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Research Article

Analysis of the genetic structure of the Persian sturgeon (*Acipenser persicus*) populations: Comparison of control region sequencing and PCR-RFLP analysis of mitochondrial DNA

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Abstract

The genetic variation and population structure of the Persian sturgeon, *Acipenser persicus* (Borodin, 1897) was investigated by means of polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) analysis of the nucleotide dehydrogenase subunit 5 (*ND*5) of mitochondrial DNA (mtDNA). We compared these data with our previous study based on mtDNA control region sequences. A total of 225 individuals were collected from 23 sample sites in the south and 4 locations in Turkmenistan, Azerbaijan, Russian Federation and Kazakhstan covering the three main geographic regions including south, middle and north part of the Caspian Sea. The PCR products were digested with 25 restriction enzymes and five enzymes revealed polymorphism patterns (*Rsa* I, *Hinf* I, *Hae*III, *Mbo* I and *Cfr*13I). Thirty two composite haplotypes were revealed with the number of haplotypes in each population sample ranging from 6 to 13. Two regional (Sefidroud River and Russia) groups were clearly identified by cluster and molecular variance model (AMOVA) analyses. Each of these groups showed dominant haplotypes that were little in populations from the other geographic areas. The mean haplotype diversity (*h*) and nucleotide diversity (π) were 0.7610±0.046 and 0.008332±0.00421, respectively. Based on heterogeneity test and Monte-Carlo with 1000 replicates, significant differences were showed for haplotype frequencies of the Persian sturgeon populations (p<0.0001). The obtained results and also F_{ST} based on kimura- 2 parameters method showed that haplotype distribution in different location were significant (p<0.0001). Results of this study determined independent populations of Persian sturgeon and will have noticeable implications for sturgeon conservation genetics in general.

Keywords: *Acipenser persicus*, Mitochondrial DNA, PCR-RFLP, Caspian Sea

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Introduction

The Persian sturgeon, (*Acipenser persicus*, Acipenseridae) is a large, cartilaginous, benthic fish, and endemic in the Caspian Sea. Its life history, characterized by long life span, late age-at-maturity and protracted spawning periodicity, is unique among sturgeon fishes of the Caspian Sea (Putilina and Artyukhin, 1985). Although once abundant throughout their range, severe overfishing in the late 1800s and early 1900s decimated most populations (Vlasenko *et al.*, 1989). Persian sturgeon is a bottom dweller occurring primarily on sand bottoms in the central and southern Caspian Sea, especially along the shores of Iran. *A. persicus* is more distributed in Iranian waters where sea fishing is permitted. (Vlasenko *et al.*, 1989; Birstein *et al.*, 1997). Commercial fishing of Persian sturgeon has diminished in most of its range, but it continues for certain populations and may increase due to the value of its caviar (Moghim *et al.*, 2006; Pourkazemi, 2006). In recent years, Persian sturgeon comprises the largest proportion of the total Iranian commercial catch (Tavakoli *et al.*, 2010) and fishery management agencies in Iran are attempting to facilitate the recovery of Persian sturgeon populations through stocking program (Abdolhay and Baradaran Tahori, 2006).

Todays, few healthy sturgeon populations remain and many anthropogenic factors continue to hamper most conservation and restoration efforts. Among these factors, hydroelectric dams that obstruct upstream access to historic spawning grounds and degrade critical downstream habitats, are the most problematic items (Ludwig, 2006; Moghim *et al.*, 2012; Waldman *et al.*, 2019). The large 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 in IUCN (Vecsei and Artyukhin, 2001; Pourkazemi, 2006) but little is known of its population structure. Thus, the range-wide population genetics of the Persian sturgeon is of interest as a recoverable example of species-wide genetic structuring of an anadromous sturgeon and as a tool for better-informed management of the species.

Mitochondrial DNA has proved to be an excellent tool for examining population genetics, above or below the species level (Avise, 1994). It has emerged as a genetic marker able to discriminate stocks (Billington and Hebert, 1991; Liua and Cordes, 2004; Roques, 2019). Because of the fast evolution and maternal mode of inheritance, mitochondrial DNA (mtDNA) has been widely used to investigate genetic differences and evolutionary history between and/or within species (Avise, 1994; Brown *et al.*, 2008). Variations of the entire mtDNA genome, individual genes, or restriction fragment length

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polymophisms (RFLPs) have been useful in characterizing taxa, establishing phylogenetic relationships, clarifying conspecific hybridizations, identification of hatchery and wild stocks and assessing stocks in many fish species (Billington, 2003; Khoshkholgh and Nazari, 2015; Khoshkholgh and Nazari, 2019).

There were several recent studies on individual mtDNA variability and its suitability for population genetic analysis and phylogenetic relationships of sturgeons. For example, Pourkazemi (1996) and Rezvani-Gilkolaei (1997) stated that the mtDNA ND5/6 gene region is very useful marker for population genetic structure of stellate, *Acipenser stellatus* and Russian sturgeon, *A. gueldenstaedtii* species. In addition, Rastorguev *et al*. (2008) based on Shimodaira and Hasegawa test analyses of several sturgeons, proposed the following classification for mitochondrial genes: very good (ND5, cytb), good (COI, ND4, ATP6), medium (ATP8, COII, COIII, ND1, ND2, ND3, ND4L) and weak (ND6).

