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

Chakmehdouz Ghasemi, F.*, Pourkazemi, M., Tavakolli Eshkalak, M., Yarmohammadi, M., Hassanzadeh Saber, M. and Baradaran Noveiri, S.

International Sturgeon Research Institute, Rasht, Iran, P.O.Box: 41635-3464

The population genetic structure of the Persian sturgeon (*Acipenser persicus*) in the Sefidrud and Gorganrud River watersheds was analyzed based on the characterization of microsatellite markers. One hundred fin clip samples of Persian sturgeon from the two regions were collected. Four microsatellite loci (*Ls*68, *Spl*168, *Spl*173, and *Afu*68) were analyzed for molecular characterization, resulting in polymorphic patterns. DNA bands were analyzed using Biocapt and GenAlex software packages. In total, 109 alleles were observed. The maximum number of alleles (17) was found in locus *Spl*168 in sturgeon from the Sefidrud watershed and the minimum number of alleles (10) in locus *Ls*68 in sturgeon from the Gorganrud watershed. No significant differences between samples of the two regions, either in average number of alleles per locus or in heterozygosities, were observed ($P>0.05$). However, based on AMOVA, the calculated F_{st} (0.07) and R_{st} (0.17) between the two regions were significantly different ($P<0.01$), showing that the two populations differ genetically. *Spl*173, *Afu*68, and *Spl*168 loci in samples from the Sefidrud watershed and *Afu*68 and *Spl*168 loci in samples from the Gorganrud watershed demonstrated Hardy-Weinberg equilibrium. The genetic distance between samples of the two areas was 0.4, which represents a significant difference. We conclude that the Persian sturgeon of two regions of the southern Caspian Sea are genetically differentiated and therefore fisheries management of this unique stock for restocking and conservation of gene pools is strongly recommended.

Key words: Persian sturgeon, *Acipenser persicus*, Caspian Sea, Microsatellite, Genetic structure, Population genetics

INTRODUCTION

Sturgeon is among the oldest known fish species, dating from 200 million years ago and frequently referred to as living fossils (Bemis et al., 1997; Grande & Bemis, 1996). There are 27 sturgeon species found throughout the world (Ustaoglu & Okumus, 2004), and most are listed as critically endangered in the 2009 IUCN Red List assessment (IUCN, 2009). Six species of *Huso* and *Acipenser*, occur in the Caspian Sea and its drainage basin and provide the bulk of the world's caviar (Pourkazemi, 2006). In recent years, *Acipenser persicus* Borodin (1897) has comprised the largest proportion of the total Iranian commercial catch (Moghim et al., 2006). This species is artificially propagated in Iranian hatcheries and the fingerlings are released into the rivers of the southern Caspian Sea for restocking.

*Corresponding author: chakmehdouz13@yahoo.com

An understanding of the genetic diversity of aquatic organisms is useful in stock conservation. Diversity is important in both natural and cultured populations, providing the necessary spectrum of genotypes for adaptive response to changing conditions. Heterozygous individuals usually are superior to less heterozygous individuals in characteristics such as growth, fertility, and disease resistance (Beardmore et al., 1997). Sturgeon population genetic studies provide important information that can be used for management, sustainability, and conservation of the species concerned (Rosenthal et al., 2006). Among molecular markers, suitable microsatellites from tissue samples harvested without endangering the life of the animal are commonly utilized for investigation in many commercially important aquatic species.

Microsatellite markers have been used to study sturgeon population structure and can also identify broodstock for management in hatcheries to conserve diversity and minimize inbreeding in artificial propagation (Norouzi et al., 2008). Pourkazemi and Skibinski (2001) and Shabani et al. (2006) studied mtDNA variation in populations of *A. stellatus* in the south Caspian Sea using RFLP, a method using ND 5/6 gene regions. They found low genetic variation among these populations and stated that $RFLP_s$ is not an adequate technique for determining genetic diversity for fishery management of this species. Using microsatellite markers, Safari et al. (2008) found a high level of variability among populations of *A. nudiventris* in the Ural River and the southern part of the Caspian Sea. Based on these results, they suggested that two independent populations of this species exist, therefore for conservation of Iranian coastline populations of ship sturgeon, it is necessary to develop a genetic based restocking programs of local populations. The population structure and relationships of sturgeon can easily be identified using microsatellite markers. Use of these methods would allow appropriate broodstock management both in aquaculture and in sturgeon restocking programs (Rosenthal et al., 2006). In recent years, genetic research has been limited to a few studies on Caspian Sea sturgeon species using a variety of molecular markers (Khoshkholgh, 2007; Norouzi et al., 2008; Pourkazemi, 2009; Pourkazemi et al., 1999; Qasemi et al., 2006; Rezvani, 1997; Safari et al., 2008; Shabani, 2005), and despite the commercial importance of the Persian sturgeon, its genetic and population structure in the south Caspian Sea is poorly understood and more studies are necessary.

