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Analysis of genetic variability and differentionin of stellate sturgeon, *Acipenser stellatus* (Pallas, 1771), in the North (Volga and Ural Rivers) and South Caspian Sea (estuary of Sefidrud)

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Abstract

In total, 140 samples of adult stellate sturgeon *Acipenser stellatus* were collected at three sites Northern (Volga and Ural Rivers) and Southern Caspian Sea (estuary of Sefidrud- Iran). Fifteen sets of microsatellite primers were tested on genomic DNA. Ten primer sets revealing polymorphic loci were used to analyze the genetic variation found in adults of the stellate sturgeon populations. The analyses revealed that the average of alleles per locus was 14.33 and all the sampled regions contained private alleles. The observed and expected heterozygosity averaged 0.677 and 0.871, respectively. Average of Fis and Fit were 0.225 and 0.250 respectively. Fst, Rst and gene flow estimates in AMOVA indicated significant genetic differentiation among regions, indicating that the populations were divergent. The genetic distance between populations indicating that the genetic difference among populations is pronounced. These results together with highly significant R_{st} of genotypic differences between these pairs of collections support the existence of different genetic populations along the Caspian Sea coasts.

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Introduction

Sevruga or stellate sturgeon (anadromous fish) is a brackish-water species, the most euryhaline among the Caspian sturgeons. In the last decade, due to higher water temperature considerable part of the North Caspian sevruga winters at the steep slopes of the North Caspian and in the north-eastern Middle Caspian. In spring, the North Caspian population migrates northward and then mature spawners enter the Volga and Ural Rivers. South Caspian population spawns in the Sefidrud and Kura Rivers. The post-spawn fish migrate back into the sea for feeding. Feeding migrations take place within the shelf zone of the sea. Food spectrum of sevruga is highly diverse. Its diet includes 16 invertebrate species: worms, crustaceans and bivalves. Its fish diet is represented by common kilka (Velikova *et al.,* 2012). Sevruga is the most fast-growing fish among other sturgeon species, and is more sensitive to impacts, including overfishing. The age structure of its spawning population is very indicative of pressures level. Thus, sevruga may be regarded as an indicator species for all sturgeons, featuring the tendencies of sturgeon stocks change and decline, in general (Velikova *et al.,* 2012). As natural reproduction in this species is extremely low, artificial propagation is practiced to rehabilitate wild Persian sturgeon stocks in the Caspian Sea. Consequently, artificial breeding of sturgeons on fish farms currently is the approach practiced to offset decreasing natural supplies of wild sturgeon (Moghim *et al.,* 2012). Stellate sturgeon was listed as an endengered species (IUCN, 1996).

Microsatellites analysis represents a very good method for individual identification and also allows the evaluation of intra-specific genetic diversity. This technique provides the ability to characterize the genetic variations in sturgeon populations from aquaculture (Georgescu *et al.,* 2013). Microsatellites are frequent in stellate sturgeon genome and can easily be amplified with PCR; they denote high levels of allele polymorphism. These features, taken together, offer the basis for a successful analysis in a wide range of fundamental and applied sectors of fisheries and aquaculture (Sekar *et al.* 2009). So far, few studies on genetic diversity, population genetic structure and demographic history of the Acipenseridae family of fishes have been carried out (Georgescu *et al.,* 2013; Zeng *et al.,* 2013; Panagiotopoulou *et al.,* 2014; Moghim *et al.,* 2012; Khoshkholgh *et al.,* 2011; Ray *et al.,* 2007) and molecular genetics research on the stellate sturgeon in the Caspian Sea have so far been limited to a few studies using RFLP methods (Pourkazemi, 2001; Shabani *et al.,* 2006) and low genetic variation was stated while no significant differences in haplotype frequency there found.

The development of management plans and the implementation of actions to restore stellate sturgeon within its native stocks can benefit from an understanding of the genetic diversity of its populations. This information is helpful in choosing donor populations to use as sources of reintroduction, as well as in formulating restoration goals regarding the population structure. This study was aimed at analyzing inter-population genetic diversity in stellate sturgeon by the analysis of microsatellites.

Materials and methods

Sample collection and DNA isolation

The fishes were caught from three different regions, including 54 samples from Volga (Russia), 43 samples from Ural (Kazakhestan), (the Norh of Caspian Sea), 43 samples from Sefidrud drainage (Kiyashahr) the South Caspian Sea in Iran (Fig.1). Fin tissue samples were prepared from 135 fishes of each location and preserved in 95% ethanol and stored at room temperature.

DNA extraction

The genomic DNA was extracted following the method described by Pourkazemi *et al.,* (1999). The quality and concentration of DNA were assessed by 1% agarose gel electrophoresis and then stored at -20 °C until use.

