Genetic Diversity and Population Structure of *Liza klunzingeri* from the Northern Persian Gulf Based on AFLP Analysis

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**Abstract**

The main purpose of this study was to investigate the genetic diversity and population structure of *Liza klunzingeri* in two regions of the Persian Gulf. In this study, the amplified fragment length polymorphism (AFLP) was employed to analyze population genetic diversity between two populations (Ziyarat & Hendijan). Seven primer combinations (E-AAG/M-CTA, E-ACT/M-CAA, E-ACT/M-CTA, E-AAG/M-CTG, E-ACT/M-CAT, E-AAG/M-CAC and E-AAG/M-CAG) were used. A total of 358 fragments were identified from 40 individuals (two populations), with percentage of polymorphic bands (PPB) being 86.63% at the population level, respectively. The average gene diversity was 0.2528 and Shannon’s information index was 0.4030. Coefficient of gene differentiation between populations (GST) was 0.0585. Analysis of molecular variance (AMOVA) revealed high genetic variations within populations (90%). The estimated number of migrants per generation (Nm) was 8.0419, indicating a high level of gene flow among populations. The UPGMA dendrogram clustered all 40 individuals into 2 groups. In some cases individuals from the same region were grouped together, but in most cases, gene exchange was observed to be common among the groups. To study the genetic relationships among populations, a principal coordinate analysis (PCoA) based on Nei’s genetic distances was performed. Results of this study showed that AFLP marker can be a useful tool for investigating the genetic diversity of *Liza klunzingeri* genotypes.

**Keywords:** *Liza klunzingeri*, Persian Gulf, Population genetic, AFLP

1. Introduction

The species belong to Mugilidae family, are distributed in various coastal aquatic habitats of the world’s tropical, subtropical and temperate regions. This family includes 17 genera and 72 species (Nelson, 2006). Mullet comprises diverse species and there are small differences between them; therefore, different classifications of them, inevitably come up with different results. One species of the mullet family which is valuable regarding its fisheries catch over the coasts of the south-western province of Khuzestan is *L. klunzingeri* (Kashi et al., 2007). This fish is distributed from the Persian Gulf to the Indian Ocean, Arabian Sea and the Gulf of Oman (Randall, 1995). *L. klunzingeri* is usually caught by purse seines, set nets, and gill nets.
Faqih Ahmadani et al. / Genetic Diversity and Population Structure of Liza klunzingeri...

It can be found in various coastal substrata, brackish water and highly saline lagoons (Golani et al., 2002). *L. klunzingeri* has mainly been studied morphemically and anatomiccally (Ismail et al., 1998; Hakimelahi, M., et al., 2011; Kashi et al., 2007; Valinasab et al., 2005). Yet, there is limited information on the genetic diversity and genetic structures of the existing populations of this species, though availability of such data is vital for biodiversity conservation, management of natural resources and fisheries.

Molecular markers are extensively employed to reveal the genetic relationships of geographic fish populations (Song et al. 2006). The informative amplified fragment length polymorphism (AFLP) (Vos et al. 1995) is a highly replicable method with significant reliability (Jones et al. 1997) and great potentiality for yielding a considerable number of polymorphic loci in a single PCR experiment for the estimation of the genetic variation among and within populations (McMillan et al. 2006). AFLP analysis is feasible without access to the sequence information in advance, thus making the research on some species possible without complete molecular and biological data being available (Han et al. 2000). In this study, two sample geographic populations of the *L. klunzingeri* were collected from the coastal waters of the Persian Gulf and the samples were analyzed by means of AFLP to determine the population genetics of the fish. This may provide the basic information required for conservation and management of the species.

2. Materials and Methods

2.1. Fish Samples

*Liza klunzingeri* (n=40) from two locations were used in this study (Fig.1). The samples were mainly collected from the coastal areas of the Persian Gulf within the Mond estuary and Bahrekan region. All individuals were then identified based on morphological characteristics, and a piece of muscle tissue was obtained from each individual and preserved in 95% ethanol or frozen for DNA extraction.

