

## Barcoding of Five Sturgeon Species in Iran

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**Abstract:** The universal barcoding gene of Cytochrome Oxidase I (COI) was utilised for molecular characterisation of five sturgeon species found in the Iranian waters of the Caspian Sea. These were *Acipenser gueldenstaedtii* (Russian sturgeon), *A. persicus* (Persian sturgeon), *A. stellatus* (Stellate sturgeon), *A. nudiventris* (Spiny sturgeon) and *Huso huso* (Great sturgeon). An additional 10 sequences from the Genbank were included in the analysis, *A. stellatus* (AJ585050), *H. huso* (AY442351), *A. baerii* (FJ205560-FJ205563), *A. fulvescens* (EU524397), *A. transmontanus* (EU523891), *A. medirostris* (EU523885) and *A. oxyrinchus* (EU524401). The data was also analysed for phylogenetic insights among the studied species. The results confirmed the utility of this gene for molecular identification and revealed monophyly of three of the species; *H. huso*, *A. nudiventris* and *A. stellatus*. However, the status of *A. gueldenstaedtii* and *A. persicus* was unresolved with Genbank sequences of *A. baerii* (FJ205560-FJ205563) also clustering in the *A. gueldenstaedtii-persicus* complex as had been reported by previous researchers investigating other genes. Thus, the results provide further evidence of the polyphylic nature of *A. gueldenstaedtii*. The data is also in agreement with several earlier reports that *H. huso* may not be a separate genus from *Acipenser* within the family Acipenserinae. The low internode bootstrap values at branch nodes did not allow for any phylogenetic inference among the *Acipenser* species to be deduced.

**Key words:** Genetic variation, barcoding, cytochrome oxidase I, sturgeon species, Iran

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### INTRODUCTION

The southern Caspian Sea of the Iranian waters is home to five sturgeon species namely *Huso huso*, *Acipenser nudiventris*, *A. stellatus*, *A. gueldenstaedtii* and *A. persicus*. As with many of their relatives in other regions, their populations are under serious threat (Rosenthal *et al.*, 2006; Ghadirnejad *et al.*, 2008). This is mainly as a consequence of their economic importance for their meat and in the caviar industry as well as habitat destruction of their spawning grounds. Sturgeons are represented by two large groups of species with different chromosome numbers based on karyological investigations of Acipenserid fishes. The low-chromosome numbered species are traditionally categorised into two subgroups, the genus *Huso* (two species) and *Acipenser* (17 species). However, several molecular studies have revealed a more complex systematic relationship that *Huso* is not a separate genus from *Acipenser* within the Acipenserinae (Birstein and de Salle, 1998; Tagliavini *et al.*, 2007; Ruiz-Rejón *et al.*, 2000). Furthermore, craniological characteristics (Vasil'eva, 2009) also grouped *Huso* within *Acipenser*.

The taxonomic status of the Persian, *A. persicus* and Russian, *A. gueldenstaedtii* has been of much controversy and debate since the 1930s (Vasil'eva, 2009). The Persian sturgeon *A. persicus* was first described as a valid species from the Ural River but have gone through many taxonomic revisions in relation to its taxonomic status to *A. gueldenstaedtii* (Ruban *et al.*, 2008) as a subspecies of *A. gueldenstaedtii*, a different race or biological group as according to divisions of spawning seasons.

The debate gained greater impetus when mitochondrial marker analysis was shown not to concur with morphological delineation of discrete taxonomic groups.

Since then the polyphyletic nature of *A. gueldenstaedtii* with respect to *A. persicus* and two other sturgeon species based on mitochondrial markers has been well documented (Birstein *et al.*, 2000; Ruban *et al.*, 2008). The phylogenetic analysis conducted by Birstein *et al.* (2000) on a 2.3 kb of mtDNA sequence data (cytochrome b, NADH5, control region) separated *A. gueldenstaedtii* into two distinct clades. One

*A. gueldenstaedtii* mitochondrial lineage comprised of *A. persicus* and *A. naccarii*, whereas the other one was more closely related to *A. baerii*. More recently (Ruban *et al.*, 2008) combined results of 28 morphometric, 6 meristic characters and the complete cytochrome b did not support the validity of the Persian sturgeon as a separate species from the Russian sturgeon. However, craniological indices demonstrated that these two could be differentiated (Vasil'eva, 2009).