Microsatellite data set

The nuclear DNA was amplified using 15 microsatellite primers designed for *Acipenser* and

Scaphirhynchus (*LS-19, 34, 39, 54, 57, 62, 68, 69,* May *et al.,* 1997; *Spl-104, 105, 113, 163, 168, 170, 173,* McQuown *et al.,* 2000). Polymerase Chain Reaction (PCR) conditions for each primer set was optimized for stellate sturgeon. Polymerases Chain Reaction (PCR) condition for each primer set were optimized for stellate sturgeon (Table 1). The annealing temperatures were: 56 °C for *LS-19* and *Spl-113*, 58 °C for *LS-34* and *Spl-163 /170*, 59 °C for *LS-54* and *Spl-105*, 61.2 °C for *LS-68*, 57 °C for *Spl-104*, and 58.5 °C for *Spl-173*. Polymerase chain reaction was performed in 20 µl volume containing 100 ng of template DNA, 0.5-1 pmol of each primer, 200 mM each of the dNTPs, 0.5 U of *Taq* DNA polymerase and 1-2.5mM MgCl2. PCR products were separated on 6% polyacrylamide gels (29:1 acrylamide: bis-acrylamide; 1X TBE buffer) and followed by silverstaining. The gels were run at 170 W for 2h and 30 min. Alleles were sized using BioCapt software, and each gel contained an allelic ladder (50bp) to assist in consistent scoring of alleles.

Data analysis

Allele frequencies were estimated using *F*-statistics and Nei's genetic distance. Fst via frequency provides a measure of genetic differentiation among populations. The total genetic diversity (heterozygosity) within and among populations can be classified as follows: *Ho* = observed heterozygosity and *He*= expected heterozygosity. Hardy-Weinberg tests of equilibrium were estimated. Wright's *F*-statistics (Wright 1965) as follows: *Fis* = inbreeding coefficient within individuals relative to the subpopulation for each locus and stellate sturgeon sampling site were assessed; *Fit* = the inbreeding coefficient within individuals relative to the total; and Fst= inbreeding coefficient within subpopulations relative to the total. Fst and Rst were calculated using analysis of molecular variance (AMOVA) to estimate genetic variation among populations and regions. AMOVA calculations and allelic richness (A_R) were performed on Arlequin 3.5 (Excoffier & Lischer 2010) using 10,000 permutations in each case. Nei's genetic identity and distance were determined using a pairwise, individual-by-individual genetic distance, with all codominant data computed in GeanAlex 6 software (Peakall & Smouse 2005). The Cornuet and Luikart (1996) programme BOTTLENECK ver. 1.2.02 was used to detect recent effective population size reduction (to assess the impact of population decline) using data from the microsatellites under the more suitable two-phased model (TPM).

Results

Amplification and Banding Patterns

Out of 15 sets of microsatellite primers, four sets (LS-69, 57, 62 and Spl-168) have not shown any flanking sites on stellate sturgeon genome. Eleven sets of primers were successfully amplified and one set (LS-39) showed monomorphic pattern. Therefore totally 10 loci were investigated at present study. All microsatellite primers where able to produce DNA bands displayed a characteristic disomic banding pattern.

Genetic variation within sampling

A total of 199 alleles were identified in 135 individuals, 71 alleles in Ural, 74 alleles Volga, and 62 alleles in Sefidrud, with frequencies >0.05 in all samples. LS-34 showed the maximum variability ranging in frequency from 0.056 to 0.567. Allele sizes ranged from 104 to 348 bp (Table 1).

The Na per locus ranged from 8 to 18, with an average of 14.33. The number of alleles in LS-19 ranged from 12 to 14 (A_R = 14), in LS-34 from 8 to 11 (A_R = 11), in LS-54 from 10 to 11 (A_R = 11), in LS-68 from 12 to 15 (A_R = 14), in Spl104 from 14 to 15 (A_R = 14), and in Spl105 from 8 to 11 (A_R = 11), in Spl113 from 11 to 18 (A_R = 15), in Spl1163 from 11 to 14 (A_R = 13), in Spl170 from 13 to 16 (A_R = 15), in Spl173 from 13 to 17 (A_R = 15), with a tendency toward being fewer in the Sefidrud samples (Table 1).

All sampled populations contained a significant number of private alleles. In total, 21 alleles were found, with the number of private alleles being Ural 13 alleles, Volga 3 alleles and Sefidrud 5 alleles, none of which was found in other sites (Table 1).

The Ho and He per locus ranged from 0.349 to 1 and from 0.785 to 0.910, with an average of 0.677 and 0.871, respectively (Table 1). The LS-19 locus had the highest level of heterozygosity, and lower heterozygosities were consistently observed in most samples screened, which may be due to the presence of null alleles or small sample sizes. Bottleneck analysis of stellate sturgeon was 0.03489 in Ural, 0.04396 in Volga and 0.35963 in Sefidrud.

