in bony fish is over 80% (Grant and Bowen, 1998). Similar results were also obtained from other studies conducted with sturgeon species (Stabile et al., 1996; Birstein and Doukakis, 2000; Doukakis et al., 2005). In this study, high haplotype diversity, between 81.8% - 96.4% for each of the three species, was observed. Despite the low number of samples for the species studied, the results obtained from this study are the same as other studies.

Furthermore, in this study, one *A. baerii*-like haplotype (Hap6) was observed within the samples obtained from the Black Sea and classified morphologically as *A. gueldenstaedtii*. Similarly, in the studies conducted on the Volga River (Jennecknes et al., 2000), the Sea of Azov (Voynova et al., 2008) and the Eurasian species (Doukakis, 2000), *A. baerii*-like mitotypes were observed within the samples of *A. gueldenstaedtii* species. According to Ludwig et al. (2002), natural sturgeon hybrids are often rare. However, possible reason for hybrids results could be intended release programs, habitat alterations or unintended escapes from hatcheries.

Different researchers indicated that Cyt-b (53-57%) and D-loop (70-80%) regions have a high A+T nucleotide content (Apostolidis et al., 1997), and an A+T nucleotide content with a 68% ratio is a general value accepted for a control region (Meyer, 1993).

The current study revealed that the A+T nucleotide ratio is 52.6% for *A. stellatus* and *H. huso*, while it is 53.2% and 53.8% for *A. gueldenstaedtii* and *A. baerii*-like, respectively, suggesting that average level of genetic diversity for studied species.

In previous studies, the Cyt-b gene was found to be rich in C, A, and T nucleotide ratios, but low in G content (Cantatore et al., 1994; Duokakis, 2000; Almodovar et al., 2000). The nucleotide frequencies of the Cyt-b gene were shown for different living groups by Johns and Avise (1998). When compared with the values given in the Table 4, the Cyt-b gene region was found to be rich in the C ratio (32%) but low in the G ratio (15%) in the current study.

The mean base composition in Cyt-b sequences was similar to those previously reported for Actinopterygian fish and the other mitochondrial protein-coding genes (Johns and Avise, 1998) with low G content (15.2%). A high A+T bias may be a common phenomenon in fish and an A+T nucleotide bias, when present, tends to accumulate in hyper variable sites (Simon, 1991).

Nucleotide diversity was calculated as 0.378%, 0.299% and 0.167% for *A. gueldenstaedtii*, *A. stellatus* and *H. huso*, respectively. Nucleotide diversity values calculated for the studied gene region was found to be different between species and generally in agreement with the values observed in other fish.

### Phylogenetic Analysis

In contrast to the known phylogenetic positions of Acipenseriforms, the phylogenetic relationships of the species in this order are still controversial (Mayden and Kuhajda, 1996; Birstein et al., 1997a; Findeis, 1997; Birstein and DeSalle, 1998; Ludwig et al., 2000).

In UPGMA, MP and ML dendrograms obtained as a result of the phylogenetic analysis prepared for *A. gueldenstaedtii* samples, it was observed that this species is divided into two groups as A (A1-A2) - B. When all *A. gueldenstaedtii* samples are compared with the samples taken from the GenBank, the samples in the "B" group are separated from the samples both in the "A" group (A1 and A2) and from the GenBank. On the other hand, the samples in the

**Table 4.** Nucleotide frequencies in the Cyt-b gene for different animals (modified from Johns and Avise, 1998)

Animals	T	C	A	G	A+T
Birds	25.0	33.9	27.6	13.5	52.6
Mammals	28.8	28.4	29.6	13.3	58.4
Reptiles	28.6	27.6	27.7	16.1	56.3
Amphibians	32.7	24.3	27.0	16.0	59.7
Fishes	29.5	29.9	25.3	15.3	54.8
Sturgeons (current study)	26.83	31.45	26.52	15.18	53.36



“A” group are not separated from the samples taken from the GenBank. In the study conducted by Birstein *et al.* (2000), based on the phylogenetic analysis of sequences of three mitochondrial gene regions (Cyt-b, ND5 and D-loop), *A. gueldenstaedtii* was separated into two main groups as NPG (*A. naccarii*, *A. persicus*, and *A. gueldenstaedtii*) and BG (*A. baerii* and *A. gueldenstaedtii*). Similar to this study, according to Almodovor *et al.* (2000), *A. gueldenstaedtii* is separated into two distinct genetic forms which are “typical” and “cryptic” (hidden) forms. The typical form phylogenetically combines *A. persicus* with *A. gueldenstaedtii* and *A. naccarii*. As a result of this data, it was specified that *A. persicus* may be a subspecies of *A. gueldenstaedtii* rather than a separate species. While the cryptic form of *A. gueldenstaedtii* is genetically very close to *A. baerii*, it is separated from *A. baerii* in 432<sup>th</sup> position of control region with C-T transition. When two mitochondrial lineage groups within *A. gueldenstaedtii* are considered, all *A. gueldenstaedtii* samples that were studied are within the group (NPG) that can called “The Black Sea Lineage Group”. The “B” group, included in the Black Sea Lineage Group, within *A. gueldenstaedtii* samples and represented by the samples on the shores of Turkey, should be finalized by working with more samples.

