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Determination of Sturgeon Species Living in the Black Sea Coasts of Turkey by Sequence, RFLP and Multiplex PCR Analysis Methods

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Abstract: In order to perform checks in the trade of fish species under the threat of extinction and with commercial importance such as sturgeon, the fish must be identified rapidly and precisely from any tissue and for this purpose morphometric and protein based determinations can be inadequate. In this study, *Acipenser stellatus*, *Acipenser gueldenstaedtii* and *Huso huso* sampled from Trabzon coasts were used. For RFLP and sequence analysis, the mitochondrial Cytochrome-b (*mtDNA Cyt-b*) gene was amplified by the help of PCR. From Cyt-b sequence data, it was observed that in the Maximum Parsimony (MP) dendrogram, 99% of the species were separated. Furthermore in the Cyt-b PCR product restriction enzyme analyses, it was determined that Hinf I and Rsa I enzymes were distinguishing for 3 species. In the multiplex PCR application in the mtDNA D-loop region, the product lengths were determined as 420 bp for *A. gueldenstaedtii*, 350 bp for *H. huso* and 260 bp for *A. stellatus* and they are distinctive for the species. The results showed that the multiplex PCR application is a method which is cheap and effective for the identification of sturgeon species in short duration.

Key words: Sturgeon, mitochondrial DNA, multiplex PCR, PCR-RFLP, sequence, species identification

INTRODUCTION

Today, 27 species belonging to the Acipenseriformes order (sturgeon and paddlefish) are known to exist and this group is the oldest to have survived from the Jurassic period until today. Sturgeons live in the rivers, lakes and seas of the world from China to Central Asia, the Aral Sea, Caspian Sea and in Northern America in the Northern hemisphere (Bemis *et al*., 1997). It was reported that 5-6 species of Acipenseriformes are distributed along Turkish coasts and the river systems of the Black Sea basin (Kizilirmak, Yesilirmak, Sakarya and Coruh) (Ustaoglu and Okumus, 2004; Celikkale *et al*., 2004). However, the species (*Acipenser sturio*, *A. ruthenus* and *A. nudiventris*) do not exist today except *Huso huso*, *A. gueldenstaedtii* and *A. stellatus*.

Since 1998, international trade in all species of sturgeons has been regulated under CITES owing to concerns over the impact of unsustainable harvesting of and illegal trade in sturgeon populations in the wild. In the context of this regulation, legal arrangements are required by all countries related to the Black Sea and the Caspian Sea for the safety of the sturgeon population. Initially, 2 sturgeon species (*A. sturio* and *A. brevirostrum*) were included in the Annex-1 list which prohibited the international trade of these 2 species. The rest was included in Annex-2 which enables any given nation to hunt a certain amount of fish from a certain place by dispensation (Raymakers, 2000, 2002). Most of the species are endangered in many parts of the world. Throughout history, sturgeon has been a valuable fish. Furthermore, the species need to be identified accurately from meat, egg and larvae samples for the protection and management practices of species with commercial value. There are some problems at this point. It is difficult to distinguish species that are similar in terms of morphometric and meristic properties. Furthermore this problem becomes particularly more apparent in the early period (larvae). A 76 similar problem is encountered in the identification of the species from sturgeon eggs.

Generally, in the identification done according to the size and colour of eggs these properties can be similar for many species (Bemis and Kynard, 1997; Bemis *et al*., 1997).

The other method is the analyses based on proteins. Among these, Isoelectric Focusing (IEF) and Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) were used for species identification. However, since SDS-PAGE protein profiles of commercial species are similar, it is not possible to distinguish *H. huso*, *A. gueldenstaedtii* and *A. stellatus*. Moreover, the studies performed using proteins are not appropriate for products treated by heating and salting (Ludwig, 2008). Together with the development and utilization of

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DNA based molecular techniques, species identification has become more definite. One of these methods is PCR based sequence analysis and three species from caviars have been identified by the sequence analysis of the *mtDNA-cyt-b* gene (DeSalle and Birstein, 1996; Birstein *et al*., 1998, 2000; Eroglu, 2008). Furthermore, species identification has been attempted by the PCR-RFLP technique (Wolf *et al*., 1999). In another study, multiplex PCR primers approaching D-loop were used for the identification of nine sturgeon species (Miuge *et al*., 2008).

