Population genetic studies of *Liza aurata* using D-Loop sequencing in the southeast and southwest coasts of the Caspian Sea

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Abstract

Genetic diversity as an important marker of the ecological status of aquatic ecosystems is considered a unique and powerful tool to evaluate biological communities. In order to evaluate the genetic diversity among golden mullet species (*Liza aurata*) in the southeast and southwest coasts of the Caspian Sea by D-Loop gene sequencing, a total of 23 fin specimens of golden mullet were collected from the Gilan (Anzali area) and Golestan (Gomishan area) provinces. Total DNA from the samples was extracted by ammonium acetate method and the quality and quantity of the extracted DNA were assessed by spectrophotometery and electrophoresis. Polymerase Chain Reaction (PCR) was conducted on the target DNA and then DNA sequencing was carried out. D- loop region in mitochondrial DNA (mtDNA) of golden mullet contained 900 base pairs (bp). Phylogenetic relationships among golden mullet were calculated by MEGA software version 5.05 and divergence time was estimated using Tahjima's test. The results obtained from this study revealed that there were high genetic differences among two regions in the Gilan and Golestan provinces. Kimura 2-parameter was used for genetic distance analysis and the genetic distance recorded between Gilan and Golestan Provinces was calculated at 0.259. The high levels of Fs_T were observed between Gilan and Golestan Provinces which indicates that genetic differences exist among present populations ($p \leq .05$). Based on the results obtained from the south Caspian Sea, probably two different populations of *Liza aurata* are living in the Gilan and Golestan Provinces.

Keywords: genetic diversity, *Liza aurata*, Caspian Sea, mtDNA , genetic distance

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Introduction

The first record of mullet fish in Iran was reported in 1933 (Shokoliukof, 1937) but its commercial catch was started only in 1942. Mullet fish are not native species of the Caspian Sea. During the years 1930-1934, three million individuals of *Liza aurata*, *L. saliens* and *Mugil cephalus* were caught from the Black Sea and introduced into the Caspian Sea. The introduction of *Liza aurata* and *L. saliens* was successful and they adapted well to the ecological conditions of the Caspian Sea in a short period of time after which they were well distributed in the Caspian Sea (Oren, 1981; Aslan Parviz, 1991; Shariati, 2006).

Mullet is migratory fish which migrates from the middle and northern parts of the Caspian Sea into the southern parts of this sea (Khoroshko, 2008). Based on the report by Tereshenko (1950), Spawning occurs from June to October in the central regions of the middle and southern parts of the Caspian Sea and peak spawning has been reported in October.

The rehabilitation of mullet fish stocks is totally dependent on natural breeding and no efforts are taken for the artificial reproduction of this species. Due to overfishing and low mean weight of mullet fish (210 g) during the years 1981-1982, severe damage was caused on the stocks of these fish (Razavi Sayad, 1990).

Information on population structure of species is required for sustainable use and management of mullet fish stocks which is provided to the researchers through genetic studies. Lack of knowledge about stock structure will contribute to the failure of fisheries management and inappropriate harvest of stocks (Papasotiropoulos et al., 2002). Apart from protecting biodiversity, harvest and exploitation of stocks can be maximized if fish stock management is based on accurate information such as molecular studies (Murgia et al., 2002).

The discovery of mitochondrial DNA sequencing has enabled the investigation of controlled genes in mitochondria and has developed a new and important approach in genetic studies. mtDNA has turned out to be an efficient and ideal genetic marker for systematic, phylogenetic and population studies because of maternal inheritance, absence of recombination and the rapid replacement of nucleotides. The pattern of DNA sequence polymorphism includes useful information on population background and it is also responsible for creation and retention of polymorphism (Li, 1997; Xian Liu et al., 2006). The nucleotide sequence of D-loop region is considered to be variable and without effect on transcription and replication. In fact mtDNA evolves 10 times more rapidly than nuclear DNA, and the D-loop is the most variable region of mtDNA. Substantial genetic variation is found in the D-loop region, even among individuals within a given species. Nucleotide variations in the D-loop among individuals have been well studied in various species. Haplotype analysis of the D-loop region is a useful tool for revealing genetic diversity, which is essential for the preservation of species. Nowdays many species are endangered as a result of the destruction of habitat. Decreases in population

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lead to reduced genetic diversity, which can cause a population survival crisis (Cecconi et al.,1995).

