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Population Genetic Structure of Stellate Sturgeon (*Acipenser stellatus* Pallas, 1771) in the South Caspian Sea Using Microsatellite Markers

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Abstract: In this study, 197 samples of adult stellate sturgeon from four fishery regions were collected. DNA was extracted using 15 pairs of microsatellite primers, Polymerase Chain Reaction (PCR) was conducted. DNA bands were analyzed using Biocapt and GenAlex software package. Out of 15 microsatellite primers, 11 loci were produced, in which 10 of them were polymorphic and 1 monomorph. Analyses revealed that average of 13 alleles per locus (range 8 to 18 alleles per locus). Average observed and expected heterozygosity were 0.650 and 0.855 and significant genetic differences between 4 regions were observed ($p \leq 0.01$). Deviations from Hardy-Weinberg equilibrium were in most cases. Maximum genetic difference were observed between regions 2 and 4 ($F_{ST} = 0.063$, Nm = 3.728). These results indicate that at least, 3 populations of stellate sturgeon exist in the South Caspian Sea. Population of stellate sturgeon in region 2 where Sefidrud drainage is located was consider as independent population, therefore management of this unique stocks for restocking and conservation of gene pools is highly recommended.

Key words: Stellate sturgeon, population genetic, microsatellite, Iranian coastline of the South Caspian Sea

INTRODUCTION

Stellate sturgeon (*Acipenser stellatus*) is one of the shard stock of the Caspian states that also distributed in the Black and Azov Seas (Keyvan, 2003). Stellate sturgeon populations play an important role in biodiversity and commercial harvest of the Caspian Sea and they were listed as a endangered species IUCN Red list assessment (Pourkazemi, 2006) and since April, 1998 its trade regulated by the Convention on International Trade in Endangered Species Founa and Flora (CITES). Overfishing and increasing illegal unaccounted catches are the main causes to decrease the stocks and adult spawners (Pourkazemi, 2006). The stocks of *A. stellatus* in the Caspian Sea are maintained through natural spawning as well as artificial breeding, which necessitates the conservation of genetic diversity of this species. Therefore identification of population and races can be considered the first measures for management and conservation of sturgeon species in the Caspian Sea (Pourkazemi, 1996). Two different ecological from of stellate sturgeon in the North (*Acipenser stellatus stellatus*) and the South (*Acipenser stellatus cynensis*) were reported in the Caspian Sea regions (Keyvan, 2003). They are identical on spawning time, growth rate and immunological characters (Keyvan, 2003). Pourkazemi (1996) investigated genetic variation of stellate sturgeon (*Acipenser stellatus*)

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population in the south Caspian Sea with allozyme electrophoresis and mtDNA using RFLP methods. Shabani (2005) studied mtDNA variation of stellate sturgeon (*Acipenser stellatus*) population in South Caspian Sea using RFLP analysis of PCR Amplified ND 5/6 gene regions and they found low genetic variation and non-significant difference in haplotype frequency. It has been concluded that mitochondrial DNA technique was not powerful techniques to detect genetic variability sufficiently population-differentiation in order to help management units of stellate sturgeon.

In population genetic studies, more genetic variability would provide a better means by which to compare sturgeon collection from different localities differentiation (McQuown *et al*., 2003). Microsatellites are hyper variable, co dominant nuclear DNA markers in which variation is partitioned in one to five base pair repeat motifs (Zajc *et al*., 1997).

In recent years, microsatellite markers as a reliable methods, have been applied for many population as well as phylogenetic studies, this is because microsatellites typically have a higher level polymorphism than traditional nuclear loci. In addition, DNA for microsatellites analysis can be easily extracted from tissues obtained by non-lethal sampling (fins, hair, faces), which is essential when working with threatened or endangered species (McQuown *et al*., 2003). Several studies have been investigated on population genetic structure of variance Acipenseriformes species: on *Acipenser*, *Huso* and *Scaphirhynchus* (Ludwig *et al*., 2001); *A. transmontanus* (Smith *et al*., 2002); *A. sinensis* (Zhu *et al*., 2002); *A. oxyrinchus oxyrinchus*, *A. oxyrinchus desotoi*, *A. brevirostrum* (Waldman *et al*., 2002); *A. oxyrinchus* (Wirgin *et al*., 2002); *A. fulvescens* (McQuown *et al*., 2003); *A. transmontanus* (Rodzen *et al*., 2004); *A. medirostris* (Israel *et al*., 2004); *A. sinensis* Gray (Zhao *et al*., 2005); and in Caspian Sea Sturgeon species. On stellate sturgeon (Pourkazemi, 1996; Shabani, 2005); on Russian Sturgeon (Pourkazemi, 1996; Pourkazemi *et al*., 1999; Rezvani, 1997; Khoshkholgh, 2007); on Beluga (Rezvani, 1997) and on Ship sturgeon (Qasemi *et al*., 2006; Safari, 2006).