The aim of this research was to determine the genetic diversity among populations of *Acipenser persicus* in the southern Caspian Sea using microsatellite DNA markers. This study could provide a genetic basis for the management of the different populations, assist future genetic improvement, and contribute to appropriate management guidelines for breeding programs in hatcheries.

MATERIAL AND METHODS

SAMPLE COLLECTION

Fin clip samples from 50 *Acipenser persicus* were collected from each of 2 regions in the southern Caspian Sea (Fig. 1). From each sample, 2-3 cm^2 caudal fin tissue was taken and placed in 100% ethanol and transferred to the Genetic Department of the International Sturgeon Research Institute, Rasht, Iran.

GENOMIC AND EXTRACTION

For each sample, approximately 50 mg of fin tissue 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 at 260 and 280 nm using UVspectrophotometry by Nanodrop (ND 1000 model), and the quality of the extracted DNA sample was checked by 1% agarose gel electrophoresis. Purified DNA was stored at -20 ^{o}C until use.

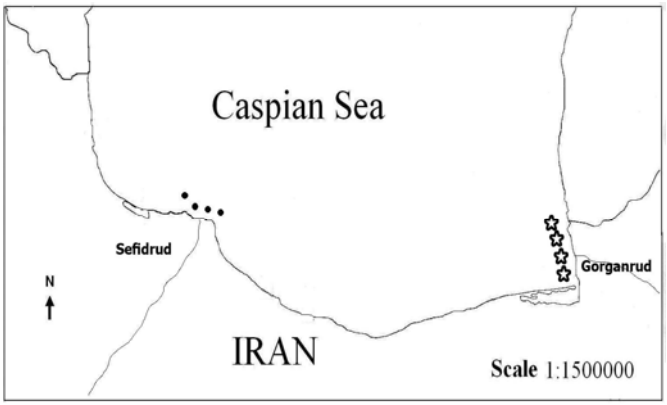

FIGURE 1. Sampling regions: Gorganrud watershed (✪) and Sefidrud watershed (●).

TABLE 1. Characteristics of four polymorphic microsatellite loci of *Acipenser persicus* used in this study.

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

PCR PROFILES AND PRIMER SEQUENCES

Four microsatellite loci were used: *Ls*68, *Afu*68 (May et al., 1997), *Spl*168, *Spl*173 (McQuown et al., 2000), which all produced polymorphic patterns. The PCR conditions, particularly the annealing temperatures, were optimized for each microsatellite locus to produce scorable amplification products (Table 1).

The PCR was performed in a 20 µl reaction volume containing 100 ng template DNA, 30 pmol 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. 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 °C followed by 30 cycles of 30 s denaturation at 94 °C, 60 s at the respective annealing temperature, and 60 s extension at 72 °C, finishing with 10 min at 72 °C as the elongation period.

GEL ELECTROPHORESIS AND STAINING

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

MICROSATELLITE DATA ANALYSIS

Following polyacrylamide 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 size. The recorded microsatellite genotypes were used as the input data for GENALEX software (Peakall and Smouse, 2006). Number of alleles per locus for each area, observed heterozygosity (H_o), expected heterozygosity (H_e) and deviations from Hardy-Weinberg equilibrium were calculated. Genetic distance between the two areas was estimated using the Nei standard genetic distance index (Nei, 1972). Also genetic differentiation between the two areas was also evaluated by the calculation of pairwise estimates of F_{st} and R_{st} values. All calculations were conducted using GENALEX version 6.

RESULTS

Allele information at all loci in samples from both areas are shown in Table 2. Overall, 109 alleles were found at the four microsatellite loci. Locus *Spl*168 presented the highest number of alleles (17) in the Sefidrud watershed, while locus *Ls*68 in the Gorganrud watershed showed the lowest (10).

