Original Article

A study of genetic population of *Alosa braschnicowi* (Borodin, 1904) in Sari and Mahmodabad coasts in the Caspian Sea, using Microsatellite loci

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Abstract: In this study, five Microsatellite loci were used to evaluate genetic diversity of *A. braschnicowi* between two populations of the Caspian Sea. Sixty samples were collected from the coasts of Mahmodabad and Sari (30 specimens for each population). Five Microsatellite loci were highly polymorphic among all samples. The number of alleles per locus observed ranged from 17 to 32 and averaged 14.1 alleles across two populations. The average observed heterozygosis in Mahmodabad and Sari were 57.1% to 60.1% and average observed heterozygosis between two populations was 58.9%. Among 10 population-locus (5 loci × 2 populations) only two tests were in the Hardy-Weinberg equilibrium, so highly deviation from the Hardy-Weinberg equilibrium was found. The average values of Fis and Nm were 0.33 and 14.19, respectively. Also AMOVA on base Fst index showed low genetic difference between two populations (2%), while the genetic diversity within population was 98%. Due to allelic diversity and estimates of heterozygosity, these markers can be useful in the genus *Alosa* for population level analysis in the Caspian Sea.

Introduction

The Caspian Sea is the largest lake in over the world. There are several species of fishes from Agnatha to Teleostomi in the Caspian Sea. *Alosa braschnicowi* (Borodin, 1904) from order Clupeiformes is one of important endemic fish in the southern Caspian Sea. All of the Caspian subspecies of A. *braschnicowi* are widely distributed in the sea but are found in limited number in the south over the winter, moving to the north to spawn in the spring and in the winter moves into deeper water of the Iranian coast (Vetchanin, 1984).

The shores of the Caspian Sea are very different based on ecological conditions such as temperature, salinity, morphology, hydrology, geography. These factors can influence the populations of fishes including *A. braschnicowi*. Generally, the species and subspecies of *Alosa* in the Caspian are larger than their related species in the Black Sea basin. These observations are attributed to the variable environment of the Caspian Sea over time, with repeated changes in salinity and temperature (Coad, 2013). There is no evidence of genetic diversity of this genus in the Caspian Sea.

Microsatellite DNA markers or simple sequence repeats (SSRs) are tandem repeated motif of 1-6 bases found in all prokaryotic and eukaryotic genomes that have been utilized in the assessment of genetic variation and population differentiation studies for a variety of vertebrates (O'Connell and Wright, 1997; Neff and Gross, 2001). Among the molecular markers available in population genetics, microsatellites are preferred due to their abundance in genomes, high levels of polymorphism, even distribution, small locus size facilitating polymerase chain reaction (PCR)-based genotyping and co-

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Table 1. Characteristics of microsatellite loci used in the present study

Locus	GenBank Accession no.	Size (bps)	Primer sequence (5′→3′)	No. of alleles	Annealing (°c)
*Asa*D030	EF014998	104-144	F:CCACAGCATCATCTCTTTACTG R: ACCTTGAATTTCTCCTTGGG	17	55
*Asa*D042	EF015000	128-192	F: ACTGGTCAATTGTAAGACACCC R:CAAGATGACCAAGGGTTAAGAC	25	50
*Asa*C249	EF014994	236-324	F:TTATTACAACGGTGAATTGAGTG R: TAAGTGCATGTTGTGTGTGATG	37	53
*Asa*C051	EF014992	260-356	F: GTAAGTCGCTTTGGACTACCAG R: TCTAAATGCCCAGGTAAAGATG	32	53
*Asa*C059	EF014993	292-388	F: CTTGGACTTACAATGCTTTTGG R: AGCAAGTGTGGAGTCAGTCG	30	53

dominant inheritance pattern (Neff and Gross, 2001; Chen et al., 2008). Genetic diversity of populations or species is a result of their evolutionary history and its decrease can reduce their adaptability and surviving potential in a changing environment (Lucentini et al., 2009). Also genetic diversity is an important factor for the conservation of endangered species (Na-Nakorn et al., 2006). *Alosa braschnicowi* is a key species in ecological pyramid and food chain. Over fishing, pollution, destroying habitats declined its population. Thus studying its genetic diversity and genetic population as one of important endemic species of the Caspian Sea may help to understand how crucial the status of this species in the Caspian Sea is.