The development of management plans and implementation of actions to restore stellate sturgeon within its native stocks can useful from an understanding of the genetic diversity of its populations. This information is helpful in choosing donor populations to use as sources of reintroduction and in formulating restoration goals regarding population structure. Therefore in this study, the population structure of stellate sturgeon from four regions in Iranian coastline of the South Caspian Sea was investigated. The objective of the present study were to analysis the population genetic structure genetic diversity and identify individual populations of stellate sturgeon using microsatellite method.

MATERIALS AND METHODS

Samples Collection

Totally 197 samples (2-3 g dorsal fin tissues) of adult stellate sturgeon were collected from four regions including 52 samples from region 1(Astara-Anzali), 43 samples, from region 2 (Kiyashahr-Ramsar), 50 samples from region 3 (Nowshahr-Babolsar), 52 samples from region 4 (Miyankale-Gomishan) in 2006 by the International Sturgeon Research Institute from South Caspian Sea in Iran (Fig. 1).

DNA Extraction

Genomic DNA was extracted from fin tissue following the method described by Hillis and Moritz (1990) with some modifications (Pourkazemi, 1996). Approximately 100 mg of fin tissue from the dorsal fin was cut into small pieces. These pieces were then placed in 1 mL STE buffer (10 mM Tris/HCl, pH 8.0; 50 mM EDTA, 200 mM NaCl and 0.5% SDS) and digested with 0.5 mg mL^{-1} proteinase K at 55°C overnight. The resulting solution was centrifuged and the DNA in supernatant that was precipitated with ethanol and dissolved in TE buffer. DNA was purified using the

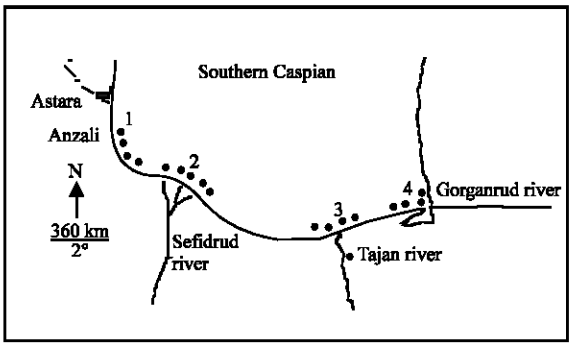

Fig. 1: Map shows the sampling sites of stellate sturgeon where 1, 2, 3 and 4 represent each region and shows sturgeon fishing station

Table 1: Loci, repeat motif, primers sequence, gene bank number and primer sources used at present study in four stellate sturgeon collections

