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Genetic diversity in the Persian sturgeon, *Acipenser percicus*, from the south Caspian Sea based on mitochondrial DNA sequences of the control region

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ABSTRACT
The Persian sturgeon, *Acipenser persicus* (Borodin, 1897), is an economically important species, which mainly inhabits the Caspian Sea. However, little is known about its population genetic structure. In this study, variation in nucleotide sequences of the mitochondrial DNA (mtDNA) control region of wild stock Persian sturgeon was determined to assess the genetic diversity among different natural populations of this species. The fish (n = 46) were collected from four sites (Astara, Sefidrood, Noshahr and Bandare-Turkaman) in the south Caspian Sea. As a result 6 haplotypes and 44 variable sites were found. The average haplotype diversity (h) and nucleotide diversity (π) were 0.640±0.028 and 0.0442±0.011, respectively. Analysis of molecular variance (AMOVA) demonstrated that most variations occurred within samples, and the difference between the populations from Astara and Noshahr or Bandare- Turkaman was not significant (p <0.001). Estimates of gene flow indicated reproductive isolation between the Sefidrood River population and the other collections. The divergence might be related to geographical isolation. The results are consistent with the findings from PCR-RFLP analysis (PCR-RFLP) and suggest considerable genetic diversity of the population from Sefidrood River.

Keywords: *Persain sturgeon; Acipenser persicus; mitochondrial DNA; genetic variation.*

INTRODUCTION

The Persian sturgeon, *Acipenser persicus* is an economically important species mainly observed in the Iranian rivers, Sefidrood and Gorgan-chaii, flowing into the Caspian Sea. It also enters to a smaller degree into the Terek, Suli and Tamur rivers in the Russian Federation and Republics, respectively. A small group of individuals live in the Volga, Kura, and Ural Rivers (Birstein *et al.,* 1997). The Persian sturgeon is concentrated in Iranian waters where sea fishing is permitted. (Vlasenko *et al.,* 1989; Birstein *et al.,* 1997). The decline in Persian sturgeon populations in the last decade is largely due to environmental changes such as the loss of spawning grounds and rearing habitats. Commercial fishing of The Persian sturgeon has diminished in most of its range, but it continues for certain populations because of the value of its caviar (Vecsei and Artyukhin, 2001; Pikitch *et al.,* 2005; Moghim *et al.,* 2006; Pourkazemi, 2006).

Molecular genetic studies using allozyme and DNA markers have provided useful insights into a number of areas of biology of some sturgeon species in the Caspian Sea, including their evolution, taxonomy, and fishery management. For example, the identification of genetically distinct populations of stellate sturgeon *A. stellatus* (Pourkazemi, 1996; Shabani *et al.,* 2003; Norouzi *et al.,* 2008), Russian sturgeon *A. gueldenstaedtii* (Pourkazemi, 1996, Pourkazemi *et al.,* 1999; Rezvani-Gilkolaei, 2000; KhoshKholgh *et al.,* 2008 and Vodolazhskii *et al.,* 2008) Beluga *Huso huso* (Rezvani-Gilkolaei, 1997) and Ship *A. nudiventris* (Pourkazemi, 1996; Qasemi *et al.,* 2004 and Safari *et al.,* 2008) are of significant value to the development of management strategies of these genetic resources in the Caspian Sea.

Mitochondrial DNA (mtDNA) has been widely used to identify both population structure and genetic variability because of

its rapid evolutionary rate (Avise, 1994; Brown, 2008). Within mtDNA, the non-coding control region has been shown to evolve five times faster than the coding region, and often has higher variability (Brown, 1985; Billington & Hebert, 1991). Therefore, the control region has been recommended for assessing intraspecific genetic variation in sturgeons (Brown *et al.*, 1993; Onge *et al.*, 1996; Wirgin *et al.*, 2000). Nowadays, analysis of the mtDNA control region (*D- loop*) is the frequently used method to resolve genetic differentiation, population structure, and intraspecific phylogenesis in sturgeon species (Pourkazemi *et al.*, 1999; Pourkazemi *et al.*, 2000; Ludwig *et al.*, 2000;Wirgin *et al.*, 2000; Grunwald *et al.*, 2002; Wirgin *et al.*, 2002; Waldman *et al.*, 2002; Wirgin *et al.*, 2005; Mugue *et al.*, 2008).