Several genetic studies have attempted to elucidate the genetic variation and population structure of the Persian sturgeon in the Caspian Sea. Rezvani-Gilkolaii (1997) investigated the genetic diversity of two wild populations of Persian sturgeon from the western and eastern of Caspian Sea using partial sequence analysis of mtDNA *NADH* 5 gene. Ataei (2004) and Khoshkholgh *et al*. (2013) found extensive genetic variability among populations of Persian sturgeon from south Caspian Sea by using PCR-RFLP technique and microsatellite markers. They found considerable genetic diversity in sampled area of distribution, western and eastern parts of the Caspian Sea. In our previous study, mtDNA PCR-RFLP analysis and mtDNA control region sequences were used to assess the genetic relationships of six Persian sturgeon populations from the south Caspian Sea along the Iranian coast and suggested that the conservation of genetic diversity of this species in the Sefidroud River should be considered (Pourkazemi *et al.,* 2012; Nazari *et al.,* 2013). Furthermore, evidence of significant genetic differentiation in microsatellite allelic frequencies among Persian sturgeon populations suggested that they are reproductively isolated and three distinct populations including Sefidroud River, middle and north Caspian Sea populations were determined (Chakmehdouz Ghasemi *et al.,* 2011; Moghim *et al.,* 2012; Khoshkholgh *et al*., 2013).

Identification of populations and subsequent grouping of genetically similar populations can be used to delineate management units and to help choose donor populations for stocking that will preserve existing genetic structure (Roques *et al.,* 2018; Wirgin *et al.,* 2018). The objective of this study was to compare the genetic diversity among Persian sturgeon samples from different geographical ranges in the Caspian Sea using mtDNA PCR RFLP

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analysis. We present the results in relation to our previous investigations of coast-wide population structure (Pourkazemi *et al.,* 2012) by using previously published data and results from new collections of Persian sturgeon from several other locations.

Materials and methods

Sample collections and mtDNA RFLP analysis

Fin clip samples of the Persian sturgeon were obtained from five sites along the Iranian coast in the south Caspian Sea: Astara-Anzali (Zone 1), Kiashahr-Chaboksar (Zone 2), Noshahr-Sari (Zone 3), Miankaleh-Bandare Torkaman (Zone 4) and Chaboksar-Noshahr (Zone 5), Sefidroud River (SEF), Turkmenistan (TUR), Azerbaijan (AZE), Russian Federation RUS), Kazakhstan (KAZ) which are depicted in Fig. 1. Twelve to thirty one individuals from each locality were collected (Table 1). Total DNA was extracted from fin tissues by standard phenol–chloroform extractions and ethanol precipitations following the method described by Hillis and Moritz (1990) as modified by Pourkazemi (1996) and Nazari et al. (2016). Extracted DNA was checked for concentration using spectrophotometer (Nanodrop ND1000, Germany) and the DNA was subsequently standardized to a specific concentration (i.e. 50 ng/µL for PCR reactions and 100 ng/µL for permanent storage in a DNA archive). The quality of each DNA specimen was confirmed visually on a 0.8% 0.5×TBE agarose gel containing ethidium bromide against a known standard.

Persian sturgeon-specific primers 5′-CCAAGTAGAAGCTATGCATTCA-3′ (forward) and 5′-GGAGGCGAATATTTGTTGA-3′ (reverse) were designed to amplify an approximate 1837 bp of the mtDNA ND5 gene (Rastorguev *et al.,* 2008). For amplification, the following reagents were added to each microtube: 2 µL of template DNA; 5 µL of 10×buffer (100 mM Tris-HCl, pH 8.3, 15 mM MgCl2, 500 mM KCl); 1 µL of each primer (10 pmol); 5 µL of a 2.5 mM solution of each deoxyribonucleoside triphosphate (dNTP); 2.5 units of Taq DNA polymerase (Fermentase). Enough ultrapure water was added to each sample to make a solution of 50 µL. Polymerase chain reaction conditions consisted of 35 cycles of denaturation at 94°C for 1 min, annealing at 58° C for 1 min and extension at 72°C for 1 min (5 min for the last extension only). The amplified samples were subjected to endonuclease digestion using the four-base recognition enzymes *Rsa* I, *Hae* III and *Mbo*I and the five-base recognition enzymes *Hinf* I and *Cfr*13I. Restriction digestion was carried out in a 10 µL volume containing 2 µL of PCR product, 2 units of restriction enzyme, 1 µL of the appropriate buffer and 7 µL of ultrapure water. RFLP digestion was performed in an incubator at 37°C for at least 16 h. Restriction fragments were separated on 6% acryl amid gel, stained with silver staining method and photographed.

