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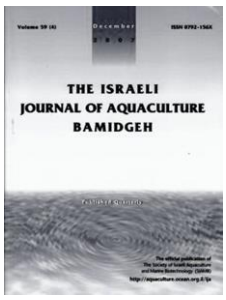
ISSN 0792 - 156X

PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL
Phone: + 972 52 3965809
http://siamb.org.il

The Israeli Journal of Aquaculture - Bamidgeh, IJA_66.2014.957, 9 pages



Characterization of *GnRH*, *ILGFRI* and *AR* Genes in Sturgeon's Genomics

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(Received 15/2/2013, Accepted 30/3/2013)

Key words: sturgeon, polymerase chain reaction (PCR) analysis, species identification, BLASTN analysis, ClustalW, DNA sequencing

Abstract

In this study, gonadotropin-releasing hormone (GnRH), insulin-like growth factor receptor I (ILGFRI), and androgen receptor (AR) genes coding, were examined by polymerase chain reaction (PCR) analysis and DNA sequencing in three Eurasian sturgeon species: *Huso huso, Acipenser stellatus* and *A. gueldenstaedtii*. Amplicons of 190 bp for GnRH, 180 bp for ILGFRI, and 200 bp for AR genes were amplified with specific primer pairs in all species genome. All amplification products were sequenced and compared to reference *H. huso* genome retrieved from NCBI database by BLASTN analysis. The ILGFRI gene had the most similar sequences (75.96-96.15%) in all species. Gene sequence similarity calculated for AR gene ranged from 48.5-95.71%. The GnRH gene had more distant sequences (23.48-83.91%) between species. There was moderate similarity between *H. huso* and the reference *H. huso* samples. An unauthorized (a sample seized from poachers) sample was more closely related to *A. stellatus*. Partial coding region of AR gene was registered in NCBI database under Accession Number: KC172108. Five nucleotide changes were detected in this sequence, two of which resulted in changes in amino acid sequence. Also, a partial sequence consisted of two microsatellite regions of two and four nucleotide repeats. Three constructed dendrograms displayed the relationship among species according to certain genomic regions. These findings indicate a high level of diversity among and even within the same species. They also show that these three genes could be useful in species' identification and characterization.

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Introduction

The family Acipenseridae includes all known sturgeons and is the oldest and most primitive group of Osteichthyan fishes. Sturgeons have been consumed for centuries and are a valuable food source in many countries of the northern hemisphere. Sturgeon products, especially caviar and meat are traded worldwide. They were commercially fished for the high value of their caviar and flesh until the implementation of the Convention on International Trade in Endangered Species (CITES) agreement.

Some species belonging to genera *Acipenser, Huso, Scaphirhynchus,* and *Pseudoscaphirhynchus* are on the brink of extinction because of their late reproductive maturation periods and long life cycles, and the effects of various factors such as water pollution and overfishing. Therefore, they must be protected and aquaculture breeding must be encouraged (Jenneckens et al., 2001).

There are six indigenous sturgeon species in the Black Sea (Ercan, 2011) but only three of them (*Huso huso, Acipenser stellatus, A. gueldenstaedtii*) are frequently found there while the others (*A. ruthenus, A. sturio, A. nudiventris*) are rarely found. Protection of sturgeon began when sturgeon populations decreased along the entire Black sea coast. Sturgeon fishing has been restricted in Turkish waters since 1971 and fishing of *H. huso* has been entirely forbidden since 1997 in Turkey (Akbulut et al., 2011).

Sturgeon genomics can be organized along four main lines of research: (i) species identification; (ii) sub-population and population characterization, especially different populations of the same species; (iii) identification of the species source, especially wild versus aquaculture; (iv) determination of genetic diversity, both at whole genome and specific gene sequence level. DNA-based techniques have become the most important tools for molecular genetics research. DNA sequencing has gained a powerful support (Ludwig, 2008).