Ghaneh et al. (2011) studied genetic diversity of *Liza saliens* in the southern coasts of the Caspian Sea by mtDNA method and find out that only one population of *Liza saliens* in these regions. Naderi et al. (2011) Studied genetic diversity of *Liza saliens* in the Caspian Sea by microsatellite method that find out more than one population of this species in southern coasts of the Caspian Sea. espite the economic and ecological importance of *Liza aurata* as a valuable species, information on its population structure in the Caspian Sea is limited that can be mention Ghodsi et al. (2011) studied genetic diversity of *Liza aurata* in the coasts of the Golestan province by microsatellite method and Nematzadeh et al. (2012) determined genetic differences and phylogenic relationships among six Mugilidae species (*Mugil cephalus*, *M. capito, Liza subviridis, L. saliens, L, aurata, Valmugil buchanani*) using PCR-sequencing. Due to the ecological changes which are currently taking place in the seas, such information is very important.

In this study, golden mullet populations were caught from the southern Caspian Sea to investigate the evolutionary history of this species. The D-loop regions of their mtDNAs were also studied. This study has provided basic information on effective and sustainable management of golden mullet stocks in Iranian waters of the Caspian Sea.

Materials and methods

In January 2012, a total of 23 fin samples of golden mullet, *Liza aurata*, were collected by beach seine from two different locations in Gilan (Anzali) and Golestan (Gomishan) Provinces. The samples were kept in absolute ethanol and then transferred to the Molecular Genetics Laboratory located at the Caspian Sea Ecology Research Center. All samples were kept at -20 ºC until DNA extraction.

Figure 1: The sampling sites in the Caspian Sea

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Table1: The geographical locations of sampling stations for golden mullet in the Caspian Sea

Location	Longitude	Latitude	Fin sample
Golestan (Gomishan)	35 º53´	37 º07´	9
Gilan (Anzali)	49° 21′ 31″	37° 29′ 00″	14

DNA was extracted from 50 mg of fin sample by ammonium acetate method (McQuown et al., 2000). The quality and quantity of the extracted DNA were assessed by spectrophotometery(biophotometer, Eppendorf company) and agarose gel (1%) electrophoresis. DNA absorption was measured by spectrophotometer at 260 and 280 nm wavelengths. Samples with a ratio of 1.8 to 2 were selected and DNA was then re-extracted from unsuitable samples.

Resolution of DNA bands on agarose gel (1%) was investigated and samples with protein contamination-free bonds and RNA were selected for PCR.

The PCR technique is used to amplify a specific region of a DNA strand and then the target gene can be recognized from the other genes by electrophoresis. Rows of nucleic acids and bases will be cleared by standard techniques for DNA sequencing (Newton and Graham, 1997). The Primers were designed to increase the number of copies of a specific region of the genome.

Amplification of D-loop was performed using the following primers that were selected according to Atabeyoglu (2007).

D-loop F1 Forward

TGGCATTTGGTTCCTACTTCAGG

12S1-H Reverse Primer

TGCGGAGACTTGCATGTGTAAGT

Different materials including PCR Buffer (1X), $MgCl_2$ (1.8 mM), $dNTP_S$ (0.1 mM), Primer F(1.5 Pmol), Primer R (1.5 Pmol), *Taq* DNA polymerase CinnaGen Company (unit 1) and DNA template (100-200 ng) was used for PCR. Adequate distilled water was mixed up to a volume of 50 µl.

DNA amplification was performed in a thermal cycler (Auto-Q, Quanta biotech, England). The programs for PCR amplification were as follow: a denaturation step at 95 ºC for 30 sec, annealing at 49 ºC for 30 sec and extension at 72 ºC for 30 sec for 30 cycles. The reaction products of the PCR were assessed on 1.5 % agarose gel. 50bp DNA ladder (Fermentas Company) was used to calculate the fragment length.