No.	Loci	Repeat motif	Primer sequence	Gene bank No.	Primer sources
1	LS-19	$(TTG)_9$	F-CATCTTAGCCGTCTGTGGTAC	U72730	May *et al.* (1997)
			R-CAGGTCCCTAATACAATGGC		
2	LS-34	$(GTT)_{10}$	F-TACATACCTTCTGCAACG	U72733	
			R-GATCCCTTCTGTTATCAAC		
3	LS-39	$(GTT)_{10}$	F-TTCTGAAGTTCACACATTG	U72734	
			R-ATGGAGCATTATTGGAAGG		
4	LS-54	$(GATA)_6$	F-CTCTAGTCTTTGTTGATTACAG	U72735	
		$(GACA)_7$	R-CAAAGGACTTGAAACTAGG		
5	LS-69	$(TATC)_{13}$	F-ATCTGAATTGANTTTCGTG	U72740	
			R-TTGGATACTGTGTTCCAAC		
6	LS-57	$(GAA)_{29}$	F-GCTTGGTTGCTAGTTTGC	U72736	
			R-GTACAGATGAGACCAGAGGC		
7	LS-62	$(GACA)_7$	F-GATCAGGAGGGCAGAGNAAC	U72738	
			R-CCCTGGATTTGAATTAACAG		
8	LS-68	$(TATC)_{13}$	F-TTATTGCATGGTGTAGCTAAAC	U72739	
			R-AGCCCAACACAGACAATATC		
9	Spl-104	$(TCTR)_{12}$	F-TTATATGGGTGGGGTGGATG	AF276173	McQuown *et al.* (2000)
			R-TCCTCTTTGGCATTTGTTCC		
10	Spl-105	$(TAGA)_{12}$	F-GCGATTTGATTGGCTCTTGT	AF276174	
			R-GGCACTGAATAAATGGACCG		
11	Spl-113	$(AGAT)_{14}$	F-TCCCACATGGCTTGTATTGA	AF276182	
			R-ACCACACCATGCGTCATAAG		
12	Spl-163	$(GATA)_{17}$	F-TGCTTGTAAACTGCCCCACT	AF276205	
			R-CCACATGCAGTTTGAGCTGC		
13	Spl-168	$(TATC)_{18}$	F-CACTGATTCGCTACAACCGT	AF276210	
			R-AGAAGGACTTGCAGTCCGAA		
14	Spl-170	$(GAT)_5$	F-GGACGCACTAGACAGGCTTT	AF276213	
		$(ATAG)_{11}$	R-CACCAAACACAGCAGATTTCA		
15	Spl-173	$(TCTA)_{10}$	F-GGCTTTTGTCTGAAACGTCC	AF276216	
			R-TGGTGTGTCATTTTGAAGGC		

phenol-chloroform extraction. The quality and concentration of DNA were assessed by agarose gel electrophoresis and spectrophotometry (model CECIL CE2040) stored at -20°C until use.

PCR Profiles and Primer Sequences

Nuclear DNA was amplified using 15 microsatellites primers designed for *Acipenser* and *Scaphirhynchus* (May *et al*., 1997; McQuown *et al*., 2000, Table 1). Polymerases Chain Reaction (PCR) condition for each primer set were optimized for stellate sturgeon. Experimental condition tested included varying the annealing temperature (49-61.2°C), $MgCl_2$ concentration (1-2.5 mM),

dinucleotide triphosphate concentration (200 μM), 1 U *Taq* DNA polymerase and primer concentration (0.5-1 pμ) and approximately 100 ng of template DNA. All reactions were performed in 20 μL.

PCR products were separated on 6% polyacrylamid gels (29:1 acrylamid:bis-acrylamid; 1X TBE buffer) and followed by silver-staining. Gels were run at 170 V for 2 h and 30 min. Alleles were sized using BioCapt software and each gel contained an allelic ladder (50 bp) to assist in consistent scoring of alleles.

Data Analysis

Allelic frequencies, observed and expected heterozygosities, genetic distance (Nei, 1978), genetic identity (Nei, 1978) were computed in GeanAlex 6.0 software (Peakall and Smouse, 2005). This package was also used to calculate F_{ST} and R_{ST}, Nm, Hardy-Weinberg (HW) tests of equilibrium, AMOVA (Analysis of Molecular Variance). The dendrograms of genetic distance (Nei, 1972, 1978) UPGMA computed in TFPGA (version 1.3).

RESULTS

Amplification and Banding Patterns

Out of 15 sets of microsatellite primers, four sets (LS-69, LS-57, LS-62 and Spl-168) have not shown any flanking sites on stellate sturgeon genome. Eleven sets of primers were successfully amplified where one sets (Spl-113; Table 2) produce 2 loci and one set (LS-39) showed monomorphic pattern. Therefore totally 11 loci were investigated at present study. All microsatellite primers where able to produce DNA bands displayed a characteristic disomic banding pattern, where heterozygous individuals showed two bands of equal intensity on the gels and some times with one band darker than the others. These banding patterns may be a result of the presence of a homologous locus that is detected under certain PCR conditions.

Genetic Variation Within Sampling Sites (Region)

The average number of alleles found at each locus was 13 and ranged from 8 (Spl-105) to 18 (Spl-113b) alleles (Table 3). The total number of alleles found in each population ranged from 122 (region two) to 154 (region four).