Two preliminary studies on population genetics of the Persian sturgeon based on the analysis of mtDNA variation have been published. Ataei et al. (2004) found extensive genetic variability among three populations of *A. persicus* from south Caspian Sea by using PCR-RFLP technique. Rezvani-Gilkolaii (1997) investigated the genetic diversity of two wild populations of *A. persicus* from the western and eastern of Caspian Sea using partial sequence analysis of MtDNA *NADH* 5 gene but this study has suffered very limited geographic sampling. They found its genetic diversity was considerable in its sampled area of distribution, western and eastern part of the Caspian Sea. Ataei et al. (2004) suggested that the conservation of genetic diversity of this species in the Sefidrood River should be considered.

In this study, the genetic variation among *A. persicus* populations from different locations of south Caspian Sea was analyzed based on the sequences of mtDNA control region. The aim of this study was to characterize the geographical patterns of genetic diversity among different wild populations of Persian sturgeon using analysis sequences of the mitochondrial DNA (mtDNA) control region.

Materials and Methods

Sampling and DNA Extractions

A total of 46 specimens of the Persian sturgeon, *Acipenser persicus*, were collected from 4 different locations in the south Caspian Sea: Astara (11), Sefidrood River (13), Noshahr (10) and Bandare Turkaman (12), (Figure 1). The position of sampling sites is shown in figure 1. All were obtained during the years 2000–2003 by the International Sturgeon Research Institute. The samples (2-3 g dorsal fin tissue) were first kept in 96% ethanol and then at -20° C until DNA extraction. Total DNA was isolated from fin tissues by standard phenol–chloroform extractions and ethanol precipitations following the method described by Hillis and Moritz (1990) with some modifications (Pourkazemi, 1996). The DNA was re-suspended in TE Buffer (10 mM Tris,10 mM EDTA, pH 8.0). The quality and quantity of total DNA were determined by agarose gel electrophoresis ethidium bromide staining and spectrophotometery, respectively.

PCR amplification and sequencing

Amplification of the mitochondrial control region was performed using the oligonucleotide primers**:** *D loop* F (5´-GCTCAACCCTCCTAATCATTT-3´) and *D loop* R (5´- AGTGTGATGAGGAGGATTGA -3´).The primers were designed based on mtDNA control region sequences of the Persian sturgeon *A. persicus* available at GenBank (Pourkazemi *et al.*, 1999; Accession No. EU714033). PCR amplification was conducted according to Pourkazemi (1996). A total volume of 25 µl containing 2 µl of template DNA (at a final concentration of 50 ng μl^{-1}), 2.5 µl 10×PCR buffer (Fermentase), 1.5 µl MgCl2 (25 mM),1 µl *D loop* F (0.4 mM), 1 µl *D loop* R (0.4 mM), 0.25 U Taq DNA Polymerase (Vio Taq™ VT1001, Fermentase) and 1 µl dNTPs (2.5 mM). The step programs for PCR amplification were as follows: a denaturation step at 94° C for 3 min, followed by 35 cycles consisting of 94° C for 30 s, 51° C for 60 s, 72° C for 70 s and a final extension at 72° C for 10 min. The reaction products of the PCR were assessed on 0.8% agarose gel in 0.5× TBE buffer.PCR products were sequenced on an ABI autosequencing machine (MegaBACE™) using a DYEnamic™ ET dye terminator cycle sequencing kit (MegaBACE™). The sequencing was performed bi-directionally and checked twice for every site of the sequence. Partial sequences of mtDNA *D*

loop were deposited in GenBank (GenBank accession numbers: *Acipenser persicus*, FJ364156–FJ364162).

