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Population structure and variation in Persian sturgeon (*Acipenser percicus*) from the Caspian Sea as determind from mitochondrial DNA sequences of the control region

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Abstract

Mitochondria1 DNA (mtDNA) control region sequences were analyzed to evaluate the population genetic structure of Persian sturgeon (*Acipenser persicus*) in Caspian Sea. A total of 45 specimens were collected from the different locations of the Caspian Sea. MtDNA control region was amplified using PCR. Direct sequencing was performed according standard method. The results showed that 12 haplotypes were observed between 45 samples in the method. The highest numbers of haplotypes were observed in Sefidroud River in which 3 haplotypes A, B and E among them were specific for the river and were not observed in the other locations. The average haplotype diversity (h) and nucleotide diversity (π) were 0.795±0.037 and 0.0062±0.0046, respectively. The results of FST based on kimura- 2 parameters method and analysis of molecular variance (AMOVA) demonstrated that most variations occurred between samples from Sefidroud River in the south Caspian Sea and that the samples include three distinct populations including Sefidrud, Russia and Azerbaijan ($P<0.001$). As mtDNA control region is hypervariable segment, this can be provide potential marker for identifying probable 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.

Key Words: *Acipenser persicus*, Caspian Sea, genetic variation, mitochondrial DNA.

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Introduction

Persian Sturgeon are distributed both in the northern and southern parts of the Caspian Sea and is one of the large sturgeons, with a maximum length of ≈230 cm and weight of 70 kg. Age at maturity varies latitudinally; females mature at 12 years in Volga river, 10-11 years in the Kura river and 12–18 years in Ural River (40, 51). At sea, the Persian Sturgeon feeds on a wide variety of benthic molluscs, crustaceans and small fish (6, 34). The Persian sturgeon is concentrated in Iranian waters where sea fishing is permitted (6, 49). Published studies illustrate that population size of all sturgeon species have declined, with some estimates suggesting 80–90% decreases in the last 30–40 years (26, 49). However, decreases in many Persian sturgeon populations have led to conservation concerns; since 2000 it has been classified under endangered Appendix II of CITES and listed as endangered species with IUCN (17, 51).

Mitochondrial DNA (mtDNA) has been used extensively in many studies for the determination of intra-specific population structure, helping to define conservation units in many endangered species (56), or to identify fisheries stocks in commercial species (4). Its application is supported from both practical and biological reasons; mtDNA is structurally and functionally simpler than nuclear DNA, has a faster mutation rate and is maternally inherited, making it ideal to detect lineages produced on relatively short time periods (2, 22). Traditionally coding genes as 16s and cytochrome b are more extensively used for phylogenetic studies (7, 9, 10, 20) because they are conservative sequences, whereas the control region has been more used for micro-evolutionary processes at the population level because it is generally the most rapidly evolving region (7, 27). Some studies based in control region in sturgeon species (13, 36, 42, 56, 59) have shown its usefulness and the control region has been recommended for assessing intraspecific genetic variation in sturgeons.

Several studies of the stock identification and genetic structure have been done for Persian sturgeon. Earlier studies on the Persian sturgeon population using electrophoresis of blood serum proteins have detected low amounts of polymorphism between and within populations of Persian sturgeon in the south Caspian Sea (3, 32). Ataei *et al*. (2004) and Khoshkholgh *et al*. (2011) found extensive genetic variability among populations of *A. persicus* from south Caspian Sea by using PCR-RFLP technique and sequence analysis of mtDNA control region, respectively. Rezvani-Gilkolaii and Skibinski, (2000) investigated the genetic diversity of two wild populations of *A*. *persicus* from the western and eastern of Caspian Sea using sequence analysis of mtDNA *NADH* 5 gene. They found its genetic diversity was considerable in its sampled area of distribution, western and eastern part of the Caspian Sea. Furthermore, evidence of significant genetic differentiation in microsatellite allelic frequencies among Persian sturgeon populations suggests they are reproductively isolated and three distinct populations including: Sefidroud River, middle and north Caspian Sea populations were determined (39).