Materials and methods
Sample collection and DNA extraction: A total of 60 specimens were collected from two regions in the coasts of Mazandaran Province, Mahmodabad and Sari (30 specimens from each region). Fin tissues were obtained from each sample and preserved in 96% ethanol. DNA was extracted using standard phenol/chloroform extraction protocol and then the DNA extract was stored at −20 °C until use (Hillis et al., 1996). DNA was evaluated for quality and quantity on 1% agaros gels and spectrophotometry (King et al., 2001).
Polymerase chain reaction and electrophoresis: Five microsatellite loci, AsaD030, AsaD042, AsaC249, AsaC051 and AsaC059 (Table 1) were used (Julian and Bartron, 2007). PCR amplification was carried out in 0.2 ml PCR tubes with an Eppendorf thermal

cycler (BIO-RAD, MJ Mini Thermal Cycler). Amplifications were performed with a reaction volume of 25 μL component (Geng et al., 2006). The profile of thermal cycling was as follows: a pre-denaturation for 3 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at the selected higher annealing temperature, 30 s at 72°C, and 5 min at 72°C.
PCR products were run on 10% polyacrylamide gels stained with silver nitrate (Rajora et al., 2000). A 50 bp molecular weight marker (Fermentas) was used as the molecular weight standard. Microsatellite allele lengths were estimated using Gel-Pro Analyzer 3.9 software (Gene, USA).
Data analysis: Scoring errors, large allele dropout and null alleles were checked using the Micro-checker (Oosterhout et al., 2004). Deviation from Hardy-Weinberg expected proportions and linkage disequilibrium between pairwise loci were analyzed using Genepop (version 3.4) (Raymond and Rousset, 2003). At this software Markov chain method was used to estimate the probability of significant deviation setting the parameters, de memorization = 10000, batches = 100 and iterations per batch = 5000. Additionally, to determine if deviations from Hardy-Weinberg equilibrium (HWE) were in the direction of heterozygote excess or deficit, HWE tests were carried out for all loci in each population. The inbreeding coefficient (Fis) and its significance were calculated using FSTAT 2.9.3 (Goudet, 2001). All cases with multiple tests, significant levels were adjusted using the sequential Bonferroni correction (Rice, 1989). Allele number (Na), expected heterozygosity (He), observed heterozygosity (Ho),

Table 2. Genetic diversity parameters for ten microsatellite loci in *A. braschnicowi*

		*Asa*C059	*Asa*C051	*Asa*C249	*Asa*D042	*Asa*D030
Mahmodabad	Na	17	15	20	10	10
	Ne	9.333	10.248	12.645	7.000	7.293
	Ho	0.321	0.714	0.571	0.536	0.714
	He	0.893	0.902	0.921	0.857	0.863
	Fis	0.651	0.226	0.395	0.391	0.190
	pHw	***	***	**	***	ns
Sari	Na	13	17	17	15	7
	Ne	8.809	10.814	9.800	11.529	4.962
	Ho	0.393	0.714	0.500	0.571	0.857
	He	0.886	0.908	0.898	0.913	0.798
	Fis	0.569	0.230	0.458	0.390	-0.055
	pHw	***	***	***	***	ns

Na = number of alleles; Ne = number of effective alleles; Ho = observed heterozygosity; He =expected heterozygosity; Fis = Fixation index; pHW = Hardy-Weinberg probability test: *$P <0.05$; **$P <0.01$; ***$P <0.001$; ns = not significant were corrected with the sequential test of Bonferroni (Rice, 1989); Significant Fis values in bold.

effective number of alleles (Ne), gene flow (Nm, = $[(1/FST) - 1]/4$) between populations were calculated using GenAlex 6.3 (Peakall and Smouse, 2006). The significance of differences among Na and He values of populations were examined using a Wilcoxon-Mann-Witney test performed in SPSS. An analysis of molecular variation (AMOVA) on base Fst index (Weir and Cockerham, 1984) was used to examine the regional structure of genetic variation within and among populations. AMOVA was carried out using GenAlex 6.3 (Peakall and Smouse, 2006). Unbiased genetic identity (I) and genetic distance (D) were calculated according to Nei (1978) using Popgene 1.0 (Yeh et al., 1999). Popgene was also used to construct an UPGMA phylogenetic tree based on the Nei' genetic distance data. Significance of an excess or defect of heterozygosity was estimated using the Wilcoxon test.