Out of 202 observed alleles, 128 occurred at frequencies of <0.05 in all samples. Spl-170 showed the highest allele number (30 allele) of which 24 alleles at frequencies of <0.05 and LS-34 showed the

Table 2: PCR condition and reaction, locus and allele size (bp) on stellate sturgeon

Locus	Actual size (bp)	Reaction consistence	Cycling condition
LS-19	132-213	200 μM each dNTPs; 1pM each primer; 1.6 mM $MgCl_2$; 2 U/Taq.	94°C/3 m [94°C/30 sec; 56°C/30 sec; 72°C/30][35] 72°C/5 m
LS-34	132-180	2 mM $MgCl_2$	[58°C][35]
LS-39	120	2 mM $MgCl_2$	[58°C][35]
LS-54	152-224	2 mM $MgCl_2$	[59°C][35]
LS-69	No amplification	1 mM $MgCl_2$	[49°C][35]
LS-57	Multiple bands and smear	1 mM $MgCl_2$	[61°C][35]
LS-62	No amplification	2 mM $MgCl_2$	[59.5°C][30]
LS-68	104-160	1 mM $MgCl_2$	[61.2°C][35]
Spl-104	184-248	2.5 mM $MgCl_2$	[57°C][25]
Spl-105	104-180	1.25 mM $MgCl_2$	[58°C][35]
Spl-113	160-212, 260-348	2.5 mM $MgCl_2$	[59°C][25]
Spl-163	160-244	2.5 mM $MgCl_2$	[56°C][35]
Spl-168	No amplification	2.5 mM $MgCl_2$	[49°C][35]
Spl-170	200-264	2.5 mM $MgCl_2$	[58°C][35]
Spl-173	176-296	2.5 mM $MgCl_2$	[58.5°C][35]

Table 3: Absolute numbers of alleles observed within 4 sampling sites (region) using 12 sets of microsatellite primers

Locus	Region 1	Region 2	Region 3	Region 4	N
LS-19	17.0	12.0	14.0	15.0	14.5
LS-68	11.0	12.0*	12.0	11.0	11.5
LS-34	9.0	9.0	9.0	11.0	9.5
LS-54	12.0	10.0	10.0	14.0	11.5
Spl-105	11.0	8.0	12.0	12.0	10.7
Spl-104	14.0	14.0	14.0	13.0	13.7
Spl-163	18.0	11.0	17.0	15.0	15.2
Spl-170	16.0*	13.0	17.0	15.0	15.2
Spl-173	17.0	13.0	14.0*	16.0	15.0
Spl-113b	14.0	11.0	16.0	18.0	14.7
Spl-113a	14.0	9.0	10.0	14.0	11.7
Total	153.0	122.0	145.0	154.0	
Average	13.9	11.0	13.1	14.0	13.0

N = Mean number of alleles observed per locus. *Loci = Loci in accordance with H-W equilibrium ($p \leq 0.01$)

Table 4: Observed (Ho) and expected (He) heterozygosities at 11 loci in four sampling regions

	Locus	Region 1	Region 2	Region 3	Region 4	Average
Ho(He)	LS-19	0.865 (0.900)	1.000 (0.888)	0.860 (0.908)	0.923 (0.879)	0.912
	LS-34	0.442 (0.713)	0.767 (0.812)	0.620 (0.796)	0.635 (0.653)	0.616
	LS-54	0.615 (0.881)	0.605 (0.783)	0.540 (0.843)	0.596 (0.839)	0.589
	LS-68	0.673 (0.862)	0.581 (0.801)	0.640 (0.865)	0.654 (0.862)	0.637
	Spl-104	0.904 (0.885)	0.860 (0.905)	0.700 (0.888)	0.808 (0.880)	0.818
	Spl-105	0.538 (0.841)	0.349 (0.785)	0.720 (0.882)	0.635 (0.849)	0.560
	Spl-113a	0.500 (0.886)	0.465 (0.869)	0.740 (0.910)	0.481 (0.883)	0.546
	Spl-113b	0.365 (0.830)	0.500 (0.759)	0.490 (0.788)	0.510 (0.875)	0.466
	Spl-163	0.462 (0.899)	0.535 (0.833)	0.460 (0.887)	0.577 (0.898)	0.508
	Spl-170	0.769 (0.898)	0.977 (0.888)	1.000 (0.902)	0.865 (0.896)	0.903
	Spl-173	0.692 (0.895)	0.581 (0.857)	0.400 (0.878)	0.577 (0.875)	0.563
	Average	0.624 (0.863)	0.656 (0.835)	0.654 (0.868)	0.662 (0.854)	0.650 (0.855)