Based on the results of previous studies and on the need to use high variable genetic markers, we used the mtDNA control region sequence analysis to compare the genetic diversity among Persian sturgeon samples from throughout their geographical range in the Caspian Sea. We also include some previously unstudied populations for a more

comprehensive analysis. These investigations allowed us to determine intra- and inter-population genetic diversity at the mtDNA level in samples from throughout the range of the Persian sturgeon and also to detect molecular genetic variations in Persian sturgeon for more extensive molecular genetic research.

Materials and methods

Sampling and DNA Extractions

A total of 45 adult Persian sturgeon, *Acipenser persicus*, were collected from 8 different locations in the Caspian Sea including: Astara (6), Sefidroud River (7), Eizdeh (5), Shiroud (5), Turkaman (5), Azerbaijan, (6), Russian Federation (6) and Kazakhstan (5) (Figure 1). All were obtained during spawning seasons by the International Sturgeon Research Institute. The samples (2-3 g dorsal fin tissue) first were kept in 96% ethanol and then at -20° C until DNA extraction. Total genomic DNA was extracted from the fin clips by digestion with proteinase K at 65°C, followed by standard ammonium acetate extractions and alcohol precipitations protocol (18). The DNA samples were resuspended in TE Buffer (10 mM Tris, 10 mM EDTA, pH 8.0) and were stored at 4° C prior to PCR analysis. The quality and quantity of total DNA were determined by agarose gel electrophoresis ethidium bromide staining and spectrophotometery (Nanodrop, ND1000), respectively.

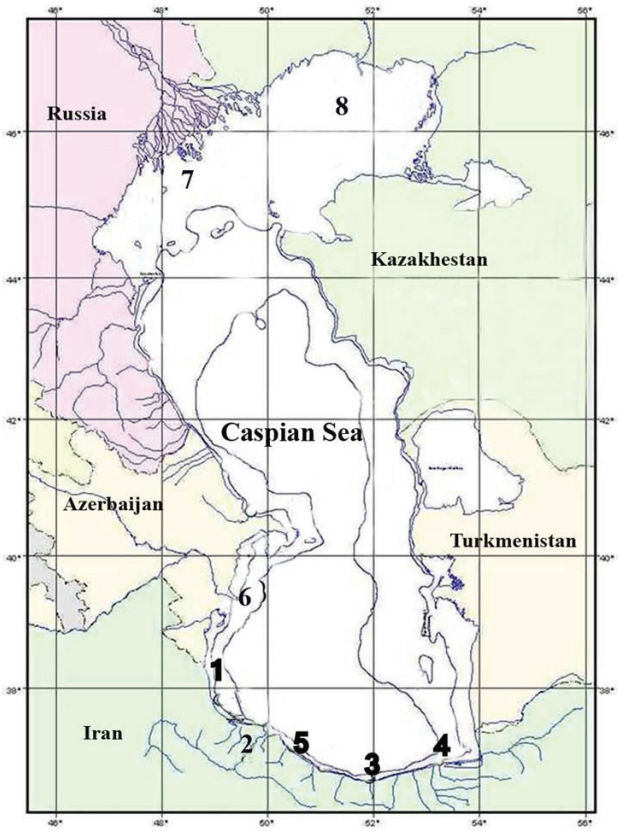

Figure 1. Map showing the locations of the Persian sturgeon sampling sites in the Caspian Sea. Sampling localities: 1, Astara (Zone1); 2, Sefidroud River; 3, Eizdeh (Zone 3); 4 Torkaman (Zone 4); 5, Shiroud (Zone 5); 6, Azerbaijan; 7, Russian Federation and 8, Kazakhstan.

PCR amplification and sequencing

DNA primers for amplifying a highly variable portion of the mtDNA control region (approximately 500 base pairs) were designed from published sequences for Persian sturgeon *A. p persicus* available at GenBank (37; Accession No. EU714033). 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′). PCR amplification was conducted according to Pourkazemi (1996). A total volume of 25 µl containing 100 ng of template DNA, 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^{Tm} 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 70s and a final extension at 72° C for 10 min. Obtained PCR products were visualized and recorded with the method of horizontal gel-electrophoresis in 0.8% agarose in the presence of ethidium bromide in 0.5× TBE buffer. PCR products were sequenced on an ABI autosequencing machine (MegaBACE^{Tm}) using a DYEnamic^{Tm} ET dye terminator cycle sequencing kit (MegaBACE^{Tm}). The sequencing was performed bidirectionally 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

