Application of microsatellite markers to determine populations of the Persian sturgeon (*Acipenser persicus*) in the South of Caspian Sea

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Abstract

The objective of this study was to analyse the population genetic structure of the Persian sturgeon **(***Acipenser persicus***)** in Sefidrud and Gorganrud rivers watershed based on the characterization of microsatellite markers during 2006 – 2008. 100 samples of Persian sturgeon were collected from two regions. Four microsatellite loci (*Ls*68, *Spl*168, *Spl*173 and *Afu*68) were analyzed for the molecular characterization of this species which resulted in polymorphic patterns. DNA bands were analysed using Biocapt and GenAlex software package. A total of 109 alleles were observed of which the maximum number of alleles (17) were found in *Spl*168 locus which belonged to sturgeons from Sefidrud river's watershed and the minimum number of alleles (10) in *Ls*68 locus belonging to the sturgeons from Gorganrud river's watershed. Results of microsatellite analysis revealed that the differences between samples of two regions were not statistically significant ($p>0.05$), neither for the average number of alleles per locus nor for observed heterozygosities. The calculated F_{st} and R_{st} between two regions was 0.07 and 0.17 showing that the genetic difference was significant ($p< 0.01$). Samples from Sefidrood river's watershed in *Spl*173, *Afu*68 and *Spl*168 loci and samples of other regions in *Afu*68 and *Spl*168 loci were at Hardy-Weinberg equation. The genetic distance was calculated as 0.4 which represents a significant genetic difference between samples of two studied areas. In conclusion, this study suggests that the Persian sturgeons in two regions of the southern part of the Caspian Sea are genetically differentiated, therefore fisheries management of this unique stocks for restocking and conservation of gene pools is highly recommended.

Keywords: Persian sturgeon, *Acipenser persicus*, Caspian Sea, Microsatellite, Genetic structure, Population genetic

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Introduction

Sturgeons are one of the oldest fishes existing from 200 million-years ago and being referred as live fossils by several authors (Grande and Bemis, 1996; Bemis et al., 1997). Recently, sturgeon populations in the Caspian Sea are endangered due to various factors, such as over-exploitation, habitat alternation, pollutions, barriers to migration and loss of spawning habitat. (Pourkazemi, 2006). Therefore all sturgeon species have been listed in the 1996 IUCN Red List assessment as an endangered species (Dugo et al., 2004; Pourkazemi, 2006). At present, 27 sturgeon species are found living throughout the world (Ustaoglu and Okumus, 2004), of which six species belonging to two genera (*Huso* and *Acipenser*), are found in the Caspian Sea and its drainage basin which provide the bulk of the world's caviar yield today (Pourkazemi, 2006). In the recent years, *Acipenser persicus* has comprised the largest proportion of the total Iranian commercial catch (Moghim et al., 2006). This species is also artificially propagated in the Iranian hatcheries and the fingerlings are released into the rivers of the south Caspian Sea for restocking.

An understanding of the genetic diversity in aquatic organisms can be useful in stock conservation. Genetic diversity is important in both natural and cultural populations because it provides the necessary spectrum of genotypes for adaptive response to changing conditions and heterozygous individuals usually are superior to less heterozygous individuals in many economically important characteristics like growth, fertility and disease resistance (Beardmore et al., 1997). Sturgeon population genetic studies provide important information which can be used for management, sustainable use and conservation of the species concerned (Rosenthal et al., 2006). Among molecular markers, microsatellite methods are new techniques which are commonly applied for many commercially important aquatic species that can be examined by using samples harvested without endangering the life of the individual. Microsatellite markers were used to study sturgeon population structure and can also identify different brood stocks for management in hatcheries to conserve diversity and minimize inbreeding in artificial propagation (Norouzi et al., 2008). Pourkazemi (2001) and Shabani et al. (2006) studied mtDNA variation of *A. stellatus* populations in south Caspian Sea by $RFLP_s$ method using ND 5/6 gene regions. They found low genetic variation among studied areas and mentioned that the $RFLP_s$ method is not a powerful technique to determine genetic diversity sufficiently to fisheries management of this species. Safari et al. (2008) using microsatellite markers showed high level of variety of *A.nudiventris* between Ural River and samples from the southern part of the Caspian Sea. Following the results, they expressed there are two independent populations of this species, so conservation policy and restocking programs are necessary. Sturgeon family structure and relationships can easily be identified using microsatellite markers. Using these methods allows appropriate broodstock management both in