The importance of DNA barcoding (Hebert *et al.*, 2003) in the various fields of biology, management and conservation has gained much attention in the past few years (Armstrong and Ball, 2005; Savolainen *et al.*, 2005). The barcode region comprising of a 650 bp segment of the 5' end of Cytochrome Oxidase I (COI) gene is now becoming the focus for species identification of fishes (Ward *et al.*, 2005; Rock *et al.*, 2008). Smith *et al.* (2008) opined that this approach is expected to become a standard tool for recognition of fish specimens. Thus the main objective of this investigation was to obtain unambiguous identification of the sturgeon species in the southern Caspian Sea and contribute towards the global effort of a standardized reference sequenced library for all fish species. In particular, we seek to clarify through this marker, ambiguities between the Russian and Persian sturgeon. However, with the inclusion of several sequences from the Genbank database we also aimed to explore phylogenetic insights of the five species of the southern Caspian Sea.

## MATERIALS AND METHODS

Individuals of the five sturgeon species namely *A. gueldenstaedtii* (Russian sturgeon), *A. persicus* (Persian sturgeon), *A. stellatus* (Stellate sturgeon), *A. nudiiventris* (Spiny sturgeon) and *H. huso* (Great sturgeon) were sampled from the Iranian waters of the Southern Caspian Sea. DNA was extracted from fin clips of five individuals of each species using Genispin™ Tissue DNA Kit (BST Tech Laboratory) or AquaGenomic™ DNA isolation Kit following the manufacturer's manual. The primer pair LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAATCA-3') was utilized to amplify partial Cytochrome Oxidase subunit I (COI) gene (Folmer *et al.*, 1994).

Amplification reactions were performed in a PTC-200, Peltier Thermal Cycler (MJ Research) in a total of 25 µL containing 19.325 µL sterile distilled H<sub>2</sub>O, 2.25 µL 10X PCR buffer, 1.20 µL of 25 mM MgCl<sub>2</sub>, 0.125 µL of 10 µM dNTPs, 0.25 µL of each 5 pM primers (forward Primer; LCO1490 and Reverse Primer; HCO2198), 0.1 µL

5U Taq DNA polymerase and 1.5 µL of DNA template. Control reaction, containing all components except genomic DNA was included in every set of amplification reaction.

**PCR reaction protocol was as the following:** Pre-denaturation at 94°C for 1 min followed by denaturation at 94°C for 30 sec (repeated for 5 cycles), annealing at 45°C for 1.30 min extension at 72°C for 1 min, denaturation at 94°C for 30 sec, annealing at 51°C for 1.30 min (repeated for 35 cycles), extension at 72°C for 1 min and final extension at 72°C for 5 min. PCR products were purified using QIA quick PCR purification kit (Qiagen) following the manufacturer's manual followed by automated DNA sequencing (ABI PRISM 377 DNA sequencer). Sequences were edited, aligned and analysed using MEGA version 4 (Tamura *et al.*, 2007). Divergences were calculated based on the Kimura two-parameter (K2P) distance model (Kimura, 1980). Phylogenetic relationships were examined using Neighbor-Joining (NJ) and Maximum Parsimony (MP). For the NJ tree, robustness was assessed by 1000 bootstrap replicates and MP tree robustness was assessed by 500 bootstrap replicates. An additional 10 sequences from the Genbank were included in the analysis; *A. stellatus* (AJ585050), *H. huso* (AY442351) *A. baerii* (FJ205560-FJ205563), *A. fulvescens* (EU524397), *A. transmontanus* (EU523891), *A. medirostris* (EU523885) and *A. oxyrinchus* (EU524401).

## RESULTS

**Sequence variation:** A total of 631 bp partial sequence of the mtDNA COI gene was sequenced from 25 samples of five sturgeon species. All studied sequences have been deposited and are available in GenBank with accession numbers FJ809715-FJ809739. Both topologies (Fig. 1 and 2) revealed similar topologies with slightly higher resolution in the NJ analysis. Three of the investigated species namely *A. nudiiventris*, *A. stellatus* and *H. huso* formed a monophyletic assemblage confirming their taxonomic identification using the barcoding gene of COI. However, haplotypes of *A. persicus* and the Genbank sequences of *A. baerii* interspersed with *A. gueldenstaedtii* assorting into two major and distinct well supported clades. One *A. gueldenstaedtii* mitochondrial lineage is interspersed with mainly *A. persicus*, while the other is more closely related to *A. baerii*. The Genbank sequences of *Acipenser transmontanus* and *A. medirostris* formed another clade with *A. oxyrinchus* as a sister taxon but with low confidence. However, detailed interspecies relationships