DNA sequence alignment was performed using 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. An UPGMA tree was constructed for all haplotypes according to Kimura 2-parameter model (Kimura, 1980) using Mega Version 3.1 (21). Haplotype (*h*) and nucleotide diversity (π) (28) were estimated using DnaSP 4.0 (45). Population structure was evaluated using the analysis of molecular variance model (AMOVA) (12) by using Arlequin Version 3.000 software package (12). Fixation indices (F_{ST}), (15) were also calculated to assess genetic divergence overall and between paired populations. 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 $P = 0.05$

Results

The aligned mtDNA sequence consisted of part of the control region containing 500 base pairs (bp). Forty four variable sites were observed in the sequences and all substitutions were transitions, and no insertions or deletions were observed. Twelve 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 (D). The haplotypes A, B and E was only seen in individuals from Sefidroud River. Five haplotypes were observed in the populations from Sefidrood river (A, B, D, E and F), one of which (D) was shared with Astara, Eizde, Shiroud and Turkaman populations (Table 2). The haplotype D was a dominant haplotype in the south coastal

waters of the Caspian, whereas the haplotype J was dominant in the middle and north Caspian Sea. Haplotype G was the only one that was found in both sites located of the middle and south Caspian Sea (i.e. in both the Azerbaijan region and Eizdeh (Zone 3). The haplotype J shared with Azerbaijan, Russia and Kazakhstan and the haplotype L was only seen in individuals from Russia. In the populations from Russia four haplotypes were observed. The mean haplotype diversity (h) and the nucleotide diversity (π) of the control region were 0.795±0.037 and 0.0062±0.0046 respectively (Table 3).

Table 1. Polymorphic sites of mitochondrial DNA control region of 12 haplotypes

Number*	1 2 3 4 5 6 7 8 9 1 0	1 1 1 1 1 1 1 1 1 1 2 3 4 5 6 7 8 9	2 2 2 2 2 2 2 2 2 2 0 1 2 3 4 5 6 7 8 9	3 3 3 3 3 3 3 3 3 3 0 1 2 3 4 5 6 7 8 9	4 4 4 4 4 0 1 2 3 4
Nucleotide site*	1 3 3 4 5 6 7 8 9 1 2 4 9 6 7 5 9 1 4 0 1	1 1 1 1 1 1 1 1 1 2 1 2 2 3 4 7 8 9 1 5 4 8 7 9 6 4 9 4	2 2 2 2 2 3 3 3 3 2 3 4 7 8 9 0 1 3 4 6 8 9 5 6 1 2 9 4 6	3 3 3 3 4 4 4 4 4 5 7 8 9 0 1 3 4 5 9 8 9 7 6 8 1 4 0	4 4 4 4 4 5 6 7 8 9 9 3 2 6 7
Haplotype					
A	C CGTATCCCC	AGTTATTAA	TCCGGTTAAT	TTATTCATCG	CCACC
B	. . . . . TTT.	. A. . G. CGG	. . . C. . A T.	. C. . . . . C. .	. T . .
C	T TA . . TT T	. C. . . . A C	CTAC.	. CG. . . T. . C	. . . . .
D	T A. . . . . T	. . . . . CGG	. A CC.	. . . AC.	T. . T
E	T A . . GG	CTGAG. . . .	CA. . . . . T.	. . . . . T. . . A	. A. . .
F	T TA.	. . GATA. . .	. . . . C G. .	. . T. . . . A. .	. . . T
G	T . . . . . T.	. . . . . CA. .	. . . . CA. . . .	. . . . .	TTC. .
H	. . . . . TTT. .	. A. . G. . . .	GT CC.	AA. . . . T. . T	. . C. .
I	. . . . . TT. .	CCC. . . A. .	A. . . . G. . . .	CC. . . . C A	. . . . .
J	. . . . . TTT. .	. A. . CGC. .	. . . . . G. .	C . . . . . T	T G T
K	. . . . . TTT. .	. . . . . GC. .	. . . AAC. . . .	. C. . . . . C. .	. . . . .
L	. . . . . TTT. .	. AG.	. TT.	C. . . . . A	TT. . .

*For sake of space, the number (of polymorphic sites) and the nucleotide positions are to be read vertically. For example, the first nucleotide site is 12, and the second is 34.