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aquaculture and in sturgeon restocking programs (Rosenthal et al., 2006). In recent years, genetic researches have been limited to a few studies on the Caspian Sea sturgeon species using a variety of molecular markers (Rezvani, 1997; Pourkazemi et al., 1999; Shabani, 2005; Qasemi et al., 2006; Khoshkholgh, 2007; Norouzi et al., 2008; Safari et al., 2008) but despite the commercial importance of the Persian sturgeon, study on its genetic and population structure in south Caspian Sea is scarce and more studies are necessary. The goal of this study was to determine the genetic diversity population and provide a genetic background of this species for management in the southern part of the Caspian Sea using microsatellite DNA markers. It can also be applied for future genetic improvement and assessment of this species in hatcheries and to design suitable management guidelines for artificial breeding activities.

Materials and methods

Sample collection

A total of 100 fin clips of adult *Acipenser persicus* were collected from 2 regions including 50 samples from Sefidrud watershed and 50 samples from Gorganrud watershed (Fig. 1). For each sample, 2-3 cm caudal fin tissue was cut and kept in absolute ethanol and then transferred to the Genetic department of International Sturgeon Research Institute, Rasht, Iran.

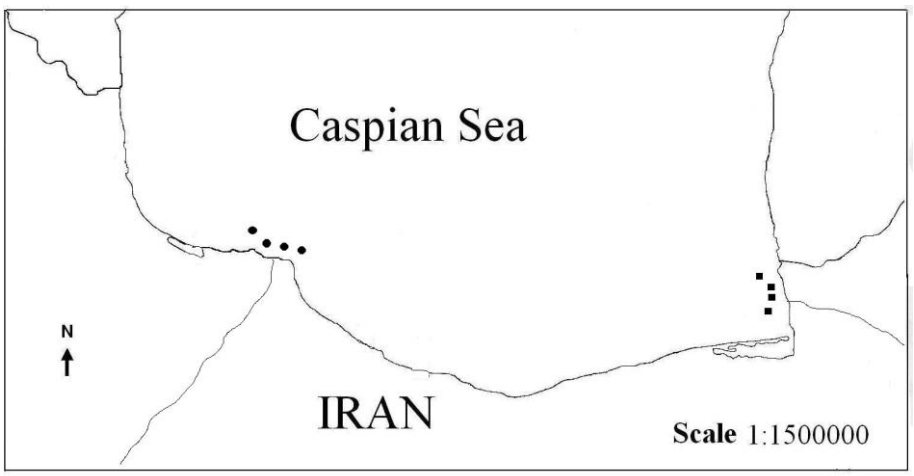


Figure1: Sampling regions map: Gorganrud watershed (■) and Sefidrud watershed (●)

Genomic DNA extraction

For each sample, approximately 50 mg of fin tissues was cut into small pieces and genomic DNA was extracted by standard SDS proteinase-K digestion, phenol: chloroform: isoamylalcohol extraction and ethanol precipitation as described by Hillis et al. (1996). The quantity of DNA was measured in 260 and 280 nm using UV-spectrophetometry method by Nanodrop (ND 1000 model) and the quality of the extracted DNA sample was checked by

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1% agarose gel electrophoresis. Purified DNA was stored at -20$^{o}_{C}$ until use.

PCR profiles and primer sequences

In this study, we used 4 microsatellite loci: *Ls*68, *Afu*68 (May et al., 1997), *Spl*168 and *Spl*173 (McQuown et al., 2000) all of which produced polymorphic patterns. The PCR conditions, especially the annealing temperatures were optimized for each microsatellite loci to produce scorable amplification products (Table 1). The PCR was performed in a 20 µl reaction volume containing 100 ng of template DNA, 30 pmol of forward and reverse primer, 200 µm of $dNTP_s$, 5 u / µl of *taq* DNA polymerase, 1 µl of 1.5 mM $Mgcl_2$ and 2 µl of 10X reaction buffer (Table 2).