The aim of this study was to distinguish three species living in Turkish coast of Black Sea by using 2 restriction enzymes (Hinf I, Rsa I), Cytochrome-b sequence and multiplex PCR analysis. It was the first molecular study performed in Turkey for the control of international trade in the context of CITES. Also, it was intended that the study would be the basis for future theoretical and practical applications by contributing, to some extent to the knowledge base.

MATERIALS AND METHODS

Fish samples: In the context of the research project carried out by the Central Fisheries Research Institute between 2006 and 2010, 15 sturgeon samples were used *Acipenser stellatus* (n = 5), *Acipenser gueldenstaedtii* (n = 5) and *Huso huso* (n = 5) which were caught off the coast of Trabzon-Rize (Fig. 1).

Different sampling methods are employed for living creatures (killing, catching without killing or sampling from the matter left behind-skin, horn, fecal matter, etc.). In this study, a sampling method was employed which was applied by taking tissue from the caudal fin of the fish without harming the fish itself.

DNA extraction: The tissue samples which were approximately 1-1.5 cm^2 taken from the caudal fins of sample fish to extract DNA were put into tubes of 1.5-2.0 mL containing 98% ethanol and brought to the laboratory to be stored at -20°C in a deep freeze. To extract DNA from tissue samples, the phenol/chloroform-alcohol precipitation method was employed (Kocher *et al*., 1989; Hillis *et al*., 1990). The purity and amount of DNA obtained was realized by reading the optical density at the wavelengths of 260 and 280 nm using a UV visible spectrophotometer.

PCR amplification: Cty-b and D-loop regions from the total DNA obtained from sturgeon samples were amplified via a Thermal Cycler using primer sets (forward and reverse) developed by other researchers. The following primers F-5'-TGACTTGAAAAACCACCGTTGTTA-3'

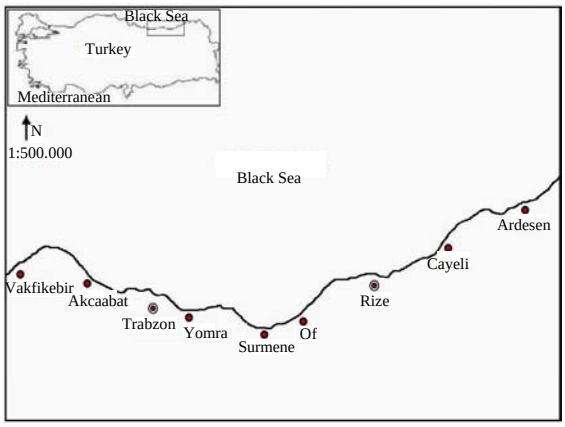

Fig. 1: Map of sampling location

(Bernatchez and Danzmann, 1993) and R- 5'-CTTCGGTT TACAAGACCG-3' (Ludwig *et al*., 2000) were used to amplify the whole *cyt-b* gene (1141 bp). For multiplex amplification of the D-loop control region, primer sets of AHR (general reverse), AGF , ABF, ABRM, HusF, DauF, NudF, RutF, SteF, SchF (Miuge *et al*., 2008) were used. Amplification reactions were carried out with 50 μL final volume and contained 1 μL of each primer (10 pM), 1 μL DNA template (200 ng μL^{-1}), 22 μL ddH_2O and 25 μL 2X PCR Master Mix (Promega) which is a premixed and ready-to-use solution containing Taq DNA Polymerase (50 unit mL^{-1}), dNTPs (400 μM dATP, 400 μM dGTP, 400 μM dCTP, 400 μM dTTP), $MgCl_2$ (3 mM) and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. The PCR reactions for Cyt-b were performed with a PTC-200 gradient thermal cycler (MJ Research, Waltham, Massachusetts, USA) with an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 60 sec, annealing at 49°C for 30 sec and extension at 72°C for 90 sec and final extention was of 5 min at 72°C. PCR amplification condition for D-loop: DNA denaturation at 94°C 60 sec, annealing at 57°C for 30 sec and primer extension at 72°C for 45 sec, repeated for 30 cycles. The first denaturation step was of 2 min at 94°C and the final extension was of 4 min at 72°C.