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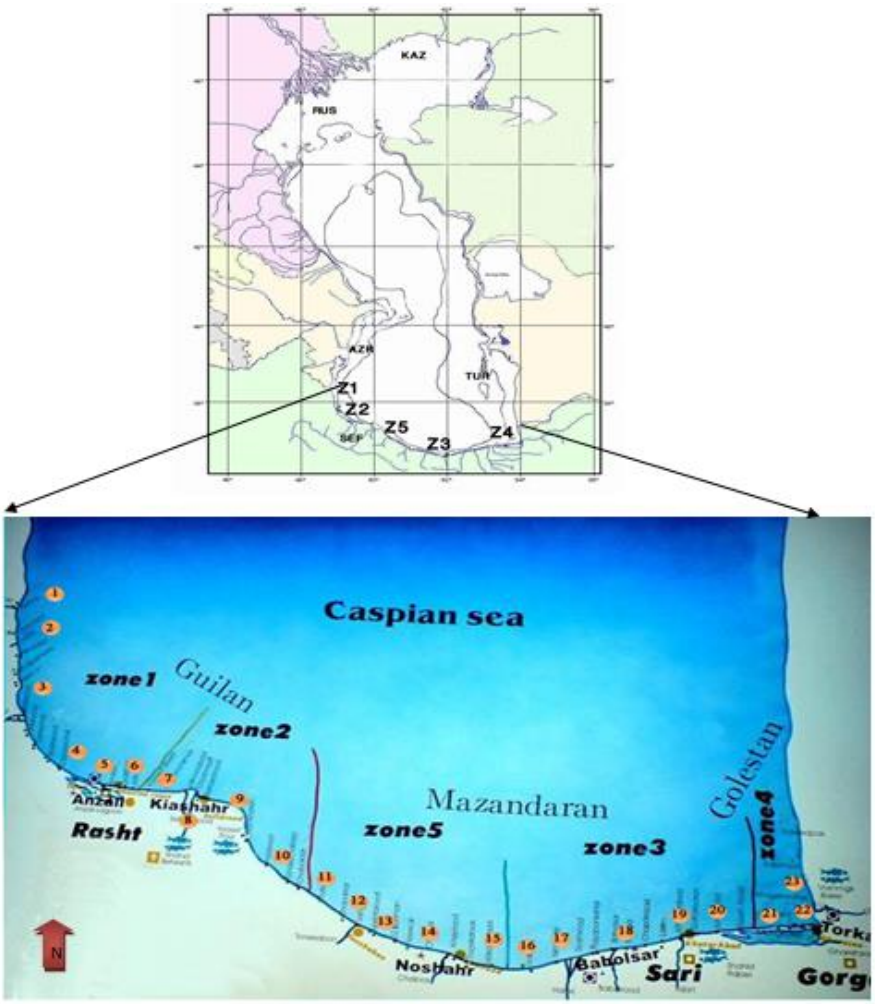

Figure 1: Map showing the locations of the Persian sturgeon sampling sites in the Caspian Sea. Sampling localities (1-23) from south Caspian Sea along the Iranian coast (Fisheries Zone 1-5). SEF, Sefidroud River; TUR, Turkmenistan, AZE, Azerbaijan, RUS, Russian Federation, KAZ, Kazakhstan.

Data analyses

A composite mtDNA haplotype, consisting of five letters that represent the fragment pattern generated by each of the restriction endonucleases was compiled for each individual. The nucleotide diversity (π) in a population was computed by (Nei andTajima, 1981):

$$\pi = 2\sum d_{ij}\,[n(n\text{-}1)]^{-1}$$

where d_{ij} is an estimate of the number of nucleotide substitutions per site between DNA sequences *i* and *j*, *n* is the number DNA sequences and also haplotype diversity (*h*) was calculated in each population. The equation of Nei and Tajima (1981) was used to estimate haplotype diversity:

$$h = 2n\,(1\text{-}\sum x_i^2)(2n\text{-}1)^{-1}$$

where *n* is the sample size, and x_i is the frequency of the haplotype in each population.

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Table 1: Persian sturgeon samples collected for population genetic analysis. Sampling ocalities (1-23) are from south Caspian Sea along the Iranian coast (Fisheries Zone 1- 5).

Region	Map no.	Sampling site	Sample size
Zone 1	1	Astara	8
	2	Lemir	3
	3	Hevigh	5
	4	Kapour-Chal	6
	5	Golshan	4
	6	Jefroud	5
Sefidroud River	7	Sefidroud	27
Zone 2	8	Yousef-Abad	10
	9	Sefidroud	3
	10	Ghasem-Abad	8
Zone 5	11	Iezdeh	8
	12	Nevisi	4
	13	Miroud	5
	14	Khazar-Abad	3
	15	Amir-Abad	5
	16	Shiroud	8
Zone 3	17	Nashtaroud	4
	18	Noursar	5
	19	Chalous	4
	20	Hasan-Abad	6
Zone 4	21	Turkeman &Faridpak	7
	22	Mian-Ghaleh	9
	23	Khajeh-Nafas	7
Turkmenistan			17
Azerbaijan			12
Russian Federation			28
Kazakhstan			14
Total			225

The estimates of nucleotide divergence (Nei and Tajima, 1981) between the mtDNA haplotypes and the samples examined were taken as standard genetic distances. The estimates were used for phylogenetic analysis performed with an unweighed pair group method with arithmetic mean (UPGMA) algorithm. Clustering robustness was estimated using bootstrap (100 iterations) (Felsenstein, 1985). Estimates of nucleotide divergence and dendrogram topology were made using the PAUP version 4.0b10 software package (Swofford, 2002).