2.2. Genomic DNA Extraction

Total DNA was extracted from 50 mg fin tissue from the ethanol-preserved samples according to Peter et al., (1993) with some modifications. Tissue was incubated in 700 µl lysis buffer (100 mM Tris-HCl, pH 7.5, 1.4 M NaCl, 50 mM EDTA, 1% SDS, 1% CTAB and 0.2% β-mercaptoethanol) containing 7 µl of proteinase K (20 mg/ml). The mixture was incubated at 55°C for 6 hours and 600 rpm. DNA was organically extracted with chloroform and precipitated with one volume of cold isopropanol. The DNA pellet was washed with 70% ethanol, air-dried for 10 - 20 minutes, resuspended in 50 - 100 µl ddH2O and preserved at 4°C for short time or -20°C long time.

![Fig. 1: Map showing sample locations of Liza klunzingeri.](image-url)
2.3. AFLP Analysis

The AFLP protocol was performed according to Vos et al. (1995) with some modifications to the manufacturer’s instructions. Genomic DNA (300 ng) was digested with 0.25 µL EcoRI (10 U/µL) and 0.25 µL MseI (10 U/µL) restriction enzymes EcoRI at 37°C for 2 hours and restriction enzymes MseI at 65°C for 2 hours in a 20 µL volume. DNA fragment was ligated by 5µl of ligation solution contained 5 µM of EcoRI (0.5µl) (EcoR I-1/EcoR I-2; Table 1) and 50 µM MseI adaptors (0.5 µl) (MseI-1/MseI-2; Table 1), in a reaction containing 50 U T4 DNA ligase (0.4 µl), 10 mM ATPs (0.5 µl), 10X T4 DNA ligase Buffer (2 µl) at 22°C for 24 min.

Pre-selective PCR was performed after diluting the ligated DNA 4-fold with sterile distilled water. A total volume of 20 µL reaction mixture containing 5 µL of the digestion/ligation mixture, 1 µL EcoRI primer (10 pmol), 1 µL MseI primer (10 pmol), 2 µL 10X PCR buffer, 1 µLMgCl2 (50 mM), 1 µL dNTPs (10 mM), 0.3 µL Taq DNA polymerase (5 U/µL) was prepared. PCR reactions were performed with the following profile: 2 min at 72 °C; 25 cycles of 30 s denaturing at 94 °C, 60 s annealing at 56°C, and 60s elongation at 72 °C, ending with 4 °C pause. After checking for the presence of a smear of fragment by 1.5% agarose gel electrophoresis, the amplification product was diluted 10 times in sterile distilled water.

A selective PCR reaction mixture (20 µL) was prepared with 5 µL of the diluted pre-amplification products, 1 µL EcoRI selective primer (10 pmol), 1 µL MseI selective primer (10 pmol), 2 µL 10XPCR buffer, 0.8 µLMgCl2 (50 mM), 1 µL dNTPs (10 mM), 0.3 µL Taq DNA polymerase (5 U/µL). PCR reactions were performed with the following profile: 30 s denaturing at 94°C, 30 s annealing at 65 °C, and 60s elongations at 72 °C, followed by reduction of the annealing temperature in each cycle by 1 °C for 11 cycles. The annealing temperature was maintained at 56 °C for the remaining 30 cycles. Seven pairs of selective AFLP primers were used to amplify the genomic DNA of 35 samples: E-AAG/M-CTA, E-ACT/ M-CAA, E-ACT/ M-CTA, E-AAG/M-CTG, E-ACT/M-CAT, E- AAG/ M- CAC and E-AAG/ M- CAG (Table 1). PCR products were run on 6.0% denaturing polyacrylamide gel electrophoresis (PAGE) for 2.5 h at 50 °C on the Sequi-Gen GT Sequencing Cell (Bio-Rad, USA), and finally detected using the silver staining technique modified from Merrill et al. (1979).

Table 1: Oligonucleotide adapters and primers used in AFLP analysis.