Table 1: Characteristics of 4 polymorphic microsatellite loci of *Acipenser persicus* used in this study

Loci	Primer (5/-3/)	Size (bp)	Annealing temp. ($^{o}_{C}$)	Reference
*Ls*68	F- TTATTGCATGGTGTAGCTAAAC R- AGCCCAACACAGACAATATC	198 – 264	60	May *et al*., 1997
*Spl*168	F- CACTGATTCGCTACAACCGT R- AGAAGGACTTGCAGTCCGAA	232 - 310	59	McQuown *et al*., 2000
*Spl*173	F- GGCTTTTGTCTGAAACGTCC R- TGGTGTGTCATTTTGAAGGC	232 - 292	61	McQuown *et al*., 2000
*Afu*68	F- AACAATATGCAACTCAGCATAA R- AGCCCAACACAGACAATATC	106 - 168	55	May *et al*., 1997

Table 2: Concentration and kind of materials used in this study

Material	Concentration	Amount for 20 µl reaction
DNA	100 ng	1 µl
PCR buffer	10 X	2 µl
$dNTP_s$	10 mM	0.5 µl
$Mgcl_2$	50 mM	1 µl
Primer (F)	30 pm	1 µl
Primer (R)	30 pm	1 µl
taq DNA polymerase	5 u / µl	0.2 µl
dH_2o	-	13.3 µl

PCR reactions were performed with an eppendorf thermal cycler (Mastercycler ep gradient, 96 plus, eppendorf, Germany) under the following conditions: initial denaturation of 5 min at 95 $^{o}_{C}$ followed by 30 cycles of 30 s denaturation at 94 $^{o}_{C}$, 60 s at the respective annealing temperature, and 60 s extension at 72 $^{o}_{C}$, ending with 10 min at 72 $^{o}_{C}$ as the elongation period.

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Table 3: Variability of 4 microsatellite loci in two areas of the Persian sturgeon (A, number of alleles; Ho, observed heterozygosity; He, expected heterozygosity; P, *P*-values of X^2 tests for Hardy-Weinberg equilibrium)

Locus	Parameters	Sefidrud watershed	Gorganrud watershed
*Ls*68	A	14	10
	H_o	0.670	0.520
	H_e	0.840	0.850
	P	0.00	0.000
*Spl*168	A	17	15
	H_o	1	0.950
	H_e	0.840	0.840
	P	0.095	0.363
*Spl*173	A	16	14
	H_o	0.708	0.710
	H_e	0.880	0.860
	P	0.1	0.008
*Afu*68	A	11	12
	H_o	0.250	0.500
	H_e	0.780	0.640
	P	0.113	0.285
Average number of alleles per locus		14.5 ± 2.64	12.75 ± 2.22
rage H_o		0.66 ± 0.31	0.67 ± 0.21
Average H_e		0.83 ± 0.04	0.80 ± 0.10

Gel electrophoresis and staining

The PCR products with a standard 100 bp DNA markers ladder (Fermentas) were separated by electrophoresis on 6% (w/v) denatured polyacrylamide gel (29:1 acrylamid: bis acrylamid; 1X TBE buffer) using a Hoefer gel electrophoresis system (Pharmacia-Biotech, USA). Gel run carried out at 120 V until the loading buffer reached the bottom of the plate. After electrophoresis, the gel was stained with silver nitrate protocol (Pourkazemi, 1996).

Microsatellite data analysis

Following polyacrylamid gel electrophoresis and silver nitrate staining, one or two clear bands were observed at each locus for each specimen. The bands representing alleles were manually scored based on their sizes. The recorded microsatellite genotypes were used as the input data for the GENALEX software (Peakall and Smouse, 2006). Allele and genotype frequencies, observed heterozygosity (Ho), expected heterozygosity (He) and test of deviations from Hardy-Weinberg equilibrium were calculated. Genetic distance between two areas was estimated using Nei standard

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genetic distance index (Nei, 1972). Genetic differentiation between two areas was also evaluated by the calculation of pairwise estimates of F_{st} and R_{st} values. All calculations were conducted using the GENALEX version 6.