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The main genetic variation indices were estimated using the REAP (McElroy *et al.,* 1992) and ARLEQUIN version 2.0 (Schneider *et al.,* 2000) software packages. Heterogeneity of haplotype frequencies between each sample pair was evaluated using the Monte Carlo method (1000 pseudorandom replicates (Roff and Bentzen, 1989) and F_{ST} statistics (Weir and Cockerham, 1984). Quantitative estimates of the geographic subdivision of mtDNA variation were performed using the AMOVA method, where molecular variance was partitioned into three hierarchical levels, including between-groups, between-population within groups, and among haplotype within population components (Excoffier *et al.,* 1992). To test statistical significance of the hierarchic components of variance, the corresponding F-statistics criteria were calculated (Weir and Cockerham, 1984).

Results

Variation within samples in total 225 individuals from different locations amplified successfully in the mtDNA *ND*5 gene. Thirty two different composite haplotypes were revealed with the number of haplotypes in each population sample ranging from 6 to 13 and 16 of them were private, that is, present in only one population sample (Table 2). Private haplotypes were found at very low frequencies (<0.005). The most common composite haplotype was haplotype 1 (AAAAA) with a frequency of 0.4177 in the pooled sample. Frequencies of the non-private composite haplotypes varied among the samples. For example, haplotype 1 was found in all samples (Table 2). In the remaining samples, it varied from 0.093 in Kazakhstan to 0.46 in the Zone 1 (Astara-Anzali) samples. Haplotype 2 (AAABA) showed a frequency distribution with 0.15 in Zone 1 samples, a frequency of 0.03 in Sefidroud River and Kazakhstan samples, and absent in Azerbaijan and Russian samples. The geographic distributions of all haplotypes in each sample are given in Table 2.

The mean haplotype diversity (*h*) and nucleotide diversity (π) were 0.7610±0.046 and 0.008332±0.00421, respectively. The samples with the highest haplotype diversity were Russia (0.8636±0.0626) and Sefidroud River (0.8144±0.0561). On the other hand, the population samples with the lowest variability were from the southern part of the Caspian Sea in Zone 1 (0.6834±0.0273) to Kazakhstan, samples of northern part of the Caspian Sea with haplotype diversities of 0.7752±0.0273 (Table 2).

Table 3 presents the results of using F-statistics method to estimate the genetic differentiation of the populations examined. The estimation was based on the number of nucleotide substitutions. Pairwise estimates of F_{ST} values ranged between 0.010 and 0.681 (Table 3). The lowest F_{ST} occurred between samples from Zone 1 and Zone 2 of Iranian coast.

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Table 2: Nucleotide diversity (π) and haplotype diversity (h) values and geographic distribution of Persian sturgeon mtDNA restriction fragment length polymorphism (RFLP) composite haplotypes derived in this study. Haplotypes are composite scores for fragment patterns produced by digestion with *Rsa* I, *Hinf* I, *Hae*III, *Mbo* I and *Cfr*13I, respectively. Abbreviations for collections are defined in Fig. 1. Numbers in parentheses indicate numbers of individuals.

		Collection										Total
		Zon1 (31)	Zon2 (21)	Zon3 (25)	Zon4 (23)	Zon5 (27)	SEF (27)	TUR (17)	AZE (12)	RUS (28)	KAZ (14)	225
	π	0.0034	0.0048	0.0059	0.0088	0.0061	0.0153	0.0056	0.0062	0.0188	0.0051	
	h	0.683	0.716	0.721	0.764	0.737	0.814	0.701	0.765	0.863	0.775	
No.	Haplotypes											
1	AAAAA	15	12	14	11	14	6	9	5	5	3	94
2	AAABA	5	3	2	2	4	1	2	-	-	1	20
3	AAAAB	4	2	1	1	3	-	2	1	1	-	15
4	AACAB	1	1	0	2	1	10	1	-	-	-	16
5	AABBB	-	1	2	-	2	-	1	-	-	-	6
6	AABAB	3	-	3	-	2	1	1	-	-	-	10
7	BBBAA	-	-	-	-	-	-	-	-	-	1	1
8	BAACA	-	-	-	2	-	-	-	-	-	-	2
9	ABABD	-	-	-	-	-	-	-	1	2	-	3
10	ABBAA	2	2	-	1	-	-	-	-	-	-	5
11	BAABC	-	-	-	-	-	-	-	-	1	-	1
12	AACAA	-	-	-	-	-	2	-	-	-	-	2
13	ABBBA	-	-	-	-	-	-	-	1	9	2	12
14	ABAAB	-	-	1	1	-	1	1	-	-	-	4
15	AAACC	-	-	-	-	-	-	-	-	1	-	1
16	ABAAC	-	-	-	-	-	-	-	-	1	-	1
17	AACBA	-	-	-	-	-	1	-	-	-	-	1
18	ABABA	-	-	-	-	-	-	-	3	-	-	3
19	ABCAA	-	-	-	-	-	2	-	-	-	-	2
20	BAAAA	1	-	1	2	1	-	-	-	-	-	6
21	BBAAD	-	-	-	-	-	-	-	-	1	-	1
22	BBBAB	-	-	-	-	-	1	-	-	-	-	0
23	BAABB	-	-	-	-	-	-	-	-	1	5	6
24	BAAAC	-	-	-	-	-	-	-	-	1	-	1
25	BACAA	-	-	-	-	-	-	-	-	1	-	1
26	BACCB	-	-	-	1	-	-	-	-	-	-	1
27	CCAAA	-	-	-	-	-	-	-	-	-	1	1
28	ABBCA	-	-	1	-	-	1	-	-	-	-	2
29	BAAAB	-	-	-	-	-	-	-	1	2	1	4
30	BAAAD	-	-	-	-	-	-	-	-	-	1	1
31	BACAB	-	-	-	-	-	1	-	-	-	-	1
32	ABBCD	-	-	-	-	-	-	-	-	-	1	1