Due to their commercial importance, there is increased interest in genetic research on sturgeons. Earlier attempts focused on sex determination and phylogenetic analysis (Dudu et al., 2009; Barbejillo et al., 2012). Gonadotropin-releasing hormone (GnRH), insulin-like growth factor receptor I (ILGFRI), and androgen receptor (AR) are the main hormone and receptors involved in growth, survival and adaptation to environmental conditions of any fish species. GnRH is a neuropeptide that mediates control of gonadal development and the release of gametes in fish. The terminal nerve GnRH system also modulates processing visual and olfactory information. This peptide and its receptor play a role in osmoregulation in anadromous fish (Guilgur et al., 2006; 2007; Maruska and Fernald, 2010). Functions, evolutions, expression patterns, amino acid sequences and cloning of coding sequences of GnRH have been examined (Lescheid et al., 1995; O'Neill et al., 1998; Maruska and Tricas, 2011).

ILGFI is a hormone affecting growth and anabolism in juvenile and adult fish, respectively. Structure, regulation, and function of this protein in fish appear similar to mammals. In addition to growth and metabolism, ILGFI has been associated with development and reproduction. Sturgeons are able to tolerate salinity ranging from fresh water to sea water and to maintain their plasma osmolarity at the same level under these conditions. The structure, function, and levels in tissue and blood systems of ILGFI and its receptors are a focus of interest (Reinecke et al., 2005; Tipsmark et al., 2007). ILGFRI is activated by ILGF1 and ILGF2 hormones. This receptor was studied in a few species and their embryos (Perrot et al., 2000; Mendez et al., 2001). Given its importance, expression analysis of ILGFRI was studied in sturgeon species (Perrot et al., 2000; Barbejillo et al., 2012).

The androgen receptor (AR), activated by the binding of androgen hormones, functions as a DNA-binding transcription factor regulating gene expression. Androgens (which are affected by binding to AR) are sex steroids that control male reproduction. They play a key role in controlling testis differentiation as well as social interactions and adaptations (Oliveira et al., 2002). To understand the molecular mechanism underlying testis differentiation in basal actinopterygian fish, AR gene expression level was investigated in juvenile fish (Berbejillo et al., 2012).

Due to their importance, we selected GnRH, ILGFRI and AR genes to compare nucleotide sequences in three Eurasian sturgeon species: *Huso huso, Acipenser stellatus* and *A. gueldenstaedtii*. Since chosen gene products are directly related to signal

transduction, they are essential to the growth, survival and adaptation of these species to the environment. Therefore, molecular characterization of them in native species and stock, obtained from artificial reproduction, can provide valuable data to be used in sturgeon conservation, stock enhancement and rehabilitation.

Materials and Methods

One specimen of *A. stellatus*, and one of *A. gueldenstaedtii,* belonging to stocks managed within the scope of FAO-TCP project, were obtained from Sapanca Inland Fish Aquaculture Research and Applied Station (SIFARAS) of Istanbul University. A DNA extract of *H. huso* from a wild fish from the Black Sea was provided by the Turkish Ministry of Food, Agriculture and Livestock, Central Fisheries Research Institute (Yomra-Trabzon, Turkey). An additional sample from a wild fish seized from poachers was obtained from Istanbul University, Faculty of Fisheries.

Genomic DNA was extracted from 50 mg of muscle tissue from one of each specimen according to the CTAB (Cetyl trimethylammonium bromide) procedure with minor modifications (Stewart and Via, 1993). Sturgeons' nucleotide sequences for the GnRH, ILGFRI and AR genes were retrieved from NCBI database.

Nucleotide homologies of these sequences were compared via ClustalW analysis. Differences in nucleotide sequences were targeted for analysis and primers were designed using Primer3 software program (GNU-General public license) (Table 1).

Table 1. Oligonucleotide primers used in this study.