Results

Allele frequencies at all loci in both areas are shown in Table 3. Overall, 109 alleles resulted in 4 microsatellite loci in which the locus *Spl*168 presented the highest number of alleles (17) in Sefidrud watershed, while the locus *Ls*68 in Gorganrud watershed was the lowest (10). The average number of alleles per locus was 14.5 for samples of Sefidrud watershed and 12.75 for Gorganrud watershed. The average observed heterozygosities (H_o) were 0.66 and 0.67 in two regions, respectively. The differences between two regions were not statistically significant (p> 0.05), neither for the average number of alleles per locus nor for observed heterozigosities. Significant deviations from Hardy-Weinberg equilibrium at the locus level in two regions are shown in Table 4. Except *Ls*68 locus in Sefidrud region and *Ls*68 and *Spl*173 loci in Gorganrud region, the other loci in both regions were at Hardy-Weinberg equilibrium. The F_{st} (0.07) and R_{st} (0.17) values from pairwise comparisons were significant (p<0.01), indicating that the populations of two regions were divergent from each other. The genetic distance computed by Nei (1972) was 0.4.

Table 4: Hardy-Weinberg equilibrium Test of 4 microsatellite loci in two areas of Persian sturgeon in this study

Regions	Locus	DF	X^2 (Chi Sq)	Level of significant	Probability of significant
	*Ls*68	21	76.11	0.00	✸✸✸
Sefidrud	*Spl*168	45	57.83	0.095	ns
watershed	*Afu*68	10	15.55	0.113	ns
	*Spl*173	22	57.51	0.1	ns
	*Ls*68	15	52.78	0.000	✸✸✸
Gorganrud	*Spl*168	36	38.37	0.363	ns
watershed	*Afu*68	6	7.41	0.285	ns
	*Spl*173	55	83.65	0.008	✸✸

p≤ 0.05 ✸, p≤ 0.001 ✸✸✸, ns: no significant

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Discussion

Anthropogenic influence and other factors caused the dramatical decrease of sturgeon stocks such as *Acipenser persicus* in the Caspian Sea. This required the development of conservation programs and genetic studies to distinguish different populations. Among molecular methods, microsatellite markers allow the evaluation of intraspecific genetic diversity and offer the possibility to distinguish differences between populations due to their high level of allelic variation (Dudu, et al., 2008). For this purpose, we evaluated the genetic diversity in Persian sturgeon from two regions in the southern part of the Caspian Sea, using microsatellite markers. Polymorphic patterns produced by primers in the present study confirmed that the microsatellite markers used are suitable for genetic diversity studies. According to the results there are no significant differences between samples of the two regions ($p>0.05$) neither for the average number of alleles per locus nor for observed heterozygosities. The high level of heterozygosity in this species can be attributed to use of most breeders in hatcheries and fingerlings released into the south Caspian Sea Rivers (Sefidrud and Gorganrud rivers) for restocking. However regular monitoring of genetic variability among the offspring of each region is essential to avoid the loss of current polymorphism due to inbreeding and outbreeding problems.

The value of F_{st} and R_{st} based on ANOVA test is a useful measure of genetic differentiation among populations (Ballox and Lugan-Moulin, 2002). Previous research has showed that low levels of F_{st} between samples of *A. stellatus* in four fishery regions are significant (Norouzi et al., 2008). In this study, the F_{st} was low (0.07) and R_{st} value was 0.17 but F_{st} and R_{st} were significant between samples of two regions ($p<0.01$). Although the lowest value of F_{st} can be considered as an important genetic difference between populations (Wright, 1978; Hartl and Clark, 1997). According to decisions of CITES (Convention on International Trade in Endangered Species), setting annual quotas of caviar in the Caspian Sea is based on sturgeon stock assessment. In the future, it is possible to determine quotas of caviar based on molecular genetic methods for the identification of sturgeon populations. The data presented here suggests that the populations of these two studied areas are genetically differentiated and do not represent a single panmictic population. The genetic distance value based on Nei, (1972) for conspecific populations averaged 0.05 (range: 0.002-0.07) and for congeneric species averaged 0.30 (range: 0.03-0.61). The distance value obtained in this study (0.4) falls within the average value of congenerics and indicated that genetic differences among these two studied populations is pronounced ($p<0.01$).