Table 1. Absolut numbers of allelles observed within 3 sampling sites using 10 sets of microsatellite primers. Observed (*Ho*) and expected (*He*) heterozygosities, number of effective alleles (*Ne*) at 10 loci in three sampling regions. loci in accordance with H-W unequilibrum *P< 0.05 ; **P<0.01; P<0.001; n.s., non-significant.

						Touchdown protocol
	Gene bank no	Ural	Volga	Sefidrud	Average	Actual size (bp)
Sample size		43	54	43		
LS-19	U72730					56 °C/35
Na (private alleles)		14	14	12(2)	13.750	
Ho		0.977***	0.963***	1.000***	0.966	132-213
He		0.881	0.884	0.888	0.883	
LS-34	U72733					
Na (private alleles)		8	11	9(1)	9.750	58 °C/35
Ho		0.442***	0.778***	0.767***	0.655	132-180
He		0.833	0.843	0.812	0.785	
LS-54	U72735					59 °C/35
Na (private alleles)		11	11	10	11.750	
Ho		0.488***	0.593***	0.605***	0.570	152-224
He		0.804	0.858	0.783	0.821	
LS-68	U72739					
Na (private alleles)		12	15	12	12.500	61.2 °C/35
Ho		0.698***	0.815ns	0.581ns	0.687	104-160
He		0.891	0.887	0.801	0.860	
Spl104	AF276173					
Na (private alleles)		14(2)	15(1)	14	14.000	57 °C/25
Ho		0.744ns	0.907ns	0.860***	0.830	184-248
He		0.900	0.910	0.905	0.899	
Spl105	AF276174					
Na (private alleles)		11	10	8(2)	10.250	59 °C/25
Ho		0.372***	0.370***	0.349***	0.431	104-180
He		0.864	0.847	0.785	0.836	
Spl113	AF276182					
Na (private alleles)		17	18	11	16.00	56 °C/35
Ho		0.488***	0.537***	0.465***	0.493	260-348
He		0.895	0.910	0.869	0.889	
Spl163	AF276205					
Na (private alleles)		14(3)	13	11	13.250	58 °C/35
Ho		0.953***	0.481***	0.535***	0.637	160-244
He		0.885	0.874	0.833	0.873	
Spl170	AF276213					
Na (private alleles)		14(6)	16(1)	13	14.500	58 °C/35
Ho		0.465***	1.000***	0.977***	0.827	200-264
He		0.903	0.907	0.888	0.899	
Spl173	AF276216					
Na (private alleles)		14(2)	17(1)	13	14.750	58.5 °C/30
Ho		0.442***	0.611***	0.581***	0.553	176-296
He		0.870	0.900	0.857	0.875	
Total of alleles (private alleles)		129(13)	140(3)	113(5)		
Ne		8.22	8.83	6.86		
Average						
Alleles		12.9	14	11.3	13.05	
Ho		0.607	0.706	0.672	0.665	
He		0.873	0.882	0.842	0.862	

Estimates of inbreeding coefficient or Fis values were positive and between LS-19, -0.108; LS-34, 0.202; LS-54, 0.304; LS-68, 0.311; Spl104, 0.075; Spl105, 0.563; Spl113, 0.188; Spl163, 0.240; Spl170, 0.095; Spl173, 0.378 (mean Fis =0.225; Fit=0.250), and and positive Fis values a relative dearth of heterozygotes and may be due to homozygotes. However, LS-19 had lower Fis and higher heterozygosity than all loci in the populations assayed.

Table 2. Pairwise estimates of genetic differentiation (via frequency) detected at 10 loci in stellate sturgeon samples, using F_{ST} values (above diagonal) and *Nm* (below diagonal).

	Samples	*Fst*		
		Ural	Volga	Sefidrud
	Ural	-	0.02	0.033
Nm	Volga	12.5	-	0.026
	Sefidrud	7.44	9.46	-

In all cases, significant deviations from Hardy-Weinberg equilibrium (p≤ 0.01) were only found at 2 loci, which were in Hardy-Weinberg equilibrium LS-68 in Volga and Sefidrud and Spl-104 in Ural and Volga samples. All departures from this equilibrium resulted from fewer heterozygotes than expected under equilibrium conditions (Table 1).

Pairwise population Fst values and estimates of Nm

The Nm and Fst via frequency ranged from 7.44 to 12.50 and from 0.020 to 0.033, with an average of 9.801 and 0.026, respectively (Table 2). In practice, Fst is rarely larger than 0.5 and often very much less. Fst, Rst and gene flow estimates in AMOVA indicated significant genetic differentiation among regions (P≤0.01), indicating that the populations were divergent from each other. Values of pairwise Rst among samples were consistently much higher (as much as an order of magnitude) than equivalent Fst values (Table 2) but differences were not significant. Nei's genetic identity ranged from 0.579 to 0.701. Consequently, Nei's genetic distance ranged from 0.356 to 0.546 (Nei, 1972; Table 3).