As seen in either the generated sequence data or the dendograms obtained as a result of phylogenetic analysis drawn by the data taken from the GenBank (Figure 3), *A. gueldenstaedtii* and *A. baerii* species are relative to *A. brevirostrum* and are close to each other. Based on the classification conducted by Almodovor (2000), North American shortnose sturgeon (*A. brevirostrum*), generally found on the Atlantic coast, was observed to be closely related to the Russian sturgeon (*A. gueldenstaedtii*), which is found in the Black Sea - Caspian (Eurasia) region (Vlasenko *et al.*, 1989), and the Siberian sturgeon (*A. baerii*), which is found in the Siberian rivers and the Lake Baikal (Ruban *et al.*, 1997). These results support the phylogenetic relationship obtained by Birstein and Desalle (1998). On the other hand, Birstein and Desalle (1998) compared the 650 bp region of the Cyt-b gene and used the 150 bp of 12S rDNA combined with the 350 bp of 16S rDNA. Furthermore, in the study conducted by Birstein and Desalle (1998), despite the use of a longer Cyt-b region in contrast to the data of other researchers, *A. naccarii*, *A. persicus*, *A. gueldenstaedtii*, *A. baerii* and *A. brevirostrum* species were found to be of a separate lineage.

The specimen that was indicated as *A. gueldenstaedtii* in samplings, but was closer to *A. baerii* according to sequence results, was found to be separated from *A. gueldenstaedtii* as a result of the comparison with *A. gueldenstaedtii* samples. The presence of *A. baerii* in different river basins, which are indeed not a natural distribution area of this species, was indicated by Jenneckens *et al.* (2000). On

the other hand, in the study conducted by Jenneckens *et al.* (2000), it was reported that the haplotype belonging to this species was observed within *A. gueldenstaedtii* samples obtained from the Volga River, but not observed in the Sea of Azov and the South Caspian Sea. In contrast to these results, the results obtained in this study indicate this sample was used in the study conducted by Jenneckens *et al.* (2000) and supports the doubts of it being of Siberian sturgeon with a haplotype shown as *A. baerii* haplotype. The most likely reason of this result is genetic contamination of *A. gueldenstaedtii* with *A. baerii* or *A. baerii* hybrids.

*A. stellatus* samples were separated as a species based on the topologies obtained with a 100% bootstrap value. In addition, when compared to the samples taken from the GenBank, it was found to be separated into its own (Clade 1 and Clade 2) and closer to *A. nudiventris*. When the studies were considered in order to determine the differences between intra-species, no distinction between species at subspecies level has been identified in the protein study conducted by Kuz'min (1994). Similarly, in the study conducted on D-loop and ND5 genes performed by Doukakis (2000), a low GST value calculated for *A. stellatus* indicated that the subspecies determined for the Ural River, the Volga River, the Caspian Sea, the Black Sea, or the Sea of Azov are the same genetically.

In light of these studies, stocking was believed to be the main factor for the intra-species embodiment of *A. stellatus*. The ongoing stocking studies ensured the continuity of populations in the Caspian, the Azov, and the Black Seas (Doukakis, 2000). Yet it was unknown whether this process had any effect on the genetic structure due to the lack of genetic studies conducted prior to these studies. This effect can be only determined by studying museum samples (outdated).

Despite the *H. huso* species belonging to the *Huso* genus, it was found to be separated from the studied samples with a 100% high bootstrap value, and when compared to the sequence data obtained from the GenBank, it was considered closely related to *A. ruthenus* (Figure 3). Similarly, in previous molecular studies (Birstein *et al.*, 1997a; Birstein and Desalle 1998; Doukakis, 2000), *H. huso* was separated and considered as a monophyletic lineage, but it is within the *Acipenser* genus and closely related to *A. ruthenus*. *H. huso* was thought as a subgenus of *Acipenser* by most nineteenth-century systematics and the genus status related to its late evolution is still controversial since then (Artyukhin, 1995; Bemis *et al.*, 1997). In contrast to these results, while *Huso* is the basis for all of other *Acipenseriforms* according to Findeis (1997), it is sister taxa within *Acipenser* according to Artyukhin (1995) and Mayden and Kuhajda (1996). Artyukhin (1995) emphasized that *Huso* may be within the *Acipenser* genus and is closely related to *A. ruthenus*,

*A. nudivetris* and *A. shrenckii*. In addition, although *H. huso* and *A. ruthenus* species have different life stories and show large variations in their morphological aspects, they were found to be closely related due to their ability to produce fertile offspring by means of easy hybridization (Bristein and DeSalle, 1998; Bristein et al., 1997b).