Samples of the two studied areas were mostly of loci at Hardy-Weinberg equilibrium test. The significant differences from Hardy-Weinberg equilibrium could be explained either by sample bias, migration, artificial breeding or the presence of null alleles. The inheritance of microsatellite null alleles

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have been reported by most of researchers (Pyatskowit et al., 2001; Welsh et al., 2003; Rodzen et al., 2004; Zhao et al., 2005; Norouzi et al., 2008). It may also be related to not using species specific primers and the most important reason, sampling from mixtures of migrating population and sampling methodology (McQuown et al., 2003). 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).

The data generated in this study showed that populations of *A.persicus* in two studied areas in the southern part of the Caspian Sea are genetically differentiated. Accurate species identification is the first step in each successful conservation program. This information can be applied for future genetic improvement and the assessment of this species in hatcheries. We strongly suggest special consideration on conservation policy and restocking program of this species in Sefidrud and Gorganrud.

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کاربرد مارکرهای مایکروستلایت جهت شناسایی جمعیت های تاسماهی ایرانی (*Acipenser persicus*) سواحل جنوبی دریای خزر

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چکیده

ساختار ژنتیکی تاسماهی ایرانی (*Acipenser percicus*) حوضه آبریز رودخانه های سفیدرود و گرگانرود سواحل جنوبی دریای خزر طی گشت ارزیابی ذخایر تاسماهیان سالهای ۱۳۸۸ – ۱۳۸۵ با استفاده از روش ریزماهواره (Microsatellite) مورد بررسی قرار گرفت. بدین منظور تعداد ۵۰ نمونه از هر منطقه (جمعا ۱۰۰ نمونه) جمع آوری و به آزمایشگاه ژنتیک مولکولی انستیتو تحقیقات بین المللی ماهیان خاویاری دکتر دادمان (رشت) منتقل گردید. پس از استخراج DNA ژنومی، از ۴ جفت آغازگر (Primer) ریزماهواره (microsatellite) جهت انجام واکنش های زنجیره ای پلیمراز (PCR) و تعیین ساختار ژنتیکی استفاده شد که همگی تولید باندهای چند شکلی (پلی مورف) نمودند. در مجموع ۱۰۹ الل مشاهده شد که بیشترین تعداد الل واقعی (۱۷) متعلق به جایگاه *Spl*168 حوزه آبریز رودخانه سفیدرود و کمترین تعداد الل واقعی (۱۰) در جایگاه *Ls*68 متعلق به حوزه آبریز رودخانه گرگانرود بود. بین نمونه های دو منطقه اختلاف آماری معنی داری بر اساس میانگین تعداد الل مشاهده شده (Na) و هتروزایگوسیتی مشاهده شده (Ho) در هر جایگاه ژنی مشاهده نشد ($p > ۰/۰۵$). میزان F_{st} و R_{st} که نشانه جدایی جمعیت ها از یکدیگر می باشد به ترتیب ۰/۰۷ و ۰/۱۷ بدست آمد و اختلاف جمعیتی بین نمونه های دو منطقه معنی دار بود ($p < ۰/۰۱$). نمونه های حوزه آبریز رودخانه سفیدرود در جایگاههای *Spl*173، *Afu*68 و *Spl*168 و نمونه های حوزه آبریز رودخانه گرگانرود در جایگاههای *Afu*68 و *Spl*168 در تعادل هاردی واینبرگ بوده و در بقیه جایگاههای ژنی نمونه های هر دو منطقه انحراف از تعادل را نشان دادند. فاصله ژنتیکی بین نمونه های دو منطقه ۰/۴ محاسبه شد که بیانگر تفاوت ژنتیکی قابل توجهی بین نمونه های مناطق مورد مطالعه می باشد. بررسی نتایج فوق نشان می دهد که جمعیت تاسماهی ایرانی مناطق مورد مطالعه از یکدیگر مجزا بوده و هریک جمعیت مستقلی را نشان می دهند. بنابراین مدیریت شیلاتی ذخایر منحصر بفرد این گونه و حفظ خزانه ژنی آنها قویا توصیه می گردد.

واژگان کلیدی: تاسماهی ایرانی، ساختار ژنتیکی، ریزماهواره، ژنتیک جمعیت، دریای خزر

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