Table2. Distribution of mitochondrial haplotypes in the Persian sturgeon collections analyzed.

Location / Haplotype	Astara	Sefidrood	Eizde	Shirud	Torkaman	Azerbaijan	Russia	Kazakhstan	∑
A		1							1
B		3							3
C				1					1
D	2	1	3	2	3				11
E		1							1
F	3	1		2	2				8
G			2			1			4
H						1			1
I						3	1		4
J						1	1	3	2
K							1	2	4
L							3		2
∑	5	7	5	5	5	6	6	5	45

The average numbers of pairwise FST values are shown in Table 4. Pairwise genetic differences ranged from 0.012 (between Shiroud and Eizde) to 0.342 (between Kazakhstan and Sefidroud River). Significant difference between Sefidroud River and all other collections pairwise FST values and significant probabilities ($P \leq 0.0001$) based on 10,000 permutations of haplotype frequencies after sequential Bonferroni correction was observed.

Table 3. Levels of genetic diversity within the eight samples of the Persian sturgeon.

Location	*n*	Molecular diversity indices	
		π	***h***
Astara	6	0.0059±0.0044	0.723±0.027
Sefidroud	7	0.0102±0.0051	0.944±0.077
Eizdeh	5	0.0052±0.0048	0.696±0.025
Shiorud	5	0.0057±0.0046	0.704±0.027
Turkaman	5	0.0057±0.046	0.789±0.034
Azerbaijan	6	0.0092±0.0043	0.874±0.039
Russia	6	0.0098±0.0049	0.936±0.047
Kazakhstan	5	0.0051±0.0035	0.701±0.026
All samples	45	**0.0062±0.0046**	**0.795±0.037**

***n*, sample size; *h*, haplotype diversity; π, nucleotide diversity. Data shown as mean ± standard error.**

Table 4. Pairwise F_{st} values between collections of *A. persicus* examined in the present study.

Location	Astara	Sefidroud	Eizde	Shirud	Turkaman	Azerbaijan	Russia
Astara	-						
Sefidroud	0. 251	-					
Eizde	0.097	0.295	-				
Shiroud	0.054	0.212	0.012	-			
Turkaman	0.035	0.192	0.009	0.021	-		
Azerbaijan	0.0171	0.268	0.139	0.178	0.180	-	
Russia	0.251	0.311	0.228	0.223	0.243	0.169	-
Kazakhstan	0.182	0.342	0.117	0.113	0.091	0.114	0.141

Chi-square analysis supported the clustering of Sefidroud River, Russia and Azerbaijan and indicated that a significant difference existed in haplotype frequencies between these populations and the other (Table 5). The Sefidroud River samples were highly differentiated from those in the middle and northern Caspian Sea ($P< 0.0001$). Among south populations, Turkeman, Shiroud, Eizdeh and Astara collections exhibited no difference in haplotype frequencies (Table 5). Similarly, samples from Kazakhstan region in northern Caspian Sea exhibited no significant difference in mtDNA haplotypes compared with fish from both Russia and Azerbaijan to the north. A marginally significant difference in haplotype frequency was observed between fish from Azerbaijan and Astara region ($\chi^2 = 14.24$, $P = 0.0431$) (Table 5).

The AMOVA indicated that there was significant differences ($P<0.001$) among eight regions. The AMOVA also partitioned of total 10.38% genetic variation among the regions and 65.39% of the total within the

regions (Table 6), indicating that much of the variation was between the regions. Results from the AMOVA analysis were supported by the high and mostly significant FST values for overall population differences ($P<0.001$) (Table 6). The most significant FST values for overall genetic structuring were observed for the Sefidroud River and Russia collections (Table 6). 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 Sefidroud River and several other samples remained significant ($P<0.0001$). No significant differences between any of the other samples were detected, either before or after Bonferroni correction.

Table 5. Pairwise comparison of Monte Carlo based chi-square values for *A. persicus* populations from 8 locations.