As a result of PCR amplification, 5 μL product was carried out in 1% agarose gel together with a 1×TBE buffer system and was visualized and controlled by a UV illuminator by being stained with ethidium bromide.

Sequence analysis: Purification of PCR product successfully amplified was done by Wizard® SV Gel and PCR Clean-Up System (Promega) kit. Then, it was sent to a commercial laboratory with bi-directional primers and sequenced.

Restriction enzyme analysis: Each mtDNA region amplified by PCR was used in earlier studies and restricted via restriction enzymes (Hinf I, Rsa I) which were observed to be polymorphic. Cyt-b PCR product is composed of the restriction enzyme and each restriction enzyme mixture consists of 7.45 μL sterile distilled water, 1.4 μL 10X restriction enzyme buffer, 0.15 μL restriction enzyme (10 μL) and 5 μL PCR product (Ciftci *et al*., 2007). After the incubation of PCR products by restriction enzyme, the restricted samples were examined on 1.5-2.0% agarose gel with ethidium bromide (1 mg 100 μL^{-1}) in a 1×TBE buffer system. The gels were run for 2-3 h at 10 V cm^{-1} for each fragment model depending on the separation desired and gel concentration. The gel images were recorded using gel documentation system (Biostep, Darkhood DH-30/32) and thermal printer (Mitsibushi, P91D).

Data analysis: BIOEDIT 7.0.9.0 (Hall, 1999) and MEGA 5.01 (Kumar *et al*., 2004) Computer Software were utilized to evaluate sequence data. The MEGA 5.01 (Kumar *et al*., 2004) Software was used to compare phylogenetic relationships to topologies obtained by method in which algorithms formed a phylogenetic tree according to Kimura 2P distances such as UPGMA, NJ and MP. The tree reliability bootstrapping value was determined as 1000 and the values which the nodes had in the common tree that was formed the second time were assumed to be 50% and over. *Polydon spathula* was used as an out group. Then, the trees obtained in this way were evaluated.

MtDNA- RFLP and Multiplex-PCR particle sizes were defined by evaluating the gel images after electrophoresis in TotalLab 1.0 Software.

RESULTS AND DISCUSSION

Sequence analysis: Sequences of 1141 bp PCR products successfully amplified were defined by forward-reverse primers. They were compared to GenBank samples to determine the species from sequence data obtained and then MP dendrogram was generated (Fig. 2). Cyt-b sequence data belonging to all sturgeon samples were used in structuring the Maximum Parsimony (MP) dendrograms. The results obtained showed that there is a clear distinction among sturgeon species sampled from the Black Sea. For all dendrograms, the *Huso* and *Acipenser* species were distinguished from each other. As the MP dendrogram generated is examined it is seen that 3 sturgeon species (*A. stellatus*, *H. huso* and *A. gueldenstaedtii)* sampled from the Turkish coasts of the Black Sea were clearly distinguished (Fig. 2).

Restriction enzyme analysis: Species-specific morphs were generated by restricting Cyt-b PCR product by the Hinf I enzyme. The sizes of each morph and DNA particles formed were defined (Table 1). Different haplotypes were identified. *A. stellatus*, *A. gueldenstaedtii* and *H. huso* samples were within A (520, 470, 210 bp), B (520, 450, 230 bp) and C morph (520, 300, 90, 70 bp), respectively (Fig. 3). While three different restriction profiles (A, B and C morphs) b were seen in the Rsa I enzyme Cyt-b region in the *A. stellatus* samples, one morph was seen in *A. gueldenstaedtii* (D morph) and *H. huso* (E morph) samples (Fig. 3). Each morph and the sizes of DNA particles formed are given in Table 1.

Determination of species by multiplex-PCR: The expected lengths of products studied were determined as 420 bp for *A. gueldenstaedtii*, 375 bp for *H. huso* and 260 bp for *A. stellatus* (Fig. 4).