Thus, three distinct genetic groups were identified (Sefidroud River, Russia and all others) and there may be four (Sefidroud River, Russia, Zone 4 and all others). The topology and bootstrap values (42–96) of Fig. 2 support the existence of these three groups, as does the high level of structure for the Sefidroud River and Russia population (Table 3).

Table 4 presents the results of the heterogeneity of the Persian sturgeon populations test (χ^2) to estimate the genetic differentiation of the populations examined. Significant differences were found in 19 of the 46 pairwise comparisons, most involving the Sefidroud River and Russian Federation samples versus those of the other locations.

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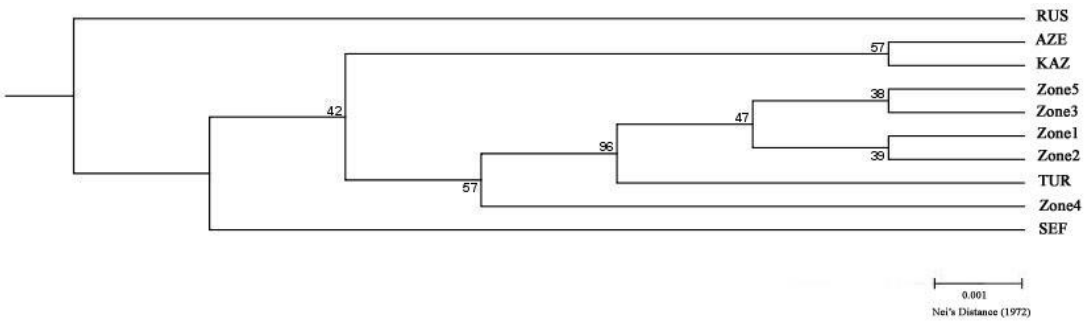

Figure 2: An UPGMA tree of the population genetic distances for the mtDNA ND5 data from Persian sturgeon of 10 Caspian Sea regions. Bootstrap values are given at each node.

Table 3: Pairwise F_{ST} values are illustrated above the diagonal. Abbreviations for collections are defined in Fig. 1. Pairwise estimates of mtDNA divergence (% of nucleotide substitutions) are illustrated below the diagonal

Location	Zon1	Zon2	Zon3	Zon4	Zon5	SEF	TUR	AZE	RUS	KAZ
Zon1	-	0.0010	0.0096	0.0264	0.0081	0.6031	0.0053	0.0002	0.6811	0.0087
Zon2	0.00214	-	0.0013	0.0097	0.0040	0.6136	0.0198	0.0042	0.6607	0.0063
Zon3	0.00201	0.00149	-	0.0025	0.0041	0.4802	0.0041	0.0098	0.4806	0.0306
Zon4	0.00215	0.00318	0.00264	-	0.0065	0.4307	0.0062	0.0131	0.3570	0.0615
Zon5	0.00178	0.00133	0.00441	0.00378	-	0.3231	0.0048	0.0392	0.4176	0.0154
SEF	0.01456	0.01344	0.01272	0.01176	0.01109	-	0.4561	0.5263	0.5631	0. 4096
TUR	0.00426	0.00366	0.00697	0.00522	0.00711	0.01366	-	0.0086	0.4133	0.0199
AZE	0.00912	0.00684	0.00794	0.00743	0.00803	0.01275	0.00924	-	0.3032	0.1016
RUS	0.02331	0.02163	0.02010	0.01885	0.01842	0.01726	0.02115	0.01995	-	0.4094
KAZ	0.00863	0.00788	0.00311	0.00216	0.00874	0.01299	0.00354	0.00368	0.01733	-

The χ^2 test of haplotype frequencies also revealed that the population of Sefidroud River and the population of Russian Federation are significantly differentiated from one another (p<0.0001). The significant differences between all population pairs are given in Table 4. As shown in Table 4, no significant differences were found between most of compared pairs. It was shown that the main contribution to the heterogeneity of the population set is made by the Persian sturgeon samples from the Sefidroud River and Russian populations.