DNA sequencing was carried out by ddNTP method (Pherson et al., 2000). DNA sequencing is based on construction of a new DNA strand by DNA polymerase which occurs at the junction of a primer to the single-stranded DNA template molecule. The fragment length of D-loop sequencing in *Liza aurata* was evaluated to be 900 bp. The purified DNAs of each sample with primer were transferred to the BIONEER Company in South Korea for sequencing.

Data were analyzed by BioEdit (ver.7.1.3.0), DnaSP (ver.5.10.01), MEGA (ver.5.05), Arlequin (ver.3.1) (Excoffier *et al.*2005) and GENEPOP (Raymond and Rousset, 1995). All sequences were aligned

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with Clustal X multiple-alignment program (Thomson et al., 1997) in BioEdit software.

Nucleotide diversity (*P*) and haplotype (*h*) for each population and fixation index (Fs_T) were estimated using DnaSP (Rozas et al., 2003).

Genetic distance within samples was estimated using Kimura 2-parameter 1980 (Kumar *et al.*, 2004). The mean difference of paired nucleotide (Tamura *et al*., 2007) within and among samples of regions and the Neighbor-Joining (NJ) tree was constructed using MEGA (ver.5.05).

Estimation of gene flow (Nm) was derived using the equation: $Nm = [(1/Fs_T)-1]/2$ (Weir and Cockerham, 1984).

Results

After D-loop sequencing in golden mullet, the fragment length of samples was evaluated to be 900 bp. One sequence from each region was recorded (Gene Bank accession numbers KF 418242, KF 465679).

Individual nucleotides were compared to identify conserved and mutated nucleotides. From a total of 912 study areas, a total of 252 protected sites and 173 variable and mutated sites were observed in the gene sequence.

The haplotype diversity (*h*) of the D-loop in the golden mullet caught in the Gilan and Golestan regions was 1.000 and the highest nucleotide diversity (0.151) was observed in the Gilan Province (Table 3). Results obtained from study revealed that there was nucleotide diversity (Nei and Kumar, 2000) among samples.

The rate of observed heterozygosity was always higher than the expected heterozygosity (Table2) and there were also significant differences between the observed and expected heterozygosity ($p \leq .05$). The populations of the two study areas were found to deviate significantly from Hardy-Weinberg equilibrium ($p \leq .05$) (Table 2).

Table 2: Haplotype diversity (*h*), nucleotide diversity (*P*), observed heterozygosity (H_o), expected heterozygosity (H_e) and Hardy-Weinberg equilibrium (HWE) of golden mullet in the Caspian Sea

Location	N	*h*	*P*	H_o	H_e	HWE	Tajima□D
Golestan	9	1.000	0.073	0.274	0.199	0.031	0.536
Gilan	14	1.000	0.151	0.291	0.277	0.021	-1.833

The genetic diversity was calculated at 1.000 in both provinces according to Nie (1987).

Genetic diversity as a marker of polymorphism included a range between 0 (where all the individuals showed the same haplotype) and 1 (where each individual had a unique haplotype).

Based on the AMOVA analysis, the highest (0.74) and the lowest (0.08) genetic variations were observed within populations and among populations within regions, respectively.

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Table 3: Analysis of molecular variance (AMOVA) of golden mullet in the Caspian Sea. Sum square (SS), p value (Pro)

Source of variation	SS	Est.var.	%	Pro
Among regions	21.72	0.021	0.18	0.034
Among populations within regions	12.25	0.085	0.08	0.025
Within populations	95.09	0.124	0.74	0.024

The Kimura 2-parameter 1980 was used to calculate the genetic differences. Depending on the amount of nucleotide differences, the interspecific and intraspecific differences are characterized by a genetic distance in this analysis.

Hence the genetic differences between Gilan and Golestan Provinces were calculated to be 0.259. Based on the Nei (1980) model, the rate of gene flow was 0.310.