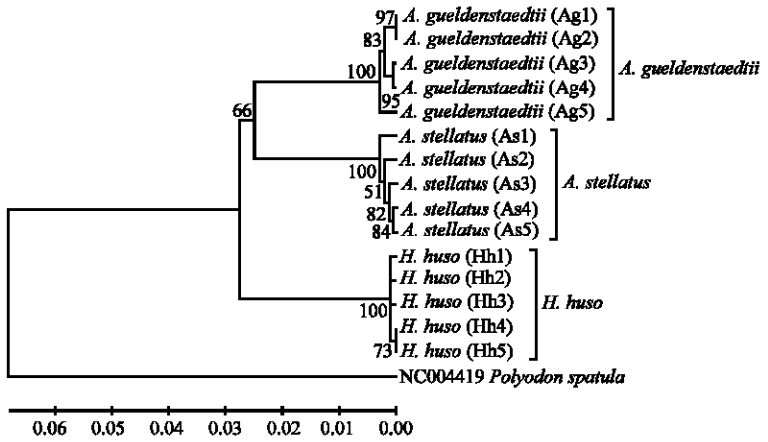

Fig. 2: Phylogenetic tree based on Maximum Parsimony (MP) Methods, numbers on branches are percentage bootstrap values based on 1000 replicates

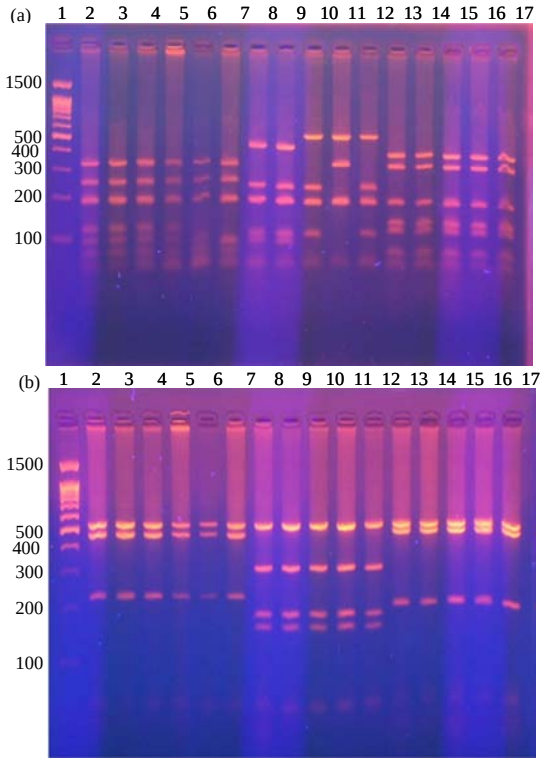

Fig. 3: Restriction enzyme pattern of *Cyt-b* gene from three sturgeon species using Hinf I (top) and Rsa I (lower). Column 1 is 100 bp DNA size standarts, columns 2-7 are *A. gueldenstaedtii*, columns 8-12 are *H. huso* and columns 13-17 are *A. stellatus* for top and bottom figures

Table 1: PCR-RFLP profiles of the cytochrome b fragment sizes (bp) from (As: *A. stelatus*, Ag: *A. gueldenstaedtii*, Hh: *H. huso*)

RsaI					Hinf I		
As			Ag	Hh	As	Ag	Hh
A	B	C	D	E	A	B	C
500	500	420	320	370	520	520	520
230	320	230	250	300	310	480	500
180	180	180	180	180	190	220	200
120	120	120	130	140	170	-	-
-	-	100	100	120	-	-	-
-	-	-	50	-	-	-	-
1030	1120	1050	1130	1110	1190	1220	1220

In the light of information obtained by the completion of phylogenetic studies on sturgeon, very different DNA markers have started to be used in species determination studies. In addition to rapid and accurate determination of species, a necessity to present an effective method using different tissues has also arisen. However, the distinguishing of fish from different origins has not been fully resolved, yet.