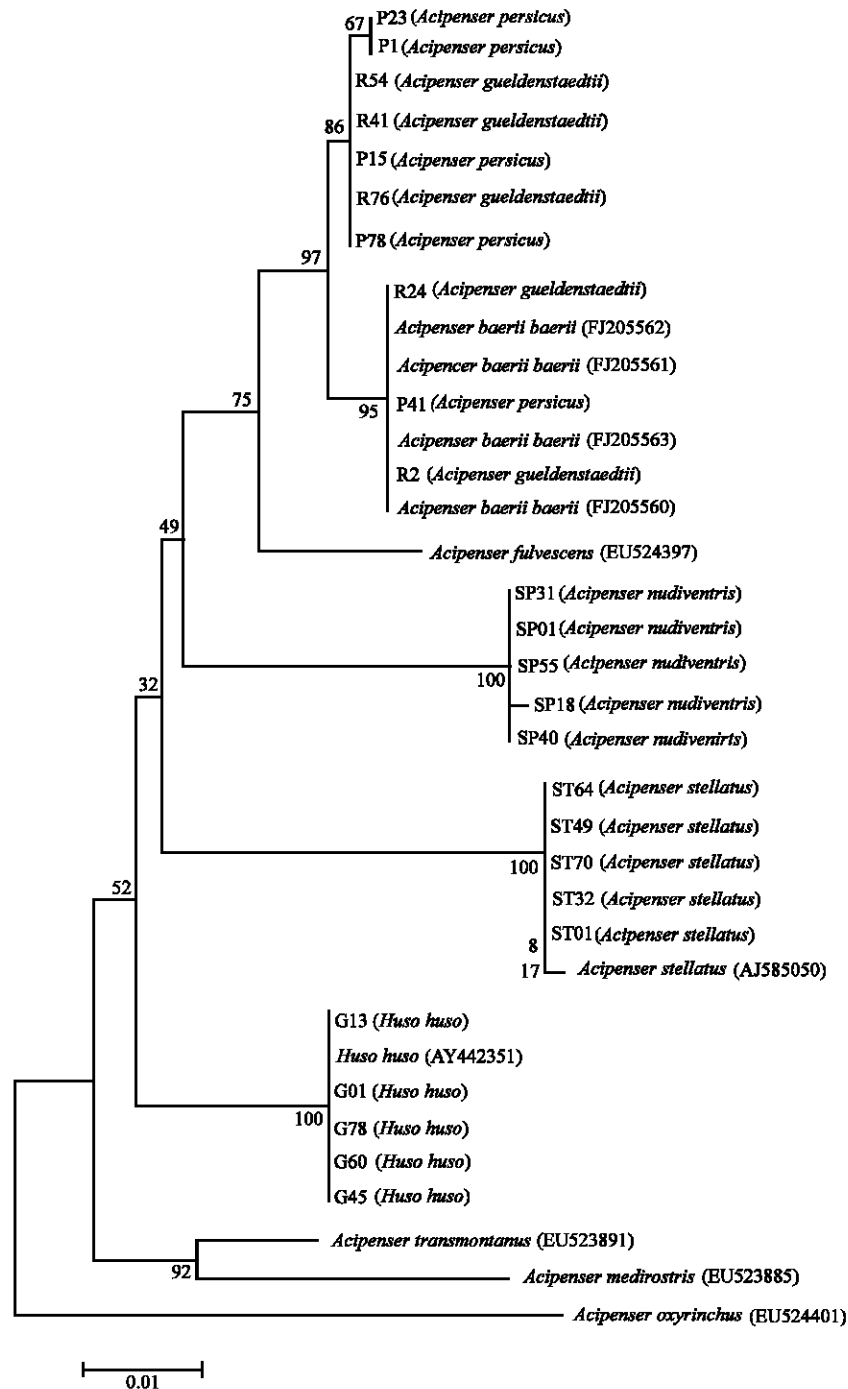


Fig. 1: Dendrogram generated through NJ method showing phylogenetic relationships among sturgeon species. Values at nodes represent bootstrap confidence level (1000 replicates)