Results

A five microsatellite loci analyzed had high polymorphism. Micro-checker showed no evidence for large allele dropout or stutter-band scoring at any of the five loci but null alleles can exist in all loci. Allele distribution and effective number of alleles as well as expected and observed heterozygosity are presented in Table 2. Across all loci, 141 different
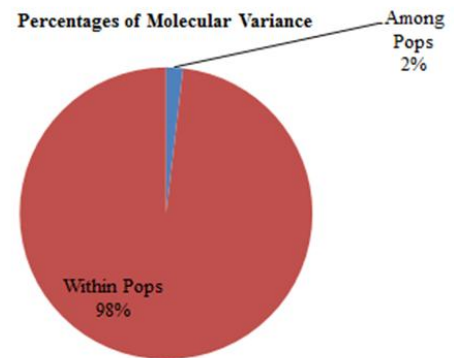

Figure 1. Scatter plot for relationship between the spawning latency and body weight of snow trout (group 1).

alleles were found within populations. The average alleles number of all loci was 14.1, ranging from the lowest 7 alleles for AsaD030 (in Sari) to the highest 20 alleles for AsaC249 (in Mahmodabad). The average number of effective alleles per locus (Ne) in Mahmodabad and Sari were 14.4 and 14.1, respectively, showing no significant difference ($P>0.05$) between two populations (Wilcoxon-Mann-Whitney test). The expected heterozygosity (He) was high, ranging from 0.80 to 0.92. The average observed heterozygosity (Ho) was the highest in Sari (0.607), followed by Mahmodabad (0.571). There was no significant difference in the average expected and observed heterozygosity between the populations (Wilcoxon-Mann-Whitney test). The highest and lowest value of observed

Table 3. Nm and Fst index of five microsatellite loci in two populations for *A. braschnicowi*

	*Asa*C059	*Asa*C051	*Asa*C249	*Asa*D042	*Asa*D030
Fst	0.015	0.021	0.012	0.024	0.020
Nm	16.607	11.825	20.371	10.206	11.950

Table 4. Nei (1978) genetic similarity (above diagonal) and genetic distance (under diagonal)

Population	Mahmodabad	Sari
Mahmodabad		0.83
Sari	0.18	

heterozygosity (0.857, 0.321) were found in locus AsaD030 (for Sari) and AsaC059 (for Mahmodabad) populations, respectively. There were significant deviations from Hardy-Weinberg equilibrium at most of the loci in the two populations. After sequential Bonferroni correction (Rice, 1989), among the 10 population-locus tests (2 populations × 5 loci) only two tests followed the Hardy-Weinberg equilibrium (Table 2). The average content of Fis index was 0.330 across all loci and ranged from -0.055 (AsaD030 loci in Sari) to 0.651 (AsaC059 loci in Mahmodabad). Most Fis values at the five loci estimated for two populations were significantly different ($P<0.05$) in heterozygosity deficiency condition except for AsaD030 locus in Sari population. The result of Genepop software did not show linkage disequilibrium after Bonferroni correction. Although analysis of pairwise genetic differentiation revealed that Fst values were small (Table 3). AMOVA showed a low percentage of variation among the regions (2%). The content of variation within populations 98% explained the majority of the variation. In addition, the content of genetic identity (I) and genetic distance (D) were obtained 0.83 and 0.18, respectively. In accordance with low percentage of variation among population, results revealed high levels of gene flow, average Nm values was 14.19.

Discussion

Biological population variation is an important foundation for evaluating species resources. In the population genetic studies, researchers use several parameters of diversity and one parameter will provide a correct judgment on genetic diversity of populations.