Together with the use of molecular techniques, species determination from any fish tissue such as meat

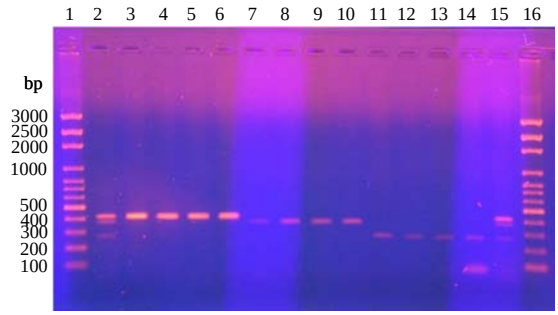

Fig. 4: Agarose gel electrophoresis of the PCR products obtained for three sturgeon species (Column 1 and 16 are 100 bp DNA size standarts, columns 3-6 are *A. gueldenstaedtii*, columns 7-10 are *H. huso*, columns 11-14 are *A. stellatus* and columns 2-15 is multiplex PCR fragments for three species)

and egg can be realized. One of these methods is PCR based applications. There have been a number of research studies oriented towards species determination by either the direct sequence analysis method or indirect analysis methods such as RFLP and Multiplex-PCR (DeSalle and Birstein, 1996; Birstein *et al*., 1998, 2000; Eroglu *et al*., 2011; Miuge *et al*., 2008).

The sequence analysis method is more descriptive than the others and is also used in the development of other methods. In this method, the reference data can easily be achieved from gene banks. However, it has restrictions in terms of time and cost. In research concerning the species determination of sturgeons, studies have been performed, in particular, by partial or complete sequencing of different genes of mtDNA. The most studied gene regions in sturgeons are Cyt-b, D-loop, 12S rDNA, 16S rDNA and ND5. However, some of the researchers stated that the *Cyt-b* gene might not be enough to distinguish similar species such as Russian and Siberian sturgeon (Jennecknes, 1999; Jenneckens *et al*., 2000, Birstein *et al*., 2000; Miuge *et al*., 2008).

Errors in morphological determinations in earlier periods emerge after genetic determinations. Phylogenetic analysis was performed on the sequence data of the *mtDNA Cyt-b* gene of fish which had been identified as *A. gueldenstaedtii* in a earlier study but it was identified later that 11 of these species belong to *A. baerii* (Jennecknes, 1999). Similarly, the *mtDNA Cyt-b* gene region of the sample, available in a museum in Spain and earlier identified as *A. naccarii* was partially sequenced. According to the results and morphological studies, it was determined that the sample earlier identified as *A. naccarii* was actually *A. sturio* and it was the only domestic species in the Iberian Peninsula

(Almodovar *et al*., 2000). Doukakis *et al*. (1999) sequenced mtDNA D-loop-cythochrome-b (*Cyt-b*) and *ND5/6* gene in *A. baerii* and *A. stellatus* and identified sub-species traditionally determined but did not find any definitive difference for them. Doukakis *et al*. (2005) studied three mtDNA regions (control, NADH5 and Cyt-b), in their study on different populations of *A. stellatus*, *A. gueldenstaedtii* and *H. huso* species. By comparing the sequences of the D-loop (control) region (631-646 bp) they attempted to determine whether there were definitive differences for sub-species or populations. In their studies, Birstein *et al*. (2000) emphasized the importance of molecular species identification methods in sturgeon and caviar trade and carried out a study on species identification from caviar for four Eurasia sturgeons (*A. baerii*, *A. gueldenstaedtii*, *A. persicus* and *A. naccarii*). In their study, approximately 2.3 kb mtDNA sequence (cytochrome-b, NADH5, Control region) data were used.