were not informative due to the associated low confidence although the data separated the *A. gueldenstaedtii*, *A. persicus* and *A. baerii* (FJ205560-FJ205563) into one cluster, while *A. fulvescens* (EU524397), *A. nudiiventris*, *A. stellatus*, *H. huso*, *A. transmontanus* (EU523891), *A. medirostris* (EU523885) and *A. oxyrinchus* (EU524401)

in another with 75 and 69% support in the NJ and MP analysis, respectively. Phylogenetic relationships within the second cluster could not be determined due to the low branch support. Interestingly, *H. huso* was embedded among the *Acipenser* species and did not form the basal clade as would be expected of a different genus.

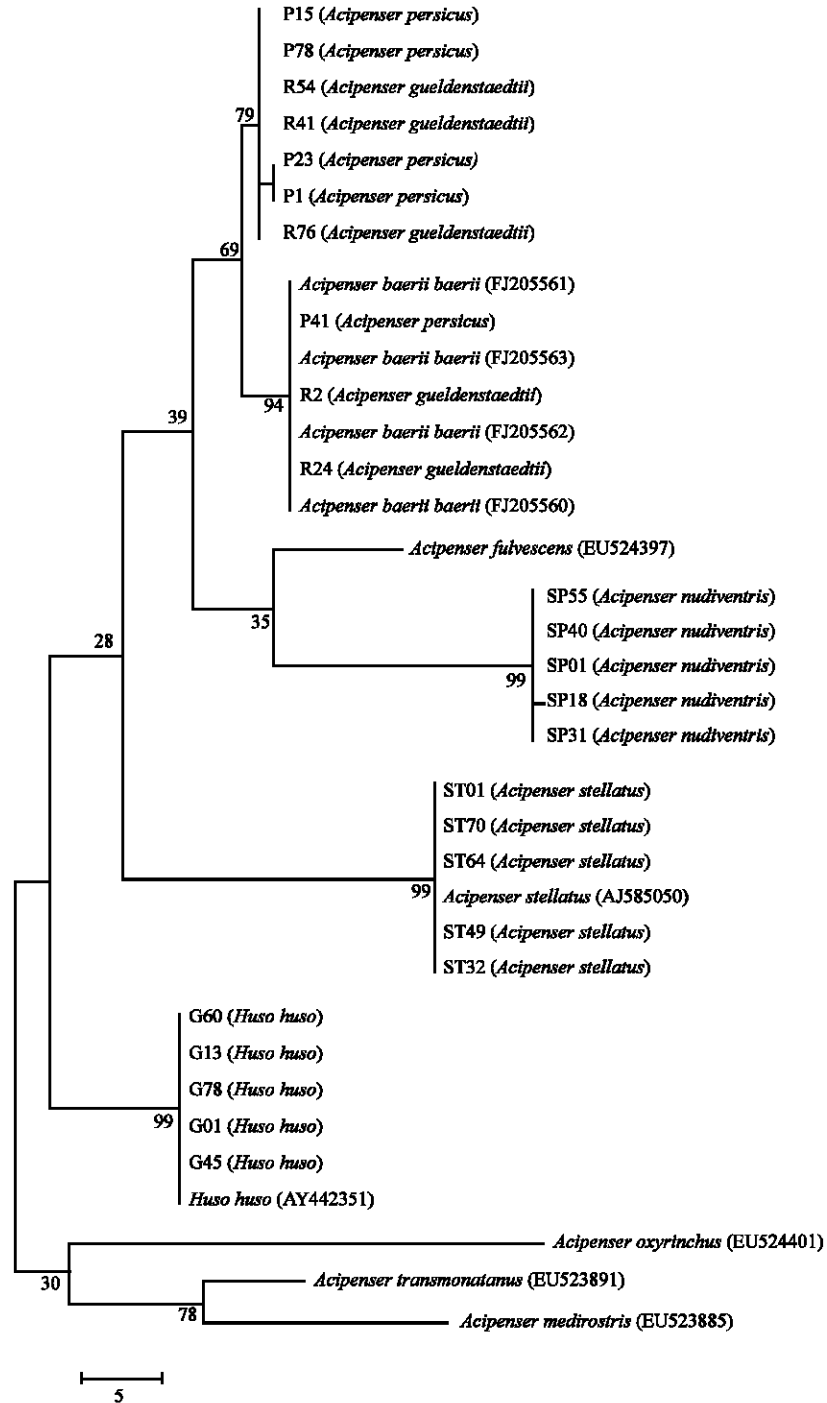


Fig. 2: Dendrogram generated through MP method showing phylogenetic relationship among *sturgeon* species. Values at nodes represent bootstrap confidence level (500 replicates)