Primer	*F/R*	*Nucleotide sequence (5'-3')*	*°C*	*Target gene*
pGR	F	CCTATGACCCCCTGAAGTCC	52	*GnRH*
	R	GGGTCTAAGTGACAAAATAA		
pIGF	F	GCAGAAAGGTATTCGGTTGG	54	*ILGFRI*
	R	CATACCGCTAAGGGCTCATC		
pAR	F	TTATTTCGGGGAGTCCAGTG	55	*AR*
	R	AAGGATACTGGAGCCTGCTG		

Polymerase chain reaction (PCR) amplifications were carried out in a volume of 25 µl. Each reaction mixture contained 50 ng DNA, 1 X PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM of each dNTPs, 10 pmol of each primer and 1 U Taq DNA polymerase (Promega, 9PIM829). PCR conditions were performed at 94°C for 5 min for pre-denaturation, 35 cycles of 94°C for 30 s, 52-55°C for 30 s, 72°C for 30 s for gene amplification, and a final 5 min extension step at 72°C.

PCR products were separated by electrophoresis in 1.5% agarose gel and stained with ethidium bromide. Images were visualized using Avegene, X-Lite 200. Amplification product sizes were calculated using Kodak 3000M software.

Amplicons were sequenced based on the chain termination method (Sanger et al., 1977). Nucleotide sequence chromatograms were evaluated using ChromasPro (Technelysium) and homology analysis was performed. ClustalW analysis was used to compare nucleotide sequence homologies among reference *H. huso* and sturgeon samples. Genetic similarity and dendrograms were computed by UPGMA (Unweighted Pair-Group Method with Arithmetic Average) via ClustalW 2.1.

Results

Partial regions of GnRH, ILGFRI & AR genes were produced with specific primers (Fig.1).

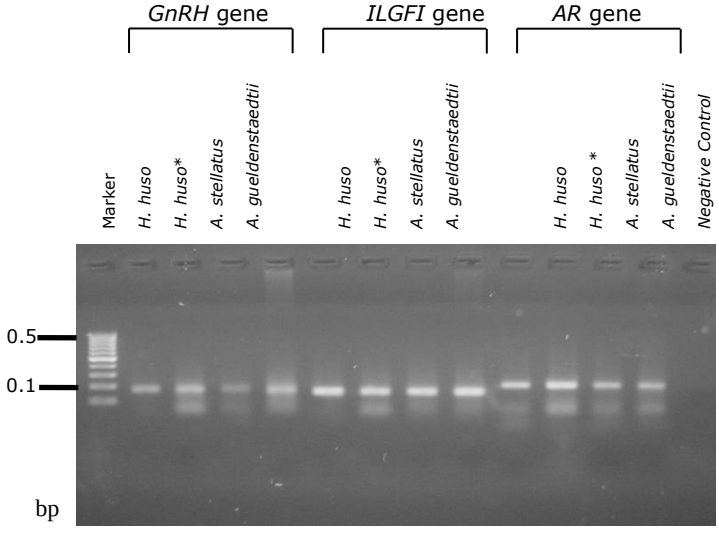

Fig. 1. PCR products amplified in sturgeon genomes. Marker is 100 bp DNA ladder and negative control contains no template DNA.
* indicates unauthorized hunting sample

The pGR primer set amplified 190 bp fragments for the GnRH gene, while the pIGF primer set amplified 180 bp for the ILGFRI gene. The pAR primers amplified 200 bp sequence segments for the AR gene.

The quality of nucleotide sequences was assessed using ChromasPro software. The chromatograms obtained allowed the necessary nucleotide alignment for use in species' comparisons. Nucleotide sequence homologies between sturgeon samples used in this study and *H. huso* reference sequences registered in NCBI database were compared with ClustalW (Fig. 2).

