Warning of Reducing Genetic Diversity of *Abramis brama* (Berg, 1905) in Gilan Coast Using SSR Markers

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Abstract

Genetic diversity is one of the three levels of biodiversity. The aim of present study was to compare levels of genetic polymorphism between wild Bream populations using seven microsatellite loci. Genetic diversity was investigated by studying samples collected from two regions, the coast of Chamkhale and Bandaranzali of Gilan province. A total of seven microsatellite loci (MFW1, MFW26, Mcs1EH, Rser10, Bl1-153, Bl1-114 and IC65) were used. The average number of alleles in Chamkhale and Bandaranzali coast were 10 and 10.71 alleles, respectively. The numbers of effective alleles were 7.05 and 7.74 alleles in each population. Allele frequency was declined in wild fish due to inbreeding and genetic drift. The mean of observed heterozygosity values were 0.66 and 0.70 in Chamkhale and Bandaranzali coast, respectively. Approximately, all of loci showed deviation from Hardy-Weinberg equilibrium. The genetic similarity and distance between the two populations were 0.316 and 0.684, respectively. The results of Molecular Variance Analysis revealed that genetic diversity within locations was 97 percent, while among them was 3 percent. The FST value was 0.024 that indicates the low genetic differentiation between the two locations which could be explained by the low number of alleles in two populations. Furthermore, the Natural Migration (Nm) between two stations was obtained 16.30. According to the analysis, it seems that *Abramis brama* has not a desirable genetic diversity in the investigated regions.

**Keywords:** Bream (*Abramis brama orientalis*), Microsatellite, Genetic diversity, Polymorphism

Introduction

There are several species of fishes from Agnatha to Teleostomi in the Caspian Sea, the largest lake in the world. The *Abramis brama orientalis* (Berg, 1906) from order cypriniformes is one of important endemic fish in the southern Caspian Sea. Furthermore, it habitat in the Anzali wetland and the neighboring Caspian coast (Kiabi, Abdoli and Naderi 1999). Considerable differences have been seen among the Caspian coast based on ecological conditions such as temperature, salinity and morphology. These factors can influence the populations of fishes including *A. brama*. Nevertheless, evidence indicate that there is loss genetic diversity among them (Ghasemi et al, 2007; Keivanshokooh and Ghasemi, 2009). Species ability to survive in the nature is determined by genetic variation that affects their ability to adapt environmental changes. Thus, genetic variation is necessary for the species survival and resistance (Bataillon et al., 1996). 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). This is mainly due to high frequency in genome, Mendelian inheritance, being semi-dominant, small loci size, ease to determine genotype by polymerase chain reaction (PCR) and great polymorphism (Chen et al., 2008; Dewoody and Avise 2000). Recently, microsatellites have been extensively used to evaluate genetic diversity and structure of wild fish species, such as *Liza aurata* (Ghodsi et al., 2011), *Rutilus rutilus caspicus* (Rezae et al., 2009), *Paraschistura bampurensis* (Askari and Shabani., 2013) and *Alosa braschnicowi* (Jafari et al., 2014). Since there is no specific SSR primers for these species from gene banks, we used 7 SSR primer pairs from specific species belongs to Cyprinidae. Nowadays, overfishing and degradation of natural environment in Iran resulted in a sharp decline in population of *A. brama*.

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Hence, they need to be protected via a restocking program (Coad, 2014). Restocking programs are a managerial strategy, in which mature individuals are caught from wild and propagated under controlled conditions. Then, fries are released to their natural habitats and this process is repeated next years (Fiumera et al., 1999). Therefore, Iranian Fisheries Organization (Shilat) tried to increase bream populations through the release of artificially bred fry. In 1986, this artificial reproduction performed using only a single pair of spawnsed for giving eggs and milt. Bream population increased annually using the generations descended from the same pair. For the prevention of loss genetic heterozygosity through inbreeding, Ghasemi et al (2007) and Keivanshokooh and Ghasemi (2009) offer Shilat to import bream stocks from Azerbaijan. Currently, stock rehabilitation program of Abramis brama is conducted by releasing artificially propagated fries with Azerbaijan stocks to the Caspian Sea. Now, after several years of implementation of conservation program, the aim of this study is to compare the levels of genetic polymorphism of Bream between the coast of Chamkhale and Bandaranzali of Gilan province. The results of this study will have implications for the conservation of genetic resources of this species. Such information is essential for effective culturing, management and conservation of fish populations. Based on this goal and to make decisions on the brood stock to be used for hatcheries, this work could be used as a guideline of the Iranian Fisheries Organization for a successful repopulation.