The divergence matrix of the Persian sturgeon populations based on nucleotide sequences is presented in Table 4. The maximum difference (about 0.02% of nucleotide substitutions) was recorded between the samples come from Russian and Zone 1. A mediocre divergence in the UPGMA tree generated from the pairwise population genetic distances was indicated between the three southern populations and those to the north Caspian Sea (Fig. 3). The most differentiated cluster included populations of the Sefidroud River and Russian populations.

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Table 4: Pairwise comparison of Monte Carlo based Chi-square values for Persian sturgeon populations from 10 Caspian Sea locations (*P*-values in parentheses).

Location	Zon1	Zon2	Zon3	Zon4	Zon5	SEF	TUR	AZE	RUS	KAZ
Zon1	-									
Zon2	1.32 (0.5504)	-								
Zon3	7.56 (0.4456)	8.32 (0.4124)	-							
Zon4	24.53 (0.0451)	18.74 (0.0948)	15.17 (0.1563)	-						
Zon5	14.89 (0.1982)	1.02 (0.5689)	20.00 0.0805	18.63 (0.0927)	-					
SEF	106.8 (0.0000)	94.5 (0.0000)	89.62 (0.0000)	77.14 (0.0000)	58.65 (0.0000)	-				
TUR	17.16 (0.1272)	10.23 (0.2653)	13.1 (0.1894)	27.2 (0.0579)	15.6 (0.1469)	95.00 (0.0000)	-			
AZE	34.28 (0.0520)	27.3 (0.0584)	19.90 (0.0840)	22.3 (0.0682)	25.74 (0.0641)	76.70 (0.0000)	36.14 (0.0502)	-		
RUS	134.00 (0.0000)	125.01 (0.0000)	119.10 (0.0000)	111.02 (0.0000)	109.04 (0.0000)	105.26 (0.0000)	115.04 (0.0000)	113.32 (0.0000)	-	
KAZ	28.60 (0.0538)	24.00 (0.0759)	12.98 (0.0981)	11.05 (0.1206)	26.22 (0.0504)	76.09 (0.0000)	7.08 (0.4016)	10.11 (0.1095)	108.66 (0.0000)	-

The collections from Turkmenistan and fisheries zone 1 to zone 5 formed a clade. The collections from Azerbaijan and Kazakhstan also formed a weak-supported clade and the node joining these collections and those from Turkmenistan and fisheries zone 1 to zone 5 was not well supported by bootstrapping. 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, zones 1 to 5-Turkmenistan-Azerbaijan- Kazakhstan (Fig. 3).

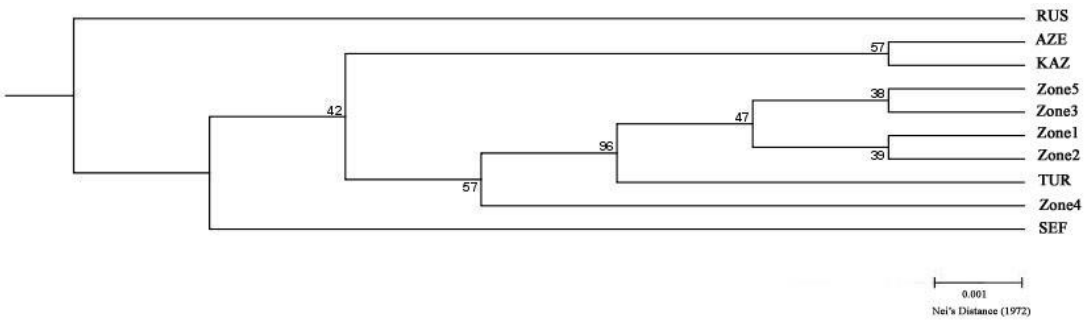

Figure 3: An UPGMA tree of the population genetic distances for the mtDNA ND5 data from Persian sturgeon of 10 Caspian Sea regions. Bootstrap values are given at each node.

The degree of genetic differentiation between all sample pairs was tested with AMOVA (Excoffier *et al.,* 2005). To make quantitative estimates of the values of genetic differences, total molecular variance of haplotype frequencies was subdivided into three hierarchical levels (Table 5). The analysis showed that molecular divergence of Persian sturgeon from the

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Caspian Sea was mostly distributed among haplotypes within populations (61.63%) (Table 5). The AMOVA also partitioned of total 36.33% genetic variation among the groups and had the low level of variance among populations within region (2.24%, $p>0.114$) However, according to the data obtained, the hierarchical levels of geographic subdivision isolated were statistically significant (95% level significance). It can be thus concluded that the most part of the mtDNA intraspecific variation is determined by differences among the haplotypes within a single population.

Table 5: Hierarchic search for haplotype differences in the Persian sturgeon.