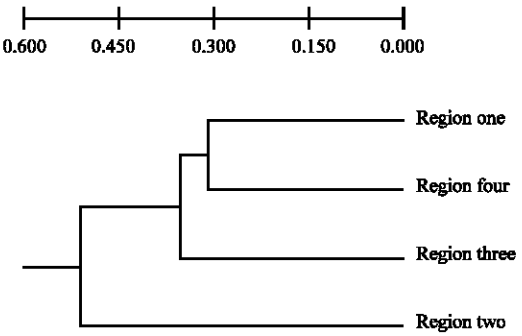

Fig. 2: UPGMA dendrogram based on the genetic distance computed by Nei's (1972) between *A. stellatus* population, according to microsatellite DNA analysis

maximum variability ranging in frequency. Average heterozygosity over the loci was 0.650 and ranged from 0.624 region one to 0.662 in Region four (Table 4).

Deviation from Hardy-Weinberg equilibrium were in all cases ($p \leq 0.01$) expect Spl-170 in region one and LS-68 in region two and Spl-173 in region three (Table 3).

Genetic Variation Among Sampling Regions

Genetic distance calculated between each pair of collections ranged from 0.337 (between Regions one and four) to 0.606 (between Regions two and four, Table 5). In general, similarities in more geographically close areas were higher than more distance one (Fig. 1, Table 5). Results of analysis

Table 5: Pairwise population of genetic distance (below diagonal) and genetic identity (above diagonal) (Nei, 1972) detected at 11 loci in stellate sturgeon samples

		Genetic identity			
	Samples	Region 1	Region 2	Region 3	Region 4
Genetic distance	Region 1	-	0.632	0.686	0.714
	Region 2	0.460	-	0.631	0.546
	Region 3	0.378	0.460	-	0.712
	Region 4	0.337	0.606	0.340	-

Table 6: Pairwise estimates of genetic differentiation detected at 11 loci in stellate sturgeon samples, using unbiased R_{ST} (below diagonal) and F_{ST} values (above diagonal)

		F_{ST} (Nm)			
	Samples	Region 1	Region 2	Region 3	Region 4
R_{ST} (Nm)	Region 1	-	0.049 (4.836)	0.035 (6.798)	0.033 (7.277)
	Region 2	0.078 (2.948)	-	0.049 (4.838)	0.063 (3.728)
	Region 3	0.131 (1.667)	0.103 (2.168)	-	0.033 (7.349)
	Region 4	0.269 (0.680)	0.287 (0.618)	0.301 (0.578)	-

Probabilities of R_{ST} or F_{ST} determined by AMOVA tests, $p \leq 0.01$

of molecular variance of genetic variation, among regions was 0.0127, among population/regions was 0.035 and individual within groups was 0.047 ($p \leq 0.01$). The highest range of genetic difference were observed between regions 2 and 4 ($F_{ST} = 0.063$, $R_{ST} = 0.287$, $p \leq 0.01$) and the lowest between regions 3 and 4 ($F_{ST} = 0.033$, $R_{ST} = 0.301$, $p \leq 0.01$). However, F_{ST} and R_{ST} estimates often differ in a pronounced manner (Balloux and Lugon-Moulin, 2002). Values of pairwise R_{ST} among samples were consistently much higher (as much as an order of magnitude) than equivalent F_{ST} values (Table 6).

Molecular Phylogeny

The dendrogram based on genetic distance Nei (1972, 1978) revealed three major cluster. One cluster consists of Region two alone, a second consists of Region three, while the third consists of Region one and four together (Fig. 2).