Materials and Methods

Samples Collection and DNA Extraction

In Autumn 2012, A total of 60 specimens were collected from two station, Chamkhale (37° 11’N, 30° 10’E) and Bandaranzali (37° 28’N, 49° 28’E) costal of Gilan province (30 specimens from each station). Total genomic DNA was extracted from fin pectoral tissue by using the traditional proteinase-K digestion and standard phenol/chloroform techniques (Hillis et al., 1996). Approximately, 100 mg tissue was treated with 25μl proteinase K (10 mg/ml) and 50μl sodium dodecyl sulfate (SDS) (10%) in a 500 μl Sodium Chloride-Tris- Ethylenediamine tetra acetic acid (STE) buffer (0.1 M NaCl, 0.05 M Tris and 0.01 M Na2EDTA, pH: 8.0) overnight at 37°C. After incubation, DNA was isolated by two phenol-chloroform (25 phenol: 24 chloroform: 1 isoamyl alcohol) steps followed by precipitation with cold absolute ethanol. The quality and concentration of DNA were assessed by 1.0% agarose gel electrophoresis and stored at -20 °C until use (King et al., 2001).

Molecular Analysis

In this study, seven microsatellite markers were amplified by polymerase chain reactions (PCR) using the following primers (Hosseini et al., 2014): (Rser10, Ic654, Bl1-114, Bl1 -153, Mcs1EH, MFW7 and MFW26) (Table 1). PCR amplification were carried out in 0.2 ml PCR tubes with an Eppendorf thermal cycler (BIO-RAD, MJ Mini Thermal Cycler). Initial denaturation was achieved at 94°C for 3 min followed by 30 denaturation cycles for 30 s at 94°C, 30 s at the respective annealing temperatures extending to 72°C for 1 min. The final step was extended to 3 min at 72°C. PCR products were separated using 8% polyacrylamide gels stained with silver nitrate (Rajora et al., 2000).

Statistical Analysis

The presence of null alleles was tested using Microchecker version 2.2.3 (Van Oosterhout et al., 2004). Allelic and genotypic frequencies, observed (Ho), expected heterozygosity (He), test for deviations from Hardy-Weinberg Equilibrium (HWE), Fst values and number of migrant (Nm) were calculated by Genealex ver. 6.5 Software (Peakall and Smouse, 2012). Deviation distribution, genetic distance, genetic identity (Nei, 1978), Difference in Ho, He and allelic variation was determined by Wilcoxon’s test using statistical software, SPSS v. 16.

Results

In this study, structure and genetic diversity of A. Brana was studied at seven microsatellite loci for two stations in Iran. 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 sizes ranged from 100 to 276 bp (Table 1). The Bl1-114, MFW26 and Rser10 primer showed the maximum allele number (13) compared to other primers. No statistically significant difference was observed between the numbers of alleles per each locus (Table 2). The average number of effective alleles per locus (Ne) in Chamkhale and Bandaranzali were 7.05 and 7.74, respectively, showing no significant difference (P>0.05) between two populations (Wilcoxon-Mann-Whitney test).
Table 1. Characteristics of *Abramis brama* microsatellite loci used in the present study

<table>
<thead>
<tr>
<th>Microsatellite locus</th>
<th>Primer sequence (5→3)</th>
<th>N</th>
<th>Size (bp)</th>
<th>Annealing (˚C)</th>
<th>Gene bank ID number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bl2-114</td>
<td>F:ATCACTGCCATTTTTAATCCA; R:CTGCTCCGCTCTGTTCCA</td>
<td>13</td>
<td>168-272</td>
<td>52</td>
<td>FJ468352</td>
</tr>
<tr>
<td>Bl1-153</td>
<td>F:GCACAGCTCTAATCGGCTACT; R:TATGGTCAAAACGGGCTAAA</td>
<td>8</td>
<td>200-276</td>
<td>53</td>
<td>FJ468350</td>
</tr>
<tr>
<td>Mcs1EH</td>
<td>F:ACCGGGCTTCTAGTGTTTGCTCA; R:TGAGACACATCCCATCACTGACTTCGAG</td>
<td>9</td>
<td>100-200</td>
<td>59</td>
<td>AY770926</td>
</tr>
<tr>
<td>Ic654</td>
<td>F:GTAGCGGAATAAGAAACAGAGC; R:GACAAAGTGACACGACAGAATG</td>
<td>9</td>
<td>128-172</td>
<td>52</td>
<td>EU252096</td>
</tr>
<tr>
<td>MFW7</td>
<td>F:CTCACCTGACGGATGCTG; R:ATCCCTGACATGAGGACACT</td>
<td>7</td>
<td>160-200</td>
<td>61</td>
<td>-</td>
</tr>
<tr>
<td>MFW26</td>
<td>F:CCCTGAATGAAACAACCAGTG; R:CACATGCGGTGACGGAGGAG</td>
<td>13</td>
<td>100-148</td>
<td>48</td>
<td>-</td>
</tr>
<tr>
<td>Rser10</td>
<td>F:TGCGTAATCGTGAAGCGGTCG; R:GCCACTAAAGCGCAGAAGCC</td>
<td>13</td>
<td>160-232</td>
<td>57</td>
<td>AJ312850</td>
</tr>
</tbody>
</table>