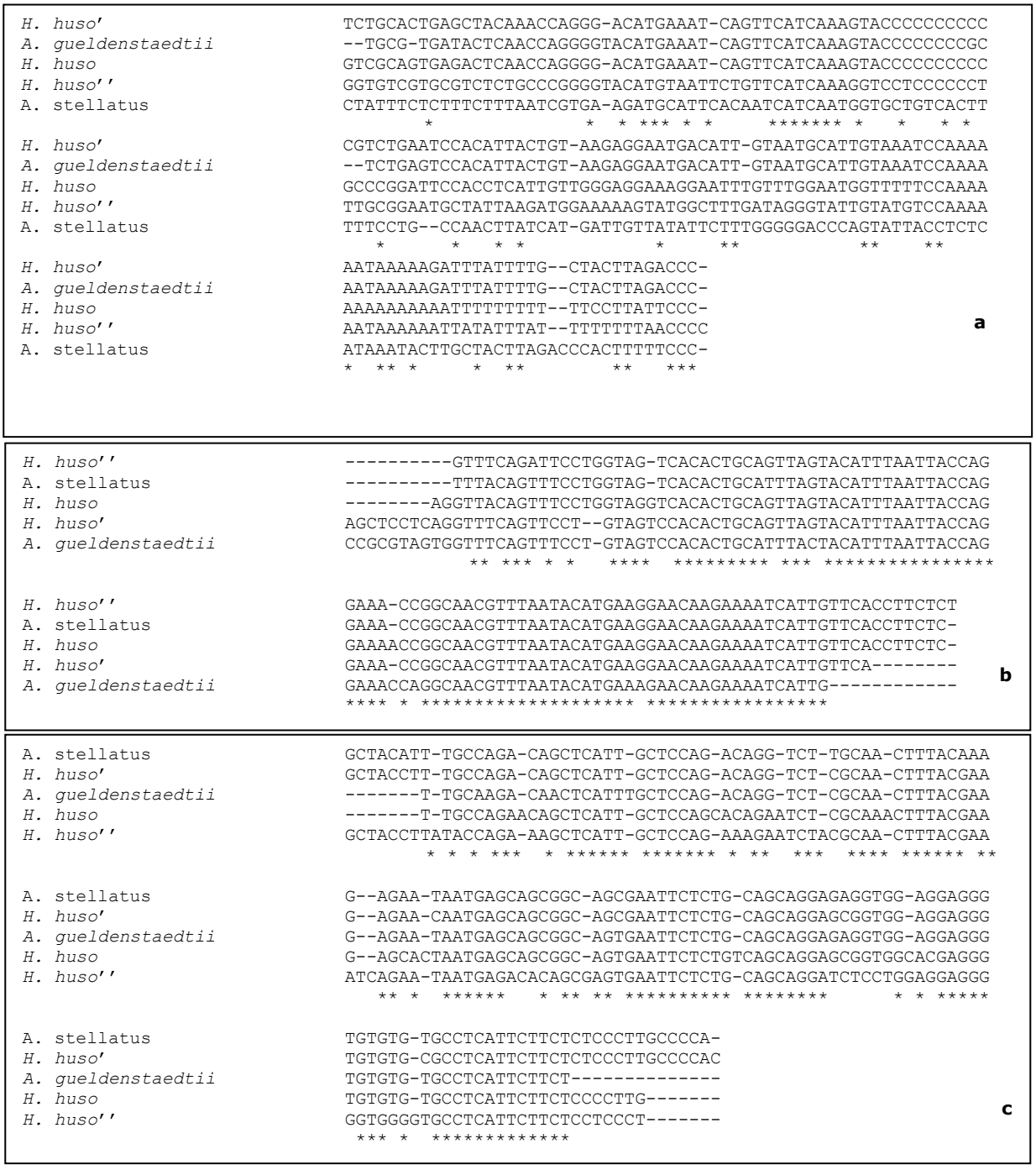

Fig. 2. ClustalW analyses of four sturgeon species and reference *H. huso* genome for (a) GnRH, (b) *ILGFRI* and (c) *AR* genes.
' shows reference species registered in NCBI
'' unauthorized hunting sample
- imply neither deletion nor no sequencing information
* point out conserved nucleotides

Table 2. Genetic similarity percentages (%) of all specimens for GnRH, ILGFRI and AR genes