The average number of alleles per locus was 14.5 for samples from the Sefidrud watershed and 12.75 from the Gorganrud watershed. Average observed heterozygosities (H_o) were 0.66 for Sefidrud and 0.67 for Gorganrud. The differences between samples from the two regions were not statistically significant ($P > 0.05$), for either the average number of alleles per locus or the observed heterozygosity. Samples at locus *Ls*68 in the Sefidrud region and loci *Ls*68 and *Spl*173 in the Gorganrud region showed significant deviations from Hardy-Weinberg equilibrium (Table 3). The F_{st} (0.07) and R_{st} (0.17) values from pairwise comparisons were significant ($P < 0.01$), indicating that the populations of the two regions were significantly divergent. The genetic distance computed by Nei (1972) was 0.4.

DISCUSSION

Anthropogenic activities, along with other factors, have led to a dramatic decrease in sturgeon stocks including *Acipenser persicus* in the Caspian Sea. Microsatellite markers allow the determination of intraspecific genetic diversity and offer the possibility of identifying differences among populations based on their high level of allelic variation (Dudu et al., 2008). Using microsatellite markers, we evaluated the genetic diversity of Persian sturgeon from two regions in the southern Caspian Sea. Polymorphic patterns produced by primers confirmed that the microsatellite markers used are suitable for studies of genetic diversity. We observed no significant differences between samples from the two regions ($P > 0.05$) either for average number of alleles per locus or for heterozygosity. The high level of heterozygosity in both sampled areas confirmed genetic diversity in this species. Increasing natural reproduction in the rivers of the two areas in order to maintain the gene pool should be considered a goal.

The values of F_{st} and R_{st} based on AMOVA are useful measures of genetic differentiation among populations (Ballox & Lugan-Moulin, 2002). Previous research has reported a low, but significant, level of F_{st} among samples of *A. stellatus* in four fishery regions (Norouzi et al., 2008). In this study, the F_{st} was low (0.07) and the R_{st} value was 0.17, but F_{st} and R_{st} were significantly different between samples of the two regions ($P < 0.01$). Data presented here suggest that the populations of the two studied areas are genetically differentiated and do not represent a single panmictic population. Shaklee et al., 1982 and Thorpe and Sol-Cave (1994) showed genetic distance values based on Nei, (1972) for conspecific populations averages 0.05 (range: 0.002-0.07) and for congeneric averages 0.30 (range: 0.03-0.61). The highest genetic distance value obtained in the present study (0.4) falls within the range of congeneric levels and indicated that genetic differences between these two studied populations are pronounced ($P < 0.01$).

TABLE 2. 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
Average H_o		0.66 ± 0.31	0.67 ± 0.21
Average H_e		0.83 ± 0.04	0.80 ± 0.10

TABLE 3. Hardy-Weinberg equilibrium Test of 4 microsatellite loci in two areas of Persian sturgeon in this study ($p \leq 0.05$ *, $p \leq 0.001$ ***, ns: no significant).

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

Significant deviations at one locus in samples from the Sefidrud region are due to deficiency of heterozygosities. The significant differences from Hardy-Weinberg equilibrium could be the consequence of sample bias, migration, artificial breeding or the presence of null alleles. The inheritance of microsatellite null alleles has been frequently reported (Norouzi et al., 2008; Pyatskowit et al., 2001; Rodzen et al., 2004; Welsh et al., 2003; Zhao et al., 2005). This may also be related to not using species-specific primers and, most importantly, to sampling from migrating populations (McQuown et al., 2003). To reliably define a population structure, samples must be collected from spawning sites. Samples from non-spawning adults may reflect mixtures of migrating populations (McQuown et al., 2003).

The data generated in this study showed that populations of *A. persicus* in two studied areas in the southern Caspian Sea to be genetically differentiated, supporting Pourkazemi (2009), who reported two populations of Persian sturgeon from the Iranian coast of the Caspian Sea. Maximum sustainable harvest and yield through fishery management requires knowledge of different age classes of resources. For this purpose, genetic studies should be undertaken to identify the composition of populations under a given species. In order to conserve each population of Persian

sturgeon, further studies on population structuring as well as the development of a conservation plan and restocking programs are required. The results of this study can be applied to genetic improvement and prevention of inbreeding and outbreeding problems of sturgeon in hatcheries. Therefore special consideration to conservation policies and restocking programs of sturgeon populations in the Sefidrud and Gorganrud Rivers is strongly recommended.

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