Na, Ne, Ho, He and PIC are all parameters of population genetic variations. The value of these parameters varies with the abundance. Results showed that the maximum and minimum number of alleles were in AsaC249 and AsaD030, respectively. In terms of observed heterozygosity, our results were higher than the average for freshwater fish (Ho = 0.54 ± 0.25, Dewoody and Avise, 2000). Also the mean number of alleles are slightly higher than that reported for fresh water fish (Na = 9.1 ± 6.1, Dewoody and Avise, 2000) and anadromous fish (Na = 10.8 ± 7.2, Dewoody and Avise, 2000). In the genetic population studies allele number is used to determine diversity and is preferred to heterozygosity, because allele number may be lost faster than genetic heterozygosity loss because low frequency alleles contribute little to overall heterozygosity (Lind et al., 2009). The genetic bottleneck could be an important factor for reduction of the number of alleles without having an obvious effect on heterozygosity (Lundrigan et al., 2005). The allelic diversity and heterozygosity are both indicative of genetic variation, but allele number is dependent on the effective population size much more than that of heterozygosity (Nei et al., 1975). Therefore, allele number can be a suitable estimate of genetic diversity in populations for enhancement, conservation or selection programmes (Diz and Presa, 2009), although there were no statistical differences between populations. At the present

study, some diversity indices such as the average values of the allele number and observed heterozygosity had a higher value than those in the study of *Alosa fallax* (μ=4.50, Ho= 0.445) and *A. alosa* (μ=4.88, Ho= 0.560) (Faria et al., 2004). There are several reasons for the high diversity in *A. braschnicowi* such as difference between number of specimens, the type of marker used and the species. The results obtained from GenAlex 6.3 (Peakall and Smouse, 2006) indicated that the mean value of Ho was lower than that of He (Ho= 0.59, He= 0.88). A high gene flow, mistake in reading alleles and a low number of specimens are some reasons for a low Ho (Li et al., 2009, Skalla et al., 2004).

Deviation from the Hardy-Weinberg expectations may be related to a small sample size, the presence of migration, genetic drift, inbreeding propagation and selection programs (Lucentini et al., 2006, Zhao et al., 2005, Dahle et al., 2006, McQuown et al., 2003, Liu et al., 2005). This species do not have any selection programs in Iran, so the deviation from the Hardy-Weinberg equilibrium may be related to the reasons referred earlier. The values obtained of inbreeding index (Fis) showed significant deficiency in heterozygosity at the all loci except AsaD030 in Sari population. The biological reasons of these shortages are not well known and many factors may be involved including null alleles, inbreeding, natural features of markers and selection (Raymond et al., 1997, Xu et al., 2001). Based on Micro-checker results, the appearance of null alleles could be the major reason for deficiency of heterozygosity at these loci. The presence of inbreeding increases the proportion of homozygot individuals. The AMOVA showed weak evidence for differentiation between populations. The average Fst were obtained 0.018. The Fst value of 0 to 0.05 indicates a low level of genetic differentiation (Wright, 1978). Our results showed high Nm between populations and may be an agent factor for low genetic diversity between populations. The genetic identity was 0.83. Thorpe (1982) calculated the values of genetic identity for different phylogenetic levels in vertebrate taxa.

Populations that belong to the same species have a genetic identity (I) between 0.80 and 0.90, on the other hand, the species that are belonged to the same genus, genetic identity values are between 0.35 and 0.85. The identity values obtained at the present study falls within the ranges of conspecific organism.

Conclusion

The present study is the first report on genetic population of the genus *Alosa* (*A. braschnicowi*) from the order Clupeiformes in the southern coasts of the Caspian Sea. Microsatellites are appropriate instruments in genetic population studies. All five microsatellite loci used in this study were highly polymorphic (100%). Hence, our primers are suggested for other researches on species of *Alosa* in the Caspian Sea. Our data showed that genetic diversity of *A. braschnicowi* was rather high in the Mazandaran coasts of the Caspian Sea, but genetic variation between two populations was very low (2%). Some reasons that may explain this low genetic variation (high gene flow) are: overfishing, oil extraction, habitats destruction, development of industries around the sea, wastewater and thermal pollution.

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