Gene	*Species*	*H.huso**	*H.huso*	*A.stellatus*	A.*gueldenstaedtii*	*H.huso***
GnRH	*H. huso**	100				
	H. huso	63.26	100			
	A. stellatus	25.17	23.48	100		
	A. *gueldenstaedtii*	83.91	60.83	23.77	100	
	*H. huso***	44.21	46.97	25.50	49.65	100
		*H.huso**	*H.huso*	*A.stellatus*	A.*gueldenstaedtii*	*H.huso***
LGFRI	*H. huso**	100				
	H. huso	77.35	100			
	A. stellatus	79.80	88.46	100		
	A. *gueldenstaedtii*	77.88	77.88	75.96	100	
	*H. huso***	81.90	87.61	96.15	75.96	100
		*H. huso**	*H.huso*	*A stellatus*	A.*gueldenstaedtii*	*H.huso***
AR	*H. huso**	100				
	H. huso	62.68	100			
	A. stellatus	95.71	59.70	100		
	A. *gueldenstaedtii*	90.08	64.46	90.90	100	
	*H. huso***	53.90	48.50	54.28	53.71	100

*shows reference species registered in NCBI
** indicates unauthorized hunting sample

Similarity percentages calculated by ClustalW 2.1 are given in Table 2.

Among the three genes, ILGFRI gene had the most similar sequences (75.96-96.15%) in all species. The most similar species were uncharacterized *H. huso* and *A. stellatus*, whereas uncharacterized *H. huso* and *A. gueldenstaedtii* were dissimilar. Homologous nucleotide sequences in *GnRH* gene were detected in four specimens. The similarity values calculated ranged from 23.48-83.91%. The highest similarity percentage occurred between reference *H. huso* and *A. gueldenstaedtii* (83.91%), whereas the most diverse species were *H. huso* and *A. stellatus*. However, reference *H. huso* and *A. stellatus* were determined to be highly similar species with respect to AR gene sequences (95.71%). The most dissimilar sequences (48.5%) were those of uncharacterized *H. huso* and *H. huso*.

Three dendrograms were constructed according to UPGMA displayed monophyletic branching. The relationship among species is shown in Fig. 3.

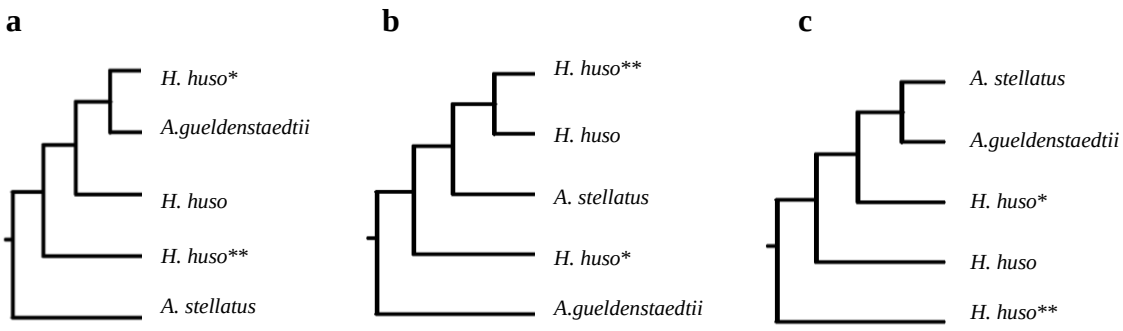

Fig.3.Dendrograms generated from UPGMA analysis for (a) GnRH, (b) *ILGFRI* and (c) *AR* genes.
*shows reference species registered in NCBI
** indicates unauthorized hunting sample

The AR gene sequence from the wild-caught *H. huso* was registered in the NCBI database under Accession No. KC172108. Partial sequences are illustrated in Fig. 4.