<table>
<thead>
<tr>
<th>Adapter or primer</th>
<th>Sequence (5´-3´)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adapter</td>
<td></td>
</tr>
<tr>
<td>EcoRI-1</td>
<td>CTC GTA GAC TGC GTA CC</td>
</tr>
<tr>
<td>EcoRI-2</td>
<td>AAT TGG TAC GCA GTC TAC</td>
</tr>
<tr>
<td>Mse I-1</td>
<td>GAC GAT GAG TCC TGA G</td>
</tr>
<tr>
<td>Mse I-2</td>
<td>TAC TCA GGA CTC AT</td>
</tr>
<tr>
<td>Primer of pre-amplification</td>
<td></td>
</tr>
<tr>
<td>EcoRI +1</td>
<td>GAC TGC GTA CCA ATT CA</td>
</tr>
<tr>
<td>Mse I +1</td>
<td>GAT GAG TCC TGA GTA AC</td>
</tr>
<tr>
<td>Primer for selective amplification</td>
<td></td>
</tr>
<tr>
<td>E-AAG</td>
<td>GAC TGC GTA CCA ATT CAA G</td>
</tr>
<tr>
<td>E-ACT</td>
<td>GAC TGC GTA CCA ATT CAC T</td>
</tr>
<tr>
<td>M-CAA</td>
<td>GAT GAG TCC TGA ACA A</td>
</tr>
<tr>
<td>M-CAT</td>
<td>GAT GAG TCC TGA ACA A</td>
</tr>
<tr>
<td>M-CTA</td>
<td>GAT GAG TCC TGA ACT A</td>
</tr>
<tr>
<td>M-CTG</td>
<td>GAT GAG TCC TGA ACT G</td>
</tr>
<tr>
<td>M-CAG</td>
<td>GAT GAG TCC TGA ACA G</td>
</tr>
<tr>
<td>M-CAC</td>
<td>GAT GAG TCC TGA ACA C</td>
</tr>
</tbody>
</table>

2.4. Data Analysis

The amplified bands were scored as presence (1) or absence (0) across all genotypes. Some genetic diversity parameters in populations i.e. percentage of polymorphic band (PPB), mean number of alleles per locus (Na), mean effective number of alleles per locus (Ne), Nei’s genetic diversity (H) (Nei, 1973), Nei’s genetic differentiation between populations (GST) were calculated by POPGENE 1.32 software (Yeh et al.,...
Analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was performed to calculate variance components within and between populations using GenAlex 6 software (Moslemi et al., 2010). Genetic differentiation coefficients (Δpt) between populations were computed from AMOVA. Principal coordinate analysis (PCoA) was also performed via distance matrix to describe the relationship between accessions in populations using GenAlex 6 (Moslemi et al., 2010).

Dendrogram was constructed by an unweighted paired group method of cluster analysis using UPGMA of NTSYS-pc program (Rohlf, 1998), in order to examine the genetic relationships at the species level.

3. Results

3.1. AFLP Polymorphism

AFLP analysis of 40 individuals using seven pairs of primers generated a total of 358 bands ranging in size from 50 to 600 bp, corresponding to an average of 51.14 bands per primer pair. Of these bands, 358 were polymorphic and the PPB value was 86.63%. The number of polymorphic bands amplified per primer pair combination varied from 10 to 21, averaging 12.28 (Table 2).

3.2. Genetic Variation within Population

The PPB populations ranged from 93.02% (Pop1) to 80.23% (Pop2). Assuming Hardy–Weinberg equilibrium, the average Nei’s gene diversity was estimated to be 0.2528 within populations.

The Shannon’s information index ranged from 0.4068 (Pop1) to 0.3428 (Pop2), with an average of 0.4030 at the population level, respectively. Estimates of Nei’s gene diversity for all bands in individual populations showed highest diversity in Pop1 (h = 0.2582) and lowest diversity in Pop2 (h = 0.2178) (Table 3). Estimates of Shannon’s information index showed different numerical values but similar trend with that of Nei’s gene diversity.

Table 2: Numbers of AFLPs detected with seven primer pairs for Liza klunzingeri populations.