The gene regions mentioned above have been commonly used in species identification. However, species identification by caviar and other products regulated by CITES can be resolved by direct sequencing of a certain part or the completion of the cytochrome-b gene which is widely preferred in inter-species distinguishing and by comparing the obtained results to reference sequences of GenBank (Wuertz *et al*., 2007). In this study as in other studies, the cytochrome-b gene -a part of the mitochondrial gene sequence- was completely sequenced and by the analysis and comparison of sequence results, the differences between sturgeons along the Turkish coasts of the Black Sea were determined. In addition to sequence analysis, attempts have been made by various researchers to identify species by the PCR-RFLP technique to achieve a faster result. As the trade has gained importance in recent years, species identification and species-specific restriction locations have been provided by restricting tRNAglu/Cyt-b PCR product by different enzymes to identify species from caviar. It was reported that this method is suitable for the identification of 10 different species of *Acipenser* and *Huso* which are among the species of Europe and Asia (Wolf *et al*., 1999). *Cyt-b* and *D-loop* gene regions were amplified by PCR in six sturgeon species in Italy and RFLP analysis was applied to particles obtained by restricting via Rsa I enzyme. It was stated that this method enabled the determination of inter-species variation and thus it was an appropriate marker for species identification (Tagliavini *et al*., 1999). More than 1 gene region as well as more enzymes might be needed in the identification of closely related species by this method. Hence, Ludwig and Kirschbaum (1998) performed PCR-RFLP analysis on the *mtDNA 12S rDNA* gene region in his study and reported that BsiYI and AciI enzymes could be used between *A. naccari* and *A. sturio* but since the restriction locations of the TspRI (315-326) enzyme are so close it could not be used for the same purpose. Ludwig *et al*. (2002) reported that species identification was not possible for 22 species of sturgeon through the restriction of the *Cyt-b* gene by using a single enzyme and depending on the species, 1-4 enzymes must be used. In this context, it was stated that 17 *Acipenseriform* species can be distinguished by identifying the species-specific restriction locations of seven different enzymes at the mtDNA level.

In this study, different to Ludwig *et al*. (2002) study, the Rsa I enzyme was used for different species. Furthermore, Hinf I enzyme which was not used in Ludwig *et al*. (2002) study was also, used for the first time and by the use of two enzymes, distinguishing restriction morphs for species along the Turkish coast of the Black Sea were determined.

It is possible to see Multiplex PCR applications in studies performed for different purposes and concerning many species. In a study performed by Miuge *et al*. (2008), the mtDNA control region (D-loop) on eight sturgeon species was defined quickly in the form of primers developed and multiplex PCR.

Since, the mtDNA control region (D-loop) region is 4-5 times more variable than the Cytochrome-b gene recycling can be expected and the polymorphism caused by nucleotide change does not provide 100% identification of species. In the study of Miuge *et al*. (2008), *A. gueldenstaedtii* and *A. baerii* were identified with only an error margin of 1% despite all false-positive results obtained and this error margin was evaluated as an acceptable result in the certification of species composition. However, since distinguishing between *A. gueldenstaedtti* and *A. persicus* available in the Caspian Sea was not provided, studying nuclear gene regions are recommended for such species. Moreover, according to the multiplex profiles obtained from Russia-origin samples which is the subject of this study, PCR product has lengths of 420, 266 and 374 bp for *A. gueldenstaedtii*, *A. stellatus* and *H. huso* species, respectively. Furthermore, according to the PCR results obtained from Turkey-origin samples while product length obtained for *A. gueldenstaedtii* (420 bp) reveals a similarity to the Russian samples, product lengths for *A. stellatus* (260 bp) and *H. huso* (375 bp) were dissimilar but the results were found to be distinguishing for three of the species.

CONCLUSION

As a result of present study, opportunities were provided for the identification and control of *A. gueldenstaedtii*, *A. stellatus* and *H. huso* species from which caviar and meat are produced.

It was determined that the restriction morphs obtained are distinguishing for the species concerned and they are consistent with other studies. Furthermore, Hinf I enzyme which was not used in earlier studies was also, used for the first time and by the use of two enzymes, distinguishing restriction morphs for species along the Turkish coast of the Black Sea were determined. Multiplex PCR applications revealed different product lenghts for *A. stellatus* and *H. huso* in comparison with other studies but the results were found to be distinguishing for three of the species.

Although, the sequence method, in particular is found to be more effective and informative, it has disadvantages in terms of time and cost. It has advantages in intraspecific and interspecies variation and phylogenetic structuring. In the RFLP Method, however, the reference data have to be generated in order to make a comparison. These profiles cannot be achieved from databanks. It was seen that the multiplex PCR application is an identification method which is cheap, effective and can be done in a short time. It can be used in the serial analysis of a high number of samples without the need to employ other methods such as sequence analysis, RFLP or other fingerprint methods.

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