ATT ATT TCG GGG AGT CCA GTG GGT GAG TTC CAG CGT GCT ACC TTT
GCC AGA CAG CTC ATT GCT CCA GAC AG**A** TCT CGC ACT AAC TTT ACG
AAG AGA A**T**A ATG AGC AGC GGC AG**T** GAA TTC TCT GCA GCA GGA GCG
GTG GAG GAG GGT GTG TG**T** GCC TCA TTC TTC TCT CCC TTG CCC CAC
TGC AGC CTG **C**AC CAG CAG GCT CCA GTA TCC TT**A**

Fig. 4. The sequence information of AR genes registered in NCBI database under Accession No. KC172108. Underlined and bold big type sized nucleotides - except both ends - show primer binding sides and nucleotide changes, respectively.

Five bases in that region differ from reference *H. huso*. Primer binding sides were found at both ends. As a result of this variation, five amino acids change in open reading frame (ORF). In addition, a partial sequence consists of two microsatellite regions: one dinucleotide [$(GT)_5$], and tetranucleotide repeat [$(CAGA)_2$] motifs.

Discussion

PCR and DNA sequencing are powerful tools for characterization and identification of fish at genera, species, and sub-species level, as well as for product species identification (Fopp-Bayat, 2007). These methods are reliable, replicable, fast and inexpensive (Hisar et al., 2006; Aksakal and Erdogan, 2007; Liu et al., 2008). There are many other approaches for genome analysis of sturgeon species (Almodóvar et al., 2000). Accurate species identification is essential and the selection of appropriate molecular marker methods will contribute greatly to breeding and species conservation programs for sturgeons. The determination of genetic diversity and its demonstrated correlation with economically important traits also provide valuable knowledge, which can be used for management, and sustainable use, as well as species conservation. Obtained data can be applied for future genetic improvement and assessment of these species in hatcheries (Chakmehdouz Ghasemi, 2011).

Since, a genome project for sturgeons has not been undertaken, and because databases still contain very limited sequence information about sturgeon genomes, our efforts focused on molecular approaches. Mitochondrial *cytochrome b* (*cytb*) sequences and *Sox 9*, coding transcription factors, were used in previous studies as targets for species identification and sex determination, respectively (Ludwig et al., 2000; Hett et al., 2005). Various molecular marker techniques have also been used to determine genetic diversity. Microsatellite loci analysis has been the preferred approach (Henderson-Arzopalo and King 2002; Israel et al., 2004; Chakmendouz Ghasemi 2011).

Other molecular marker techniques such as RAPD, AFLP, and ISSR have been used in diversity studies (Wuertz et al., 2006; Pourkazemi and Razikazemi, 2011; Yarmuhammadi et al., 2012). A research project based on mitochondrial *cytb* and *tRNA^{pro}-Dloop* region analysis in sturgeon species was carried out in Turkey (Akbulut et al. 2011). Other studies have focused on growth performance, acclimation to farm conditions, adaptation to cold water, and transfer to natural environments (Çelikkale et al., 2002; Memiş et al., 2011a; 2011b).

Because GnRH hormone, ILGHRI, and AR receptors play an important role in growth, survival, behavioral adaptations, and response to environmental change, there have been many studies about hormones and their receptors (Méndez et al., 2001; Guilgur et al., 2007; Maruska and Fernald, 2010; Barbejillo et al., 2012).

In this study, PCR, DNA sequencing and bioinformatic tools were used to conduct genomic analysis. GnRH, ILGFRI, AR genes were selected, and partial regions of these genes were compared in different sturgeon species. The discrimination power of chosen regions in these three genes for species identification was investigated. We successfully amplified partial regions of the selected genes by PCR. Once the DNA sequences were

obtained, we compared nucleotides to detect similarity using ClustalW software. We found high similarity and moderate diversity among sturgeon genomes. *Acipencer gueldenstaedtii* and *A. stellatus* were the two species most closely related to reference *H. huso*. Similarity between the reference genome and the wild *H. huso* from the Black Sea was 62.68-77.35% for all genes. The uncharacterized *H. huso* sample was more closely related to *A. stellatus* than the other specimens. Relationships among all specimens were revealed in three monophyletic dendrograms. They were differently grouped in nearly all species. Dendrograms especially constructed using molecular marker methods were able to provide informative and comprehensive data for species discrimination. Establishment of an accurate relationship among species in the higher eukaryotic organisms depends on selection of targeted regions in the genome. Therefore GnRH, ILGFRI, and AR genes are suitable genomic regions for genetic characterization of sturgeon species.