Data Analysis

All nucleotide sequences were aligned with Clustal X 1.8 multiple-alignment program (Thompson *et al.*, 1997) with subsequent refinement by means of the Chromas 2.23 program (Technelysium, Tewantin, Australia). Sequence polymorphisms and genetic distances within and between the populations were estimated. A Neighbor-Joining (NJ) tree was constructed for all haplotypes according to Kimura 2-parameter model (Kimura, 1980) using Mega Version 3.1 (Kumar *et al.*, 2004). Haplotype (*h*) and nucleotide diversity (π) (Nei, 1987) were estimated using DnaSP 4.0 (Rozas *et al.*, 2003). Population structure was evaluated using the analysis of molecular variance model (AMOVA) (Excoffier *et al.*, 1992) by using Arlequin Version 3.000 software package (Excoffier *et al.*, 2005). Fixation indices (F_{st}), (Hudson *et al.*, 1992), were also calculated to assess genetic divergence overall and between paired populations. Estimates of gene flow were derived using the equation: Nm = [(1/Fst)-1]/2 (Weir and Cockerham, 1984). The statistical significance of the total and pairwise fixation indices was estimated by comparing the observed distribution with a null distribution generated by 10,000 permutations. Statistical significance was at $P = 0.05$.

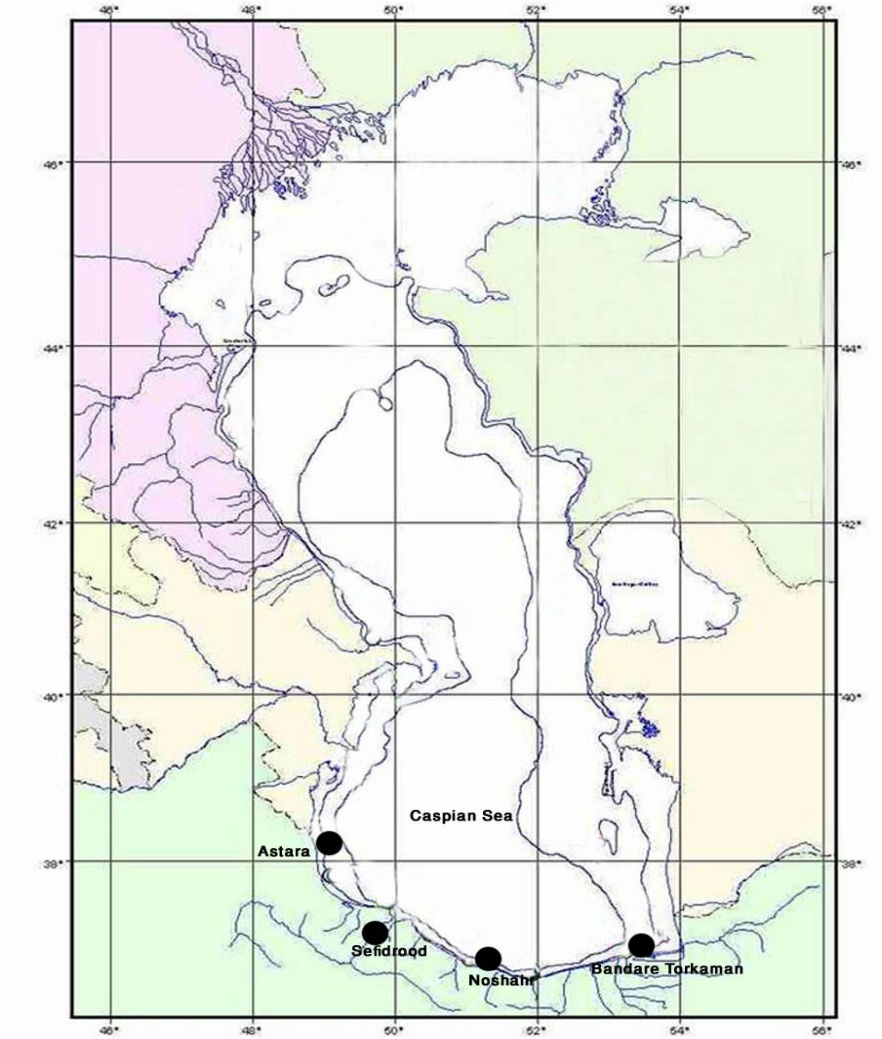

Fig 1. Sampling sites in the south Caspian Sea from which the Persian sturgeon *Acipenser persicus* specimens were obtained. The four sampling sites are indicated by black dots for the Persian sturgeon.