Source of variation	df	Percentage of variation	Fixation indices	*P*
Among groups	2	36.13	F_{SC} =0.0132	<0.001
Among populations within groups	7	2.24	F_{CT} =0.0016	>0.141
Among haplotype Within population	215	61.63	F_{ST} =0.0332	<0.001
Total	224			

Discussion

The results of F_{ST} and analysis of molecular variance (AMOVA) demonstrated that samples between Sefidroud in the south Caspian Sea and Russia are significantly different ($p<0.0001$). Therefore, two distinct populations were identified. Heterogeneity test of Persian sturgeon populations for haplotype frequencies and Monte-Carlo with 1000 replicates in RFLP analysis of *ND*5 gene showed significant differences ($p<0.0001$) and these results showed that haplotype distribution in different location were significant and populations of Sefidrud River and Russia revealed statistically significant differences ($p<0.0001$). Detecting genetic variation in mtDNA between organisms mainly depends on the evolutionary rate of genes and the number of nucleotide bases surveyed (Moritz *et al.*, 1994; Billington, 2003; Barmintseva *et al*., 2017). We found that the large mtDNA gene region (*ND*5 gene) has the high haplotype diversity.

Pourkazemi *et al.*, (2012) indicated that intraspecific population structure between Persian sturgeon using RFLP in mtDNA NADH5 gene and the number of haplotypes (17 haplotypes) which is clearly showed a high level of polymorphism in this region of mitochondrial DNA. In the previous study by Ataei (2004), for which the analyzed segment of control region is almost the medium size as that for other genes (such as cytochrome *b*) and no significant differences in variation were observed. Other mitochondrial genes such as cytochrome *b* are intraspecifically conserved but contain enough interspecific heterogeneity to produce species-specific patterns (Ludwig and Kirschbaum, 1998; Panagiotopoulou *et al.*, 2017). In addition, several differences have been reported among sturgeon species that inhabit in the Caspian Sea by

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researchers using the nucleotide variation of *ND*5/6 and control region segments such as Stellate sturgeon, *A. stellatus*, (Pourkazemi, 1996), ship sturgeon, *A. nudiventris*, (Qasemi *et al.*, 2004), and Russian sturgeon *A. gueldenstaedtii* (Pourkazemi, 1996, Pourkazemi *et al.*, 1999; Rezvani-Gilkolaei, 1997).

Sturgeon species in the Caspian Sea are characterized by weak or mediocre population differentiation on a large spatial scale using mtDNA variation (Pourkazemi *et al.*, 1999; Khoshkholgh *et al.*, 2011). However, even in those species contradictory results have been obtained. For instance, Stellate sturgeon have shown a lack of genetic differentiation within the south Caspian Sea populations (Pourkazemi, 1996), while pronounced genetic differentiation has been found between south populations and Ural River populations of Ship sturgeon (Qasemi *et al.*, 2004). Another example is the Persian sturgeon, for which no genetic differentiation has been reported by researchers using partial mtDNA *ND*5 gene sequencing analysis (Rezvani, 1997), while microsatellite loci has revealed significant genetic heterogeneity between two nearly adjacent populations (Zone 3 and Turkmenistan) in the south Caspian sea as well as in middle and north parts of the Caspian sea (Khoshkholgh *et al.*, 2013). PCR- RFLP analysis of *ND*5/6 genes in the Russian sturgeon, *A. gueldenstaedtii* also revealed significant mtDNA variation, with high haplotype diversity between southwest and southeast parts of the Caspian Sea. In case of Persian sturgeon, the observed genetic differentiation in the Sefidroud River and Russia population despite the marine habits of the species is consistent with homing behavior. In light of the natural history of the Persian sturgeon, more populations with defined population structure are expected; however, the use of finer scale markers should be considered in order to detect such levels of heterogeneity (Wirgin *et al.*, 2018; Roques, 2019).

Ataei (2004) by RFLP analysis of Persian sturgeon mtDNA had found haplotype frequency differences among three collections in regions in South Caspian Sea with no significant differences (p>0.05). However, this was not true for the present mtDNA RFLP study in which a greater number of haplotypes was revealed and additional populations were included. Indeed, the 32 composite haplotypes we found were more than the numbers identified by Ataei (2004). Throughout the geographic regions included in this study, the Persian sturgeon shows considerable genetic diversity and differentiation among populations. The three coarse regional divisions of sturgeon populations (northern, middle, southern parts of the Caspian Sea) that previously identified by microsatellite analysis (Chakmehdouz Ghasemi *et al.*, 2011; Pourkazemi *et al.*, 2012; Khoshkholgh *et al.*, 2013) are supported by this study. The identification of far

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more haplotypes, the addition of samples from more estuaries on the different coasts, and larger sample sizes in rivers in the Caspian sea have provided additional resolution in distinguishing populations.