Location	Astara	Sefidrood	Eizde	Shirud	Turkaman	Azerbaijan	Russia
Astara	-						-
Sefidrood	35.60 (0.0000)	-					
Eizde	8.34 (0.1073)	42.86 (0.0000)	-				
Shirud	7.21 (0.1156)	19.56 (0.0000)	2.86 (0.1624)	-			
Turkaman	5.36 (0.1456)	25.10 (0.0000)	7.65 0.1220	4.12 (0.1589)	-		
Azerbaijan	14.24 (0.0431)	38.15 (0.0000)	25.22 (0.0000)	21.18 (0.0000)	12.68 (0.0000)	-	
Russia	34.41 (0.0000)	55.14 (0.0000)	37.20 (0.0000)	29.11 (0.0000)	33.13 (0.0000)	24.15 (0.0000)	-
Kazakhstan	14.11 (0.0610)	67.33 (0.0000)	12.14 (0.0786)	9.98 0.0909)	11.21 (0.0686)	9.84 (0.0994)	18.19 (0.0006)

***P*-values are shown in parentheses.**

Table 6. Analysis of molecular variance (AMOVA) of mitochondrial DNA composite haplotypes for the eight Persian sturgeon collections.

Source of variation	df	Percentage of variation	Fixation indices	*p*
Among populations	7	24.23	0.1456	<0.001
Among samples	7	10.38	0.09855	0.0432
Within populations Within samples	38	65.39	0.2231	<0.001

The UPGMA dendrogram (Figure 2) reinforced the regional clustering of Persian sturgeon populations into Sefidroud River, Russia. The most differentiated cluster included populations of the Sefidroud River and Russian populations. The collections from Turkeman, Shiroud, Eizde and Astara in the south Caspian Sea formed a clade. The collections from Azerbaijan and Kazakhstan also formed a medium-supported clade and the node joining these collections and those from Turkeman, Shiroud, Eizde and Astara in the

south Caspian Sea were not well supported by bootstrapping.

The dendrogram further suggests that the populations in Azerbaijan region are more closely related to those in Kazakhstan rather than Russia. Note that the population positions on the dendrogram inferred from the mtDNA variation did not show clear differentiation among the Persian sturgeon populations from Azerbaijan and Kazakhstan. Thus, the UPGMA tree suggested three distinct groups; Sefidroud River, Russia-Azerbaijan-Kazakhstan and Turkeman-Shiroud-Eizde-Astara (Figure 2).

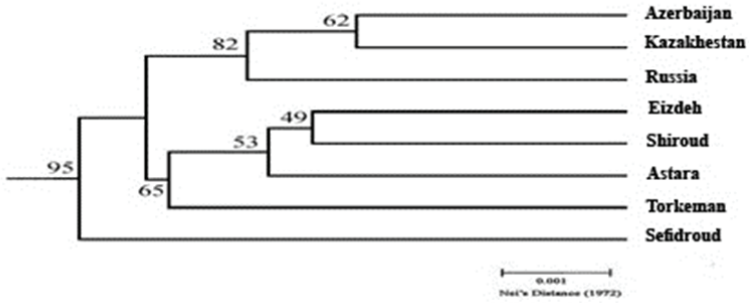

Figure 2. An UPGMA tree of the population genetic distances for the mtDNA control region sequence data from Persian sturgeon of 8 locations. Bootstrap values are given at each node.

Discussion

The results indicate that there is a genetic structure in the Persian sturgeon populations along the Caspian Sea coast and three distinct populations including Sefidroud River, Russia and Azerbaijan were identified. In this study, nucleotide sequences of the partial control region were determined to investigate the genetic relationships among local samples. The occurrence of 2 or more highly variable nucleotide segments flanked by conserved (invariable) segments is a hallmark of the mtDNA control region in animals, including many fish species (4, 22). The location of the region of highest variability, however, differs among fish species (4). In the Persian sturgeon, the DNA segment of greatest variability was located near the 3´ end of the control region (42) and termed the hypervariable region. This may be due to the high mutation rate of HVR-1 which elevates within population diversity levels for this marker. Analysis of this region has proven adequate for resolving the relationships among closely related taxa, such as local races, sub-species and sibling species (7, 27, 54). For example, genetic population structures have been investigated using the control region in several sturgeon species such as Atlantic sturgeon *Acipenser oxyrinchus* (13; 31, 54, 57), shortnose sturgeon, *Acipenser brevirostrum* (59), Chinese sturgeon, *Acipenser sinensis* (61) (Table 6).