Results

The aligned mtDNA sequence consisted of part of the control region containing 390 base pairs (bp). Forty four variable sites were observed in the sequences and all substitutions were transitions, and no insertions or deletions were observed. Six control-region haplotypes were found among the sequences (Table 1). The haplotypes tend to be restricted to separate populations and regions. All the individuals of *A. persicus* in the south Caspian Sea shared a common haplotype (Ha4). The haplotype (Ha6) was only seen in one individual from Sefidrood River. Four haplotypes were observed in the populations from Sefidrood river (Ha1, Ha3, Ha4 and Ha6), one of which (Ha4) was shared with Astara, Noshahr and Bandare Turkaman populations (Table 1). The Bandare Turkaman and Noshahr regions shared two haplotypes (Ha4, Ha5) that differed by only one transition (Table1).

Table 1. Distribution of mitochondrial haplotypes in the Persian sturgeon collections analyzed in this study

Location / Haplotype	Astara	Sefidrood river	Noshahr	Bandare Turkaman	∑
Ha1		6			6
Ha2		3			4
Ha3		2			2
Ha4	8	1	9	10	28
Ha5	3		1	2	5
Ha6		1			1
∑	11	13	10	12	46

Genetic structure analysis among and within regions

The haplotype diversity *(h)* of the control region within the regions Astra, Bandare Turkaman ,Noshahr and Sefidrood River were 0.559±0.027, 0.449±0.014, 0.598±0.015 and 0.955±0.057 respectively. Whereas the nucleotide diversity (π) was 0.0413±0.013, 0.0221±0.017, 0.0324±0.006 and 0.0810±0.009, respectively (Table 2).

Table 2. Levels of genetic diversity within the four samples of the Persian sturgeon (*n* sample size; *h* haplotype diversity; π nucleotide diversity). Data shown as mean± standard error

Location	*n*	Number of haplotypes	Molecular diversity indices	
			π	*h*
Astara	11	1	0.0413±0.013	0.559±0.027
Sefidrood river	13	3	0.0810±0.009	0.955±0.057
Noshahr	10	1	0.0324±0.006	0.598±0.015
Bandare Turkaman	12	1	0.0221±0.017	0.449±0.014
All samples	46	6	0.0442±0.011	0.640±0.028

The AMOVA indicated significant differences (P<0.05) and low gene flow (N_m) among four regions. The AMOVA also partitioned of totally 58.03% genetic variation among the regions and 41.97% of the total within the regions (Table 4), indicating that much of the variation was between the regions.

Results from the AMOVA analysis were supported by the low and mostly insignificant Fst values for overall population differences (P<0.05) (Table 4). The only significant Fst values for overall genetic structuring were observed for the Sefidrood river (Table 4). After Bonferroni correction for multiple independent tests (adjusted P = 0.003), some of the differences were no longer significant. However, differences between the sample collected in Sefidrood River and several other samples remained significant (P<0.05). No significant differences between any of the other samples were detected, either before or after Bonferroni correction.

Table 3. Pairwise F_{st} values between collections of *A. persicus* examined in the present study.

Location	Astara	Sefidrood River	Noshahr
Astara	-		
Sefidrood river	0.732	-	
Noshahr	0.097	0.825	-
Bandare Turkaman	0.054	0.966	0.082

Haplotype Ha4 was the most common and widespread, being detected in all the studied sites (Table 1). Haplotype Ha5 was the only one that was found in both sites located of the Western Caspian Sea (i.e. in both the Astara region and Sefidrood River).The haplotype neighbor - joining tree (Figures 2) reveals two distinct clades. Individuals in clade 2 were widespread and occurred in three regions Bandare Turkaman, Noshahr and Astara, while clade 1 was totally restricted to the Sefidrood River.