Table 2. Genetic diversity parameters for seven microsatellite loci in *A. Brama*

<table>
<thead>
<tr>
<th>Location</th>
<th>Bl2-114</th>
<th>Bl1-153</th>
<th>MF17</th>
<th>MF16</th>
<th>Rser10</th>
<th>IC654</th>
<th>Mcs1EH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni</td>
<td>12</td>
<td>13</td>
<td>8</td>
<td>7</td>
<td>13</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Ho</td>
<td>0.70</td>
<td>1.00</td>
<td>0.55</td>
<td>0.30</td>
<td>0.65</td>
<td>0.70</td>
<td>0.75</td>
</tr>
<tr>
<td>He</td>
<td>0.894</td>
<td>0.901</td>
<td>0.824</td>
<td>0.701</td>
<td>0.880</td>
<td>0.786</td>
<td>0.850</td>
</tr>
<tr>
<td>Fis</td>
<td>0.217</td>
<td>-0.110</td>
<td>0.332</td>
<td>0.615</td>
<td>0.261</td>
<td>0.110</td>
<td>0.118</td>
</tr>
<tr>
<td>Fis</td>
<td>0.217</td>
<td>-0.110</td>
<td>0.332</td>
<td>0.615</td>
<td>0.261</td>
<td>0.110</td>
<td>0.118</td>
</tr>
<tr>
<td>Nst</td>
<td>13</td>
<td>13</td>
<td>10</td>
<td>7</td>
<td>13</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Ho</td>
<td>0.85</td>
<td>0.85</td>
<td>0.90</td>
<td>0.60</td>
<td>0.85</td>
<td>0.55</td>
<td>0.65</td>
</tr>
<tr>
<td>He</td>
<td>0.906</td>
<td>0.896</td>
<td>0.859</td>
<td>0.803</td>
<td>0.901</td>
<td>0.818</td>
<td>0.838</td>
</tr>
<tr>
<td>Fis</td>
<td>0.062</td>
<td>0.052</td>
<td>-0.048</td>
<td>0.252</td>
<td>0.057</td>
<td>0.327</td>
<td>0.224</td>
</tr>
</tbody>
</table>

The number of observed allele (Na), Ne, observed heterozygosity (Ho), expected heterozygosity(He) and fixation index (Fis) are shown in Table 2. Hs and expected He means for all samples were 0.50-1.00 and 0.701-0.906 respectively. In the Chamkhole sample, the mean for heterozygosity and expected heterozygosity values were 0.664 and 0.833, respectively. In the Bandaranzali samples, these values were 0.75 and 0.860, respectively, but there was no significant difference in the average expected and observed heterozygosity between the populations (Wilcoxon-Mann-Whitney test). All seven loci were tested for deviation from the HWE (Table 2). There were significant deviations from Hardy -Weinberg equili-brum at most of the loci in the two populations. After sequential Bonferroni correction (Rice, 1989), among the 14 population-locus tests (2 populations × 7 loci) six out of 14 possible HWE tests were statistically significant. Fixation index (Rst , Fst) were calculated 0.010 and 0.024 between two wild fish populations; respectively. These values were calculated according to the formula [Nm = [(1 / Fs) - 1] / 4] between populations. The mean Nm was obtained as 15.509, between Chamkhole and Bandaranzali station, and the minimum and maximum amount calculated for loci MFW7 and loci Bl1-153 were 0.138 and 15.978, respectively (Table 3).

Table 3. Fst and Nm for tested

<table>
<thead>
<tr>
<th>Location</th>
<th>Bl2-114</th>
<th>Bl1-153</th>
<th>MF17</th>
<th>MF16</th>
<th>Rser10</th>
<th>IC654</th>
<th>Mcs1EH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fst</td>
<td>0.016</td>
<td>0.015</td>
<td>0.038</td>
<td>0.007</td>
<td>0.015</td>
<td>0.016</td>
<td>0.039</td>
</tr>
</tbody>
</table>

http://jcmr.fum.ac
The average content of $F_{st}$ index was 0.223 across all loci. Furthermore, the analysis of molecular variance and index $F_{st}$ in 99% showed the high genetic diversity (97%) within populations and the low genetic variation among populations (3%) (figure 1).