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Total AFLP bands</th>
<th>No. of polymorphic bands</th>
<th>Percentage of polymorphic bands (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-AAG/M-CTA</td>
<td>59</td>
<td>11</td>
<td>18.64%</td>
</tr>
<tr>
<td>E-ACT/M-CAA</td>
<td>43</td>
<td>10</td>
<td>23.25%</td>
</tr>
<tr>
<td>E-ACT/M-CTA</td>
<td>31</td>
<td>12</td>
<td>38.70%</td>
</tr>
<tr>
<td>E-AAG/M-CTG</td>
<td>65</td>
<td>10</td>
<td>15.38%</td>
</tr>
<tr>
<td>E-ACT/M-CAT</td>
<td>47</td>
<td>11</td>
<td>23.40%</td>
</tr>
<tr>
<td>E-AAG/M-CAC</td>
<td>50</td>
<td>21</td>
<td>42%</td>
</tr>
<tr>
<td>E-AAG/M-CAG</td>
<td>63</td>
<td>11</td>
<td>17.46%</td>
</tr>
<tr>
<td>Total</td>
<td>358</td>
<td>86</td>
<td>-</td>
</tr>
<tr>
<td>mean</td>
<td>51.14</td>
<td>12.28</td>
<td>25.54%</td>
</tr>
</tbody>
</table>

Table 3: Genetic diversity statistics for the two natural Liza klunzingeri populations and the species based on AFLP data.

<table>
<thead>
<tr>
<th>Population</th>
<th>Sample size</th>
<th>PPB(1) (%)</th>
<th>na(2)</th>
<th>ne(3)</th>
<th>h(4)</th>
<th>I(5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pop1</td>
<td>20</td>
<td>93.02</td>
<td>1.9302</td>
<td>1.4031</td>
<td>0.2582</td>
<td>0.4068</td>
</tr>
<tr>
<td>Pop2</td>
<td>20</td>
<td>80.23</td>
<td>1.8023</td>
<td>1.3467</td>
<td>0.2178</td>
<td>0.3428</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>86.62</td>
<td>1.8662</td>
<td>1.3914</td>
<td>0.2528</td>
<td>0.4030</td>
</tr>
</tbody>
</table>

1- Percentage of polymorphic bands.  
2- Observed number of alleles.  
3- Effective number of alleles.  
4- Nei’s gene diversity.  
5- Shannon’s information index.
3.3. Genetic Structure of Populations

Nei’s (1978) genetic distance among the two populations was 0.0312 and Nei’s genetic identity among the two populations was 0.9693. The coefficient of genetic differentiation among populations (GST) was 0.0585 as estimated by partitioning of the total gene diversity.

The results of AMOVA analysis indicated that 90% of the genetic variation existed within populations and 10% among populations.

The estimated number of migrants per generation (Nm) was 8.0419 among populations.

Two-dimensional diagram of PCoA (Principle Coordinates Analysis) that were drawn for the relationships between the samples (Fig.2) show that Mond estuary and Bahrekan region fishes were in two separate groups.

3.4. Cluster Analysis

The UPGMA dendrogram was constructed on the basis of the inter-population genetic similarity. Cluster analysis results produced by UPGMA method in NTSYS-pc software revealed that low similarity exists in both groups of fishes (Fig.3).

Figs. 2: Two-dimensional diagram of PCoA for Mond estuary and Bahrekan region migrating forms of Persian Gulf. Pop 1: Mond estuary and Pop2: Bahrekan region.