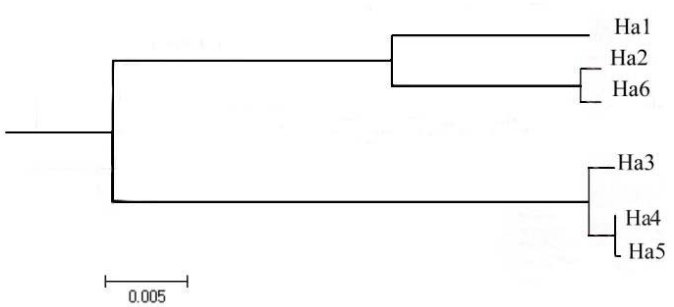

Fig 2. Neighbor-Joining tree of the mtDNA control region haplotypes of Persian sturgeon using Kimura 2-parameter distance method.

Table 4. Analysis of molecular variance **(**AMOVA) of mitochondrial DNA composite haplotypes for the four Persian sturgeon collections

Source of variation	**df**	**Percentage of variation**	**Fixation indices**	***p***
Among populations	3	10.23	0.09564	0.08321
Among samples within populations	3	7.26	0.03245	0.00321
Within samples	40	36.21	0.31411	0.01234

The average number of pairwise F_{ST} values are shown in Table 3. Pairwise genetic differences ranged from 0.054 (between Bandare Turkaman and Astara) to 0.966 (between Bandare Turkaman and Sefidrood River). Significant differences between Sefidrood river and all other collections pairwise F_{ST} values (0.732 to 0.966, $p \leq 0.05$) and significant probabilities ($p \leq 0.05$) based on 10,000 permutations of haplotype frequencies after sequential Bonferroni correction were observed.

Discussion

Data from the present study indicate that within the south Caspian Sea, there is little evidence to suggest that *A. persicus* is divided into discrete populations. Only the samples from Sefidrood River showed some differences between the samples collected in Southwest and Southeast Caspian Sea.

The mtDNA control region sequences of the Persian sturgeon revealed 6 haplotypes based on the nucleotide variation. The total haplotype diversity and nucleotide diversity were 0.640±0.028 and 0.0442±0.011, respectively. Grunwald et al. (2002) suggested that the nucleotide diversities of the control region were low to moderate for shortnose sturgeon, *Acipenser brevirostrum* (ranging from 0.0022 to 0.0057), and that the diversity of haplotypes was moderate to high (ranging from 0.641 to 0.817). According to these results, the haplotype and nucleotide diversity of the

control region were moderate to high in the studied Persian sturgeon. Nevertheless, the haplotype and nucleotide diversity obtained in this study were higher than those resulted in Atlantic sturgeon *Acipenser oxyrinchus oxyrinchus* (Wirgin *et al.,* 2000). This could be due to the differences in the sequence length analyzed. In the present study, 390 bp of control region were sequenced and analyzed, which could reveal more variable sites than previous study based on 203 bp in Atlantic sturgeon (Wirgin *et al.,* 2000). Also, the limited population size examined in this study, compared to the larger population size in Atlantic sturgeon, (Wirgin *et al.*, 2000) and shortnose sturgeon, *A. brevirostrum* (Grunwald *et al.*, 2002) might have affected the estimates for haplotype and nucleotide diversity.

The F_{st}-value in the three populations of the Persian sturgeon was 0.0837, which compared to that estimated by RFLP markers (Ataei, 2004), indicates a low genetic differentiation. Besides, the overall F_{st} of the four collections inferred from the mtDNA control region was much higher than that from RFLP analysis, indicating a higher level of genetic differentiation at the mtDNA sequences. Pairwise population comparisons of F_{st}-values between the populations from Sefidrood River and Bandare Turkaman (0.966), and Sefidrood River and Noshahrregion (0.825) indicated strong population structure. The results inferred from mtDNA data should be integrated with those obtained from other types of markers such as RFLP or microsatellites and occurrence of gender biased dispersal of genetic structure should be examined.