In the present study, significant genetic difference in the control region was found among Persian sturgeon samples from various locations. One possibility is that the observed genetic structure of Persian sturgeon might be affected by life history. The species' life history plays a significant role in influencing contemporary levels of spatial population structure. Although life history characteristics for the Persian sturgeon has not been well studied but some life history characteristics, including delayed sexual maturity (approximately 10–12

years), long periods between spawning events (3-6 years), and extensive dispersal throughout the Caspian Sea between spawning periods were reported (25, 40). Differences in the genetic structure among local populations have been observed in several sturgeon species, such as stellate sturgeon *A. stellatus* (30, 47), Russian sturgeon *A. gueldenstaedtii* (44, 53) Beluga *Huso huso* (43) and Ship *A. nudiventris* (35, 41, 46).

Table 7. Population genetic studies in sturgeon species used control region previously published.

Species	No. of base pairs	No. of specimens	Method	Reference
A. brevirostrum	440	275	sequencing	(54)
A. brevirostrum	550	691	sequencing	(59)
A. gueldenstadtii	1004	145	RFLP	(36)
A. oxyrinchus	203	23	sequencing	(31)
A.r oxyrinchus	220	165	/sequencing RFLP	(48)
A. oxyrinchus	203	322	sequencing	(57)
A.r oxyrinchus	245	477	sequencing	(54)
A. oxyrinchus	440	487	sequencing	(58)
A. oxyrinchus	580	739	sequencing	(13)
A. sinensis	419	106	sequencing	(61)
A. persicus	500	45	sequencing	**Present study**

The mtDNA control region sequences of the Persian sturgeon revealed 12 haplotypes based on the nucleotide variation. 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 was higher than those resulted in Atlantic sturgeon *Acipenser oxyrinchus oxyrinchus* (57). This could be due to the differences in the sequence length analyzed. In the present study, 500 bp of control region were sequenced and analyzed, which could reveal more variable sites than previous study based on 203 bp in Atlantic sturgeon (57). Also, the limited population size examined in this study , compared to the larger population size in Atlantic sturgeon, (57) and shortnose sturgeon, *A. brevirostrum* (13) might have affected the estimates for haplotype and nucleotide diversity.

The FST-value in the eight populations of the Persian sturgeon was 0.117, which compared to that estimated by RFLP markers (2), indicates a high genetic differentiation. Besides, the overall FST of the eight 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 FST-values between the populations from Sefidrood River and other collections from Turkeman, Shiroud, Eizde and Astara in the south Caspian Sea indicated strong population structure as well as those collections from Russia and Azerbaijan. Although the Kazakhstan in the north Caspian Sea samples were expected to be geographically and genetically isolated from those of the other locations in the Caspian Sea, the haplotype composition of the Kazakhstan collections were not significantly different from those of other locations (Table 4).The results inferred from mtDNA data should be integrated with those obtained from other types of markers such as RFLP or microsatellites.

The results inferred from mtDNA control region sequences were mostly in according with those from microsatellite analysis (39) but in contrast with partial sequence analysis of mtDNA ND5 gene reported by Rezvani-Gilkolaii and Skibinski, (2000). Nevertheless, the estimates of the genetic differentiation slightly differed between the two molecular makers. The present study reinforced the finding that the genetic diversity of these eight wild populations of Persian sturgeon was considerable (39) and suggests that the genetic conservation of populations in the Sefidroud 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.

In conclusion, the present mtDNA sequencing analyses provided some evidence for the existence of a significant genetic differentiation among some sample populations, suggesting that the Persian sturgeon does not comprise a single panmictic population. Although sample sizes were limited in the present study, our data are consistent with the hypothesis of minimal, if any, geographic structuring of mtDNA diversity among Persian sturgeon in the Caspian Sea. This is important information for a stock enhancement program because there may be a lower risk of detrimental genetic impact when attempting to enhance a population of fish having limited genetic structure and high diversity. The high haplotype and nucleotide diversities observed here suggest that the hypervariable region might be useful as a genetic monitoring tool in stocked Persian sturgeon populations. In particular, these data provide a prestocking baseline that will be useful in tracking temporal genetic changes, should they occur, in stocked populations of Persian sturgeon. In that case, nuclear DNA in addition to mtDNA data could provide a superior tool for testing for genetic differentiation of subpopulations, since mitochondrial genomes are mostly transmitted maternally.

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