DISCUSSION

Out of 15 pairs of studied primers, 4 pairs was not amplified in the PCR reaction, which may be because of the lack of flanking sites in these primers due to high genetic distance of primers source species and the stellate sturgeon. One primer pair was monomorphic and the rest of them were polymorph. One pairs of polymorphic primers amplificated two loci. Few samples showed few additional bands in some loci, which may related to non-exclusive primers of this species. The long-term persistence of an endangered fish species can be investigated by allelic diversity, gene diversity, effective population size and population structure (Yue *et al*., 2004). At present study, comparison of the Ho values of *A. stellatus* (0.650, Table 4) it was slight lower than anadromus species (Ho = 0.68, Dewoody and Avise, 2000) and the mean number of alleles in this study (13.6) was higher than anadromus species (11.3) for microsatellites. The high genetic variability of the stellate sturgeon from region two, may reflect different population colonization origins. Region two populations not only presented the least similar genetically to other regions, but also showed the lowest number of alleles (122 alleles) due to bottleneck population such as over fishing and deterioration in the breeding environment resulting in less recruitment, dams, pollution. Many alleles with low frequency (<0.05) confirm that. The number of alleles for a given locus is more sensitive to demographic fluctuations (Nei *et al*., 1975; Maruyama and Fuerst, 1985) and is therefore more appropriate for testing whether a bottleneck has occurred in a population (Luikart and Cornuet, 1998). Therefore it needs immediate attention for its genetic conservation and overall variability (Table 3). AMOVA test revealed the

populations of each region differ gather presenting a significant difference ($p \leq 0.01$) among the samples of the various regions in the Iranian coast line South Caspian These results stand in stark contrast to previous studies used mtDNA. Low levels of variation were detected and little differentiation among sampling site was detected over a comparable geographic range (Pourkazemi, 1996; Shabani, 2005). This may result from the more rapid rate of mutational change at microsatellite loci compared with that at even the most rapidly evolving area of the mitochondrial genome (Wirgin *et al*., 2002). At present study deviation from the Hardy-Weinberg equilibrium observed in most loci. Maybe, it is due to presence of null alleles. Similar results have been reported the null alleles in inheritance of microsatellite loci in lake and white sturgeons (Rodzen *et al*., 2004; Zhao *et al*., 2005; McQuown *et al*., 2003; Pyatskowit *et al*., 2001; Welsh *et al*., 2003) and it also may be related to not using species specific primers and the most important reason sampling from mixtures of migrating population and sampling methodology. To detect such a population structure, samples must be collected from spawning sites. Samples from non-spawning adults may reflect mixtures of migrating population (McQuown *et al*., 2003). At present study F_{ST} in all sampling site was low but significant ($p<0.001$). So in each river in the South Caspian sea have different population. The lowest of F_{ST} on AMOVA was between region 4 and 3 with the high gene flow. But the highest of genetic distance and lower genetic identity was between region 2 and 4 (Table 5). Regarding the fishery returning, it is possible that the caught samples are a combination of the various generation and different birth places which have formed a gathering to feed.

Phylogenetic tree of stellate sturgeon using 12 microsatellites loci in four regions of south of Caspian Sea showed three clarified clusters included cluster of region two, three, one and four. As it shown in Fig. 2, the samples of the region two is in a separate cluster, which is possibly the samples that have caught before entering spawning sturgeon into the Sefidrude river and suggested the existence of a separate population in Sefidrud. The obtained results of the phylogeny show that 3 populations of stellate sturgeon exist on South of Caspian Sea originated from Sefidrud, one of population is in Sefidrud and other populations are derived from it. However, it should be mentioned that usually phylogeny sanders has not recommended distinguishing different population for a species. Stellate sturgeon is an anadromus species which immigrates to river for spawning and returns to the sea for feeding and growth, but the comprehensive study has not been performed up yet. In comparison with other molecular methods performed in the stellate sturgeon species, microsatellites studies showed the higher genetic variation and heterozygosity than mtDNA. Although this fish has the various allelic number in each of the under-studied regions in the Iranian cost line south Caspian Sea varies greatly. As microsatellites method has higher capacity to show the genetic variation, using above primers compared to other methods and with regard to the severe reduction of these fishes reserves before performing the artificial reproduction, identify the caught sample, then take the required actions for artificial reproduction and transfer among the various workshops. The present study showed that the genetic variation in the stellate sturgeon compared to the other sturgeons is in acceptable level, but the effect of over fishing have caused the reduction stocks in this species. Over fishing and ecologic changes in the Caspian Sea have altered the population structure of this species effectively. With population differentiation in region two especially Sefidrud drainage, we suggest special consideration on conservation policy and restocking program of this species in Sefidrud area.

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