![percentage of genetic diversity](image)

**Figure 1.** The distribution of genetic diversity on $F_{st}$ standard

Genetic distances and similarities (Nei et al., 1975) computed between the Chamkhale and Bandarananzali fish populations were 0.316 and 0.684, respectively (table 4).

<table>
<thead>
<tr>
<th>Area</th>
<th>Chamkhale coast</th>
<th>Bandarananzali coast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chamkhale coast</td>
<td></td>
<td>0.68</td>
</tr>
<tr>
<td>Bandarananzali coast</td>
<td>0.31</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.** Genetic identity (regular font) and genetic distance (bolded font) of *Abramis brama* originated from Chamkhale and Bandarananzali coast

**Discussion**

Genetic diversity is important for ecological and evolutionary processes ranging from individual fitness to ecosystem function. Heterozygocity and allele number are important parameters in population genetic variation which determine the ability of organism to compete and survive in natural habitats (Reed, 2009; Hakansson and Jensen, 2005; Frankharn, 2008; Diz and Persa, 2009). In genetic variation studies, allelic richness is more worthy than heterozygosity. In fact, higher allelic richness shows higher effective population size and use of allelic richness is suitable for populations which are treated by selection or conservation programs (Diz and Persa, 2009).

In the case of allele number, Ho and He, the present study is in line with the previous report on *Abramis brama* with the different primers (Keivanshokooh and Ghasemi, 2009). The results of this study indicated that the average number of alleles per locus and observed heterozygosity were comparable, but there was no significant difference between two populations ($p>0.05$). In our result, observed heterozygosity were higher than the average for freshwater fish ($Ho = 0.54 \pm 0.25$, Dewoody and Avise, 2000). The mean number of alleles are little more than that noted for fresh water fish ($Na = 9.1 \pm 6.1$, Dewoody and Avise, 2000) and anadromous fish ($Na = 10.8 \pm 7.2$, Dewoody and Avise, 2000). 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). The Iranian Fisheries Organization produces and releases up to 15 million bream fry into the Anzali wetland annually. Because the hatchery population used for restocking has been founded with a small effective number of parents (Ne), it is likely that these populations have lost some alleles during restocking. Loss of allelic variation has also been reported with (Ghasemi et al., 2007; Keivanshokooh and Ghasemi, 2009). 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, and low frequency alleles contribute little to overall heterozygosity (Lind et al., 2009). It seems that artificial interbreeding of the Iranian and Azeri broodfish in hatcheries slightly improve the genetic heterozygosity of the Iranian stocks. Unfortunately, number of alleles is not satisfying. As showed in table 2, Ho was less than He ($Ho= 0.70$, $He= 0.83$). A high gene flow and a low number of specimens are some reasons for a low Ho (Li et al., 2009, Skalla et al., 2004).

Eight of 14 tests showed a significant deviation from HWE. Beyond the hypothesis of null alleles, genetic drift and inbreeding are likely to be the causes for deviation from the H–W disequilibrium. In addition, the values obtained from inbreeding index ($F_{st}$) showed significant deferrrence ($p< 0.05$), indicating increased inbreeding and nonrandom mating (Xu et al., 2001).

Analysis of molecular variance (AMOVA) is a suitable criterion to assess population structure, and to determine the differentiation and genetic similarity between populations (Grassi et al., 2004). According to our obtained $F_{st}$ index, the genetic diversity between the two populations was 3%. The $F_{st}$ index mean was about 0.024, which represents the low differentiation between two populations. According to Wright (1987), $F_{st}$ value of less than
0.05 indicates low differentiation among communities.
In this study, number of migrant’s average was reported as 15.50, that was in line with previous study on Abramis brama populations which had Nm = 16.30 (Keivanshokooh and Ghasemi, 2009). Li et al. (2007) reported that when Nm> 1, it can be an agent factor for number of migrant and low genetic diversity between populations.
In present study, the genetic identity was 0.68. Shaklee et al, (1982) and Thorpe and Sol-Cave (1994) showed that genetic distance values (Nei, 1972) for censpecies populations averaged 0.05 (range: 0.002- 0.07), averaged 0.30 for congeneric species (range: 0.03-0.61), and ranged from 0.58 to 1.21 for confamilial genera. The genetic distance between the Chamkhale and Bandaranzali populations falls within the range of congeneric species, suggesting their genetic convergence.

Conclusion

The results of this study suggested that genetic diversity of A. brama was rather high in Gilan coasts of the Caspian Sea, but genetic variation between two populations was very low (3%). It seems that mode of reproduction and no appropriate release of larvae are main reasons for genetic differentiation and also, genetic bottleneck in the Bream populations. Restoration of rivers where this species spawned there and providing natural reproduction of brood stocks are best ways for preventing this process.

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References


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