## DISCUSSION

This study confirms the utilization of the COI barcoding gene for diagnosis of three southern Caspian

Sea sturgeons namely *H. huso*, *A. nudiiventris* and *A. stellatus* through their monophyly. However, the data failed to resolve the taxonomic status of *A. gueldenstaedtii* and *A. persicus*. The COI barcoding

gene has been successfully utilized in a vast range of organisms ranging from insects (Smith *et al.*, 2005; Janzen *et al.*, 2005) to fishes (Ward *et al.*, 2005; Rock *et al.*, 2008) and extinct ratite birds, the moa of New Zealand (Lambert *et al.*, 2005). However, even within these studies, ambiguities in several taxonomic groups were detected. As suggested by Rock *et al.* (2008) in their investigation of the Scotia Sea fishes, failure to discriminate taxonomic groups may reflect shared haplotypes possibly representing previous introgressive hybridization between species with overlapping ranges. In cases, where this marker set lacked sensitivity then other markers (Savolainen *et al.*, 2005; Monaghan *et al.*, 2005) should be utilized as set out in the barcoding guidelines in Consortium of Barcode of Life, CBOl (<http://barcoding.si.edu/>).

In a recent publication, Rach *et al.* (2008) discussed the pitfalls in relying on phenetic approaches and tree-building methods for defining species boundaries due to the difficulties in determining a universal threshold of genetic distance values to distinguish taxonomic groups. They suggested DNA barcoding approaches of character based, whereby species are identified through the presence or absence of discrete nucleotide substitutions (character states) within a DNA sequence as another alternative. Their investigation demonstrated the potential of using this technique not only to delineate species within this group but also population level differentiation. A total of 54 species and 22 genera of Odonata were discriminated through unique combinations of character states within only a single mitochondrial non COI gene region (NADH dehydrogenase 1). Thus, this could be a future useful option to address the complex status of *A. gueldenstaedtii* group. Needless to add, distinguishing between these species is vital as they are endangered and is subject to a high degree of commercial exploitation (Birstein *et al.*, 2000).

Beluga sturgeon, *H. huso* and its close relative the kaluga sturgeon *H. dauricus* have been traditionally considered as representatives of a separate genus *Huso* within the subfamily Acipenserinae of the family Acipenseridae based on morphological studies (Bemis *et al.*, 1997). However, since the mtDNA studies of Birstein *et al.* (1997) and Birstein and de Salle (1998), there has been accumulating evidence of *Huso* being synonymous with *Acipenser* within the Acipenserinae. Similarly in this study, the clustering of *H. huso* within the *Acipenser* clade of the four investigated species and additional Genbank data of *Acipenser* species is in agreement with this. As pointed out by Tagliavini *et al.* (2007) based on their investigations of the cytochrome b (cyt b) gene, the generic status of the *Huso* within

Acipenserinae should be reviewed, a view supported by this data. Although, phylogenetic relationships insights may be obtained from barcoding studies (Ward *et al.*, 2005), the investigations highlights the limitations of this short fragment of the COI in inferring phylogenetic relationships among the *Acipenser* genus. This is perhaps due to the more conserved amino acid sequence within the COI gene in the *Acipenser* relative to other taxonomic groups where phylogenetic relationships have been deduced. Other phylogenetic analyses using longer length sequences or combined markers have been more efficient in resolving the phylogenetic relationships of this group (Birstein and de Salle, 1998; Tagliavini *et al.*, 2007) and is definitely a more useful approach.

## CONCLUSION

This study confirms the utility of the COI barcoding gene in accurate identification on three of the five sturgeon species in the southern Caspian Sea but has yet again failed to resolve the *A. gueldenstaedtii*-*A. persicus* controversy based on distance methods of single mtDNA.

## RECOMMENDATIONS

It is suggested that future studies should incorporate character-based methods. In addition, the data supports the view that *H. huso* may not be a separate genus from *Acipenser* within the Acipenserinae.

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