### Divergence Times and Evolutionary Scenario

The possibility of inferring divergence times more accurately has been promoted by the idea that the accumulation of genetic change between lineages can be used as a molecular clock (Zuckerkanndl and Pauling, 1965). Molecular sequence data allow an estimation of distances only. Under the assumption of a constant rate over time, the distance is a linear function of time. To convert distances into absolute times, an external time (called a calibration point) is used, and obtained from fossil data or geological events that mark the separation of the species. From a biological perspective, sturgeons are one of the oldest and most primitive existing Osteichthyan fishes (Ludwig, 2006). Acipenseriformes have existed since at least the Lower Jurassic period (approximately 200 MYBP), and all fossil and recent taxa arose in the Holarctic period. Phylogenetic relationships among Paleozoic and Early Mesozoic actinopterygians are problematic, but most researchers agree that Acipenseriformes are monophyletic and derived from some component of 'paleonisciform' fishes (Bemis et al., 1997). In order to estimate the divergence times among species from the mtDNA data set, published fossil data were used to calibrate the ages of the nodes on the ML tree. A number of previous clock studies have suggested that the estimated time for splitting between sturgeons and paddlefishes is 135-150 mya (Birstein et al., 1998a). Therefore, 135–150 mya was used as the range of dates for the C1 calibration node.

All available paleontological information indicates that Acipenseriforms originated in the Tethys basin (Birstein et al., 1997a). The geological events during the formation of the Tethys region are among the main factors playing a role in both the diversity of many species and the distribution of sturgeon fishes. The Tethys Sea sank, and the Black Sea, the Caspian Sea and the Aral Sea were formed approximately 120 mya, and as a result of these events, the sturgeon lineage originated (171 mya). Therefore, ongoing separation resulted in the North American *A. oxyrinchus*, and *A. sturio* from Europe. However, this scenario completely eliminates the other possibilities. Other reasons such as increased salinity in waters between continents should be considered. Another example for the distribution of sturgeon fish in the sea is the Vicariance Scenario, observed due to continental drift. The opening of the Atlantic Ocean between Europe and North America formed about 100 mya, and the difference between the two main lineages of sea sturgeon supports this

event. Sea sturgeons are the anadromous fish, well adapted to the marine environment, and seen on both sides of the Atlantic Ocean. The adaptation of sturgeons to the marine environment is probably a primitive feature for these groups of fish. The loss or reduction in the ability to enter the marine environment is observed, depending on the formation of ecologically suitable freshwater locations.

Six different species were used for calibration, and the status of polyploidy was taken into account in the study conducted by Peng et al. (2007). In this way, the differentiation time of *H. huso* and *A. stellatus* species from *A. gueldenstaedtii* was calculated as 86.4 mya (48.2-132.6) and 59.7 mya (29.4-99.8), respectively. These values support the results obtained in this study. In other words, the distance from *A. gueldenstaedtii* to *H. huso* was calculated as 7.15-58.8 mya, while it was calculated as 6.89-58.1 mya from *A. gueldenstaedtii* to *A. stellatus* in this study.

According to previous studies on the molecular phylogenetic of sturgeon fish, two different mtDNA lineage groups in *A. gueldenstaedtii* complex and *A. baerii*, *A. persicus* and *A. naccarii* originated from one monophyletic. They are relatively younger and *A. gueldenstaedtii* is a more fundamental taxa. Two different mtDNA lineage groups in *A. gueldenstaedtii* indicate that the other three species may have originated from the populations of this species (Bristein and DeSalle, 1998).

Geological factors have a definite role in the structure of *A. baerii* intra-species. According to Berg (1928) in Doukakis (2000), ancestral *A. baerii* which belong to the BG group of *A. gueldenstaedtii* may have migrated from the Black Sea-Caspian region to the Siberian rivers and lakes system during the great ice age or by means of post-glacial melt (flood) (Doukakis, 2000). The connection of the lakes with the Siberian rivers occurred approximately 10,000-15,000 years ago (Arkhipov et al., 1998). It has been reported that the sea and the basins of Siberian Rivers were connected by way of a lake system during the last glacial period (Arkhipov et al., 1998). The last connection occurred approximately 18,000-20,000 years ago (Laukhin, 1997). From this relatively new connection to the present, there has not been enough time for change so that the nucleotide changes can be stabilized into different subspecies of *A. baerii* (Doukakis, 2000). In contrast to these geographic structures, according to the evaluation of the molecular clock indicated by Peng et al. (2007), *A. baerii* (24 mya) and *A. naccarii* (5.3 mya) appear to have diverged from *A. gueldenstaedtii* earlier than this period. These values support the results regarding the time of separation of *A. baerii* (0.5-12 mya) obtained in this study.

According to assessments of the researchers, the two species (*A. naccarii* and *A. baerii*) originated from different genetic forms of *A. gueldenstaedtii* at approximately the same time. The differences in

genetic and morphological characters between species are very small due to the proximity of the past historical process.

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