Figs. 3: UPGMA dendrogram showing the phylogenetic relationship among all individual of L.klunzingeri. Number(s) 1-20 Mond estuary, 21-40 Bahrekan region.
4. Discussion

The conservation of genetic diversity is important for the long-term interest of any species (Falk and Holsinger, 1991). The AFLP method due to generation of a large number of polymorphic DNA fragments has been used to investigate genetic diversity of many different fishes species (Song et al., 2010). In this study using seven primer pair combinations, 358 bands were detected. The percentage of polymorphic bands per primer pair ranged from 18.64% (E-AAG/M-CTA) to 42% (E-AAG/M-CAC) with an average of 25.54% (Table 2). Liu et al. (2009) in their studies on Mugil cephalus diversity using AFLP technique observed a polymorphism of 53.91% respectively. The genetic diversity (h=0.2528±0.1433, I=0.4030±0.1843) of L. klunzingeri was relatively high compared with other fish species, such as Mugil cephalus (0.2074) (Liu et al, 2009), Ictalurus punctatus (0.135) (Mickett et al., 2003) and Synechogobius ommaturus (0.0794) (Song et al., 2010). Menezes et al. (1992) found an average heterozygosity of 0.043 in 131 striped mullet at eight presumptive loci. Rossi et al. (1998) reported an average heterozygosity of 0.05 for 284 individuals of M. cephalus collected from ten populations worldwide. Both estimates are close to the range of average heterozygosities (0.051–0.058) reported by means of allozyme for many fishes (Ward et al., 1992). By analyzing mitochondrial control region DNA sequences for 140 striped mullet collected from six populations worldwide, Rocha-Olivares et al. (2000) detected high levels of mitochondrial haplotypic and nucleotide diversity, which were much higher than the average of 37 marine teleosts (Avise et al., 1987; Grant, 1998). The present value of heterozygosity is considerably higher than the results of isozymes analysis, but lower than the mitochondrial analysis.

This could be attributed to the geographic difference between samples and the oceanographic conditions of the study area. In addition, AFLP DNA markers should be neutral, whereas allozyme variation was inferred to be subjective to selective pressures (Hallerman et al., 1986), and AFLP can resolve more loci and detect greater levels of polymorphism than isozyme (Mickett et al., 2003).

The genetic structure patterns of L. klunzingeri populations using both Nei’s genetic diversity analysis (GST = 0.0585) and Analysis of Molecular Variance (AMOVA) showed the difference within populations (0.90%) were significant, while in similar results difference within and between Mugil cephalus populations were calculated 60.7% and 39.3%, respectively (Liu et al., 2009).

Based on these biological traits, L. klunzingeri had maintained a high level of within populations genetic diversity and low population differentiation, and there existed a strong gene flow (Nm=8.0419) among populations. A migration rate of Nm =1.0 is theoretically necessary to counter population divergence due to genetic drift (Wright, 1931); in the case for L. klunzingeri populations in the present study, the Nm value was much higher than one successful migrant per generation, suggesting that genetic drift may have not played any major historical roles in determining the genetic structure of L. klunzingeri populations.

In this study, samples from Hendijan station were more diverse than those collected from Ziyarat estuary station. This can be due to ecological difference such as the variety of estuaries the consequent genetic diversity. Furthermore, population genetic theory predicts that larger populations tend to keep genetic diversity at higher levels (Zhao, et al 2007); therefore there is the possibility that larger population of L. klunzingeri might inhabit Hendijan region.

Station Ziyarat estuary and Hendijan are 270 Km away from each other, and thus intermingling of the two populations through migration seems very unlikely. Analysis of the current results confirms the low gene flow between the fish of the two regions.

Many of marine creatures have pelagic larvae with the capability to spread widely through sea currents.
(Han et al., 2009). The general circulation of water in the Persian Gulf influenced by the Density Driven Current in a way that waters of Oman sea after entering the Persian Gulf pushes forward until it reaches Bushehr Coast, then changes its direction to South and never reaches Khuzestan Coast and Hendijan. This can be a determining factor in migration and distribution of adult and larvae of L. klunzingeri in a way that it acts like a physical barrier separating the two populations of the two regions (Kampf and Sadrinasab, 2006).

Considering the genetic distance between the two stations based on Nei coefficient (Nei, 1987) and the PCA diagram, the population can be distinguished and it can be said that samples from stations Hendijan region and Ziyarat estuary belong to two distinct populations.

Results of the present study prove that genetic analysis based on AFLP technique is a suitable method for studying the phylogenetic relations at the species level; and since this molecular technique does not require background taxonomic data, it can be used for phylogenetic study of many creatures.

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