The results of AMOVA supported our hypothesis of available genetic structure. Most of the genetic structure could be explained by the differentiation between the clades, and their vicariate geographical distribution. The population structure found in the Persian sturgeon was relatively strong in comparison to the Stellate sturgeon (Pourkazemi *et al.,* 1996; Florescu *et al*., 2019), and ship sturgeon (Qasemi *et al.,* 2004). The population of Persian sturgeon in the Sefidroud River is genetically distinct from those in tributaries of the Volga, Ural, and Kura rivers. Significant haplotype frequency differences exist between samples from the Sefidroud River and those from other localities. The genetic structuring of anadromous Persian sturgeon revealed by mtDNA haplotype frequencies is consistent with the obtained results from microsatellite analysis (Chakmehdouz Ghasemi *et al.,* 2011; Khoshkholgh *et al.,* 2013). One possible explanation for this is that the Persian sturgeon is anadromous species and migrates for spawning sites in different rivers entering the Caspian Sea (e.g. Sefidroud, Volga, Tajan, Gorgan, Ural, and Kura), and these rivers may represent isolated populations in each geographic region. In the Azerbaijan and Kazakhstan populations, χ^2 analysis revealed a deficit of heterogeneity (Table 4) and this phenomenon can be explained by the Wahlund effect (Balazik *et al*., 2017; Gilbert and Whitlock, 2015), since this population includes spawning subpopulations in Ural and Kura. Since the samples were collected in the sea, identification of individual fish by the origin was impossible. It seems likely that deficit of heterogeneity in some populations was associated with a dramatic decrease of its number during the last decades, as well as by inbreeding, as a result of the limited number of producers upon artificial breeding (Tavakoli *et al.,* 2010; Khoshkholgh *et al.,* 2013). Although a further assessment is required by collecting the samples from the north and middle part of the Caspian Sea regions.

Across all collection sites in the south Caspian Sea, there was no significant subdivision at the level of individual populations. F_{ST} values of these collections were below 0.05 except for collections from Zone 4 and these values are generally considered to indicate little genetic differentiation. Although nearly the miniscule values of F_{ST} among pairwise comparisons were observed between geographically distant samples of the Persian sturgeon in the south Caspian Sea, there was no significant pattern of isolation by distance in the data. The absence of apparent genetic structure among fisheries zones along the south Caspian Sea populations based on RFLP analysis of mtDNA *ND*5 gene may

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reflect high gene flow and homogenization within this area. The proximity of Persian sturgeon populations would promote the admixture of these populations. Some unique haplotypes in locations like Zone 4, Azerbaijan and Kazakhstan may reflect some differentiation among these populations, but the presence of these haplotypes is not extensive enough to be considered subpopulations of the species. Moreover, the lower number of total haplotypes in each collection and the absence of statistical differences in haplotype frequencies between the five fisheries zones specimens in south Caspian Sea may be due to a higher level of gene flow between these populations or population bottlenecks that might have reduced haplotypic diversity by eliminating some rarer haplotypes.

The haplotypic patterns observed and significant nucleotide divergence among Persian sturgeon populations may be related to both the life history and contemporary gene flow. According to the life history style of sturgeon fishes, the Persian sturgeon is long lived species and lives to the age of 40 and reaching 200 to 220 cm in length with a weight of 60-65 kg (Vlasenko *et al.,* 1989). Around 57% of the sturgeon catch in the past (1991) as well as nowadays (91% in 2010) is belonging to the Persian sturgeon (Tavakoli *et al.,* 2010). Therefore one reason for the high diversity of this species could be due to the large population size and long age of fish, some rare haplotypes that have been observed in the present study may be derived from an old lineage that presented within the populations. Because the Persian sturgeon also has two races of spring and autumn (Vlasenko *et al.,* 1989), this is another possible explanation of the haplotype frequencies differences between the collections. The presence of two races of this species was reported in brood fish migrating to the Sefidroud River for the first time by Ataei (2004). In addition, Putilina and Artyukhin (1985) stated that the spring race of the Persian sturgeon can be distinguished from the autumn race in the presence of two specific antigens.

In comparison of all 10 samples, the heterogeneity test of the Persian sturgeon showed significant differences. Our results indicate that, in spite of the significant genetic heterogeneity in mtDNA structure of Persian sturgeon on the range part examined, population divergence in general is low, although in some cases the differences between populations were statistically significant. More recent studies on Stellate and Russian sturgeon revealed lower (0.011%) and higher (0.052%) level of divergence, respectively, in comparison with the percentage of divergence in Persian sturgeon (around 0.02%).

In conclusion, the significant values of F_{ST} observed in the Sefidroud River and Russian populations and also the existence of unique haplotypes in the most surveyed populations are

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consistent with some degree of structure among the analyzed populations. Although the pairwise F_{ST} values support the existence of only three groups (Sefidroud River and Russia population and the other populations), conservative management may recognize four groups (Sefidroud River and Russia, Zone 4, and the other populations) based on the topology of the UPGMA tree. These preliminary observations are proposed to guide management and promote further studies to clarify the geographic and temporal details of this apparent structure. The results of this study show that mitochondrial DNA is a powerful tool for determination of the genetic structure among Persian sturgeon samples, which is important for a proper management policy designed to protect and to cultivate this species. Furthermore, a comparative analysis involving both mtDNA and finer scale nuclear markers (microsatellites or DNA sequencing) will be necessary to corroborate the observed structure and establish proper management and conservation programs for the Persian sturgeon along the Caspian Sea.

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