Table 3. Genetic Distance (Nei, 1972) detected at 10 loci in stellate sturgeon amples.

	Samples	Ural	Volga	Sefidrud
Genetic Distance	Ural	0.000		
	Volga	0.356	0.000	
	Sefidrud	0.546	0.406	0.000

Discussion

Although DNA-dependent methodologies such as microsatellite markers are important tools in fishery management and aquaculture, the application of population genetic data to management of the stellate sturgeon is at an early stage and little information exists about the genetic population structure subdivision. Ten out of fifteen primer sets designed originally from *Acipenser and Scaphirhenchus* DNA sequences (Table 1) amplified in *A. stellatus*.

These results suggest that there is evolutionary conservation of the flanking regions for these loci among related taxa. The cross-amplification between lake sturgeon, shovelnose sturgeon and stellate sturgeon is consistent with earlier findings related species (May *et al.,* 1997; McQuown *et al.,* 2000). Totally four sets of primers were not amplified in the PCR reaction. There is a significant and negative relationship between microsatellite performance and evolutionary distance between the species. The proportion of polymorphic loci among those markers that amplified decreased with increasing genetic distance (Cui *et al.,* 2005).

The average number of alleles per locus and observed heterozygosities were comparable in North and South Caspian Sea's populations as reported earlier in the RFLP analysis of the same populations by Shabani *et al.,* (2006). In fact, although the populations do not differ in the amount of genetic variation expressed as heterozygosity or alleles per locus, they are very different in the nature of the genetic variation, which depends on the private alleles and genotypes. Unfortunately, most commercial catched adults are being used for caviar production (Abdolhay and Baradaran Tahori, 2006) the losses of alleles and heterozygosity may increase with bottlenecking and inbreeding through time in the artificial propagation center stocks. On the other hand, reduced genetic diversity may increase the susceptibility to disease and other selective factors, resulting in further decline in population size (Shen and Gong, 2004). Regarding the fact that the natural propagation is very low in Caspian Sea for restocking, regular monitoring of genetic variability among the progenies is essential to avoid the loss of current polymorphism due to inbreeding and outbreeding problems.

At present study deviation from the *H-W* equilibrium observed in most loci and there were no significant differences in the average expected and observed heterozygosities among the populations (P>0.05). The significant deviations from *H-W* equilibrium could be explained either by sample bias or not using species specific primers, the presence of null alleles in these populations. In the presence of null alleles, heterozygotes possessing a null allele could be erroneously recorded as homozygotes for the variant allele leading to a deficiency of heterozygotes in the respective population. Similar results have been reported in lake and white sturgeons (Rodzen and May, 2002; McQuown *et al.,* 2003; Welsh and May, 2006), Chinese sturgeon (Zhao *et al.,* 2005) and It also may be related to sampling from mixtures of migrating population.

Fst and Rst are very commonly used to describe population differentiation at various levels of genetic structuring, (Balloux *et al.,* 2002). In our study, Fst in all sampling site was low but significant (P≤0.01), suggesting that at least three populations are genetically differentiated and do not represent a single panmictic population.

In fact, in the great majority of cases, Fst is low, because the effect of polymorphism (due to mutations) drastically deflates Fst expectations (Balloux *et al.,* 2002). In fish, negative correlation has been demonstrated between Fst values and dispersal capability (Waples, 1987). According to this, *A. stellatus* might present high dispersal capability presumably due to the absence of physical or ecological barriers to individuals. Feeding and spawning migrations are defined as continuous movements of fish from one part of the sea to another. However, the loss of genetic variability also might be caused by sampling error and releasing fingerlings with hatchery-origin returning to rivers to spawn may also contribute to the loss of regional genetic differentiation (Vasema¨gi *et al.,* 2005).

The genetic distance between populations has presented at Table 3. Shaklee *et al.,* (1982) and Thorpe and Sole-Cava (1994) showed that genetic distance values (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 the present study falls within the average value of congenerics, which indicates that the genetic difference among the studied populations is pronounced.

Conclusions

This study provides preliminary evidence for the existence of at lest three differentiated populations in the Caspian Sea included Ural, Volga and Sefidrud populations. Probably in each river more than one population exist (spring migration, fall migration …) and more samples from these rivers should be investigated to find out the exact population numbers. Characterizing the genetic structure of *A. stellatus* currently being used in the aquaculture industry will aid in for future bloodstock development and improve management plans that aim to conserve

diversity and minimize inbreeding in artificial propagation.

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