The results inferred from mtDNA control region sequences were mostly in according with those from RFLP analysis but in contrast with partial sequence analysis of mtDNA ND5 gene. Rezvani Gilkolaei (1997) in his study suggested that ND5 gene might not be useful for population genetic analysis, but it would be useful for phylogenetic analysis of sturgeon fishes. Nevertheless, the estimates of the genetic differentiation slightly differed between the two molecular makers. This study reinforced the finding that the genetic diversity of these four wild populations of Persian sturgeon was considerable (Ataei, 2004). This study suggests that the genetic coservation of populations in the Sefidrood River is still possible despite many anthropogenic disturbances imposed on this river system. In the same manner, the genetic homogeneity revealed among southeast and southwest Caspian Sea populations suggests that these populations could be designated as one unique unit for conservation. However, the use of other molecular markers such as microsatellites could further resolve the genetic structure of these geographically distinct populations.

The mtDNA control region might be useful as a genetic marker for aquaculture purposes such as planning for selective breeding, maintaining stock diversity and distinguishing hatchery stocks from the wild populations. The high genetic diversity in the control region could be due to the high mutation rate (Billington and Hebert, 1991). A high rate of mtDNA mutation suggested can explain the high genetic diversity and divergence in this species using different tools. Such mtDNA diversity has been reported for several other Acipenserid species (Wirgin *et al.,* 2000; Grunwald *et al.,* 2002; Waldman *et al.,* 2002; Wirgin *et al.,* 2005; Vodolazhskii *et al.,* 2008).

In conclusion, the present study clarified that there were some genetically distinct Persian sturgeon populations in the south Caspian Sea. These results provide useful information for identifying populations and for determining their management and conservation units, leading to the useful application of molecular genetics in investigating conservation biology of the Persian sturgeon.

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بررسی تنوع ژنتیکی جمعیت تاس ماهی ایرانی (*Acipenser persicus*) حوضه جنوبی دریای خزر با استفاده از روش توالی یابی منطقه کنترل DNA (D loop) میتوکندریایی

م. خوش خلق، م. پورکاظمی، س. نظری ، ل. عزیززاده

چکیده:

تاس ماهی ایرانی (*Acipenser persicus*) یکی ازگونه های با ارزش تاس ماهیان است که در دریای خزر پراکنش دارد و تاکنون مطالعات کمی در مورد ژنتیک جمعیت آن انجام شده است. در این مطالعه به منظور تعیین ساختار ژنتیک جمعیت تاس ماهی ایرانی در مناطق مختلف حوضه جنوبی دریای خزر روش توالی یابی قطعه D-Loop DNA میتوکندریایی به کار گرفته شد. در مجموع تعداد ۴۶ نمونه باله تاس ماهی ایرانی از ۴ منطقه (آستارا، رودخانه سفیدرود، نوشهر و بندر ترکمن) حوضه جنوبی دریای خزر جمع آوری گردید. توالی یابی با استفاده از روش استاندارد انجام پذیرفت و در مجموع بین ۶ هاپلوتیپ متفاوت و ۴۴ جایگاه متغیر بدست آمد. تنوع هاپلوتایپی و نوکلئوتیدی در نمونه های کل مناطق به ترتیب برابر با ۰/۰۲۸ ± ۰/۶۴۰ و ۰/۰۱۱ ± ۰/۰۴۴۲ بدست آمد. نتایج آنالیز F_{st} بر اساس روش دوپارامتری و آنالیز واریانس مولکولی (AMOVA) نشان داد بیشترین تنوع در درون نمونه ها می باشد و اختلاف بین نمونه های مناطق آستارا، نوشهر و بندر ترکمن معنی دار نبود. برآورد جریان ژنی نشان داد که بین نمونه های رودخانه سفیدرود و دیگر مناطق جدایی تولید مثلی وجود دارد که ممکن است در نتیجه جدایی جغرافیایی باشد. نتایج این بررسی موافق مطالعه PCR-RFLP بر روی این گونه بوده و همچنین پیشنهاد می دهد که تنوع ژنتیکی جمعیت تاس ماهی ایرانی رودخانه سفیدرود مورد توجه قرار گیرد.