Variations may be used as molecular markers for discrimination of populations belonging to the same species (Dudu et al. 2011). However these markers have intraspecific discriminative power due to high mutation and low specimen numbers. Gene flow within and among same and different sturgeon populations due to mating and reproduction also limits discrimination efficiency of molecular markers.

Hybridization between different sturgeon species occurs both in natural waters and under aquaculture conditions (Birstein et al., 1977). Hybrids between sturgeon species with the same chromosome number are fertile. Only a genetic study can provide the necessary proof that nuclear genes from both parental species are present in the hybrid (Fopp-Bayat, 2007; Dudu et al., 2011). Thus, definitive species identification and accurate relationships among species cannot be established with complete certainty.

Presence of auto- and allopolyploid individuals causes problems when using markers for species discrimination in sturgeons. This is due to the high ploidy levels in nearly all Acipenseriformes. Species discrimination in Acipenseriformes is challenging because nearly all sturgeons are either fertile interspecific or intergeneric hybrids and contain high ploidy levels. Further studies on population structuring as well as the development of conservation plans and restocking programs for sturgeons are needed (Chakmehdouz Ghasemi, 2010).

AR gene sequences generated through this study were registered in NCBI database under Accession No. KC172108. We found five base changes in ORF region of this gene. We think that three variations caused by a deamination mechanism resulted in the conversion of C base to T. One transition and one transversion were also observed. As a result, one purine base converted to another purine (G→A), and one purine to primidine (G→C). Changes found in ORF, affect the genetic coding of five amino acids. Among them, three codons have transformed different codons encoding the same amino acids (AGG→AGA encoding for arginine, AGC→AGT for serine, and TGC→TGT for cysteine). Conversions of ACA to ATA, and GAC to CAC, have resulted in encoding isoleucine instead of threonine, and histidine instead of aspartic acid, respectively. The existence of polymorphism in the highly conserved cyt *b* gene in *H. huso* samples has been detected Dudu et al. 2009). Although four codons ORF were affected by conservative missense mutations, they reported that biological function of cyt *b* protein did not change.

There is no difficulty in the classification and identification of sturgeons based on morphological characteristics (Costache et al., 2012). Sturgeon are now an endangered species and are protected in certain countries since poaching for caviar creates serious challenges to conservation. At present there is no genome project for sturgeons therefore information about genomes of Acipenseriformes species is limited and scientific research on sturgeon genomes using molecular approaches is of vital importance.

Our findings suggest that DNA sequence regions in the GnRH, ILGFRI and AR genes could be useful in the identification and characterization of three Eurasian sturgeon species (*H. huso, A. stellatus* and *A. gueldenstaedtii*). Partial AR gene sequence information was registered in NCBI database under Accession No. KC172108. Nucleotide variations in the ORF region were detected between *H. huso* and reference *H. huso*. We have shown that there is no differentiation potential of the three genes among species. This report uses the GnRH, ILGFRI, and AR genes in sample comparisons of sturgeon species. Data obtained from molecular characterization of native sturgeon species, artificial

reproduction stock, and valuable wildlife products will contribute to sturgeon conservation, stock enhancement and rehabilitation programs.

Acknowledgements

We are grateful to Prof. Dr. Devrim Memiş for providing fingerling tissue samples (*A.gueldenstaedtii* and *A. stellatus*), and to Dr. Atilla Özdemir and M.Sc Oğuzhan Eroğlu for providing DNA extract of *H. huso*.

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