Results of gene flow revealed that there was low gene flow (Nm) between two regions indicating that there was reproductive isolation between these two regions. Fixation index (Fs_T) was used to determine genetic differentiation. The differentiation was estimated either directly or through relationship with effective migration. The high rate of Fs_T (0.499) was observed between Gilan and Golestan Provinces which indicated that there was genetic differentiation among populations in the Gilan and Golestan Provinces.

Phylogenetic relationships among golden mullet were calculated by MEGA software and divergence time was estimated using Tahjima's test (Tajima, 1993). The results obtained from genetic differences showed that there were significant differences among regions in the Gilan and Golestan Provinces. Based on the Neighbor-Joining tree, the Gilan samples appeared in a single branch, indicating that two different groups of golden mullet (*Liza aurata*) which belong to weast and east of south coasts of the Caspian Sea.

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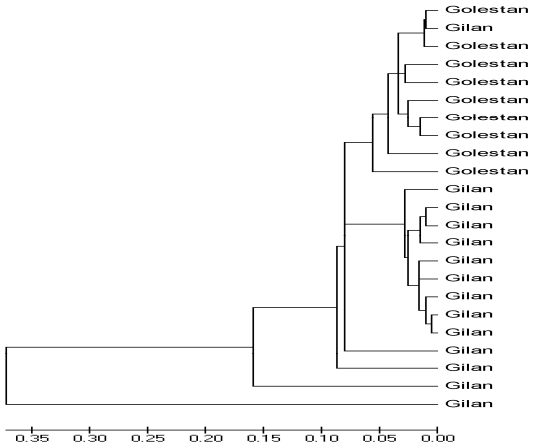

Figure 2: Neighbor-Joining (NJ) tree drawn for golden mullet in the Gilan and Golestan Provinces

Discussion

Identification of population structure and interspecific variations are essential requirements to apply a sustainable management in the exploitation of fish stocks. Genetic markers and identification of diversity at the DNA level provide the opportunity to investigate the correct genetic differences between individuals. mtDNA is applied to identify fish stocks and determine stocks contribution in mixed catches. mtDNA also provides useful information to study the genetic differences in fish (Murgia et al., 2002).

D-loop, a displacement loop in mitochondrial DNA, is applied as a mediator at the beginning of replication. Nucleotide sequence from D-loop region reveals diversity occurring without any effects on translation and replication. Nucleotide sequence in mtDNA occurs 10 times sooner than DNA and D-loop is the most changeable region of mtDNA (Cecconi et al.,1995).

Genetic diversity is considered as one of the three essential elements for species conservation (Miller, 1997). Hence the study of the genetic structure of the economically valuable species golden mullet the population of which has declined considerably (from 6599.4 tons in 2002-2003 to 3865.5 tons in 2011-2012) is essential (Daryanabard, 2012).

All the study samples of *Liza aurata* from two regions showed high level of haplotype and nucleotide diversities. The recorded haplotype diversities for both provinces were 1.000 and the highest haplotype diversity (0.151) was recorded in the Gilan Province.

The total halplotype was 23 and a total of 173 variable sites were found which indicated the high population diversity within and among populations. The results inferred from halplotype diversity in this study are in agreement with the results on mullet fish of Mediterranean Sea (Erguden et al., 2010) and on the other golden mullet (Rossi et al., 1998; Caldara et al., 2002; Papasotirpoulos et al., 2007). The large number of mtDNA haplotypes and the comparatively high values of mtDNA nucleon diversity observed within and among the red drum samples in Gold and Richardson, 1991 investigation that studied

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population structure in this species using mitochondrial DNA. The more appropriate measure of genetic diversity, nucleotide diversity consider in Liu et al. (2006) that studied differential population structuring of two closely related fish species in Northwestern Pacific. Naderi (2011) also found a high level of halplotype diversity and more than one population of *Liza saliens* in these regions of the southern coasts of the Caspian Sea by microsatellite method, this observation is in agreement with current study. Due to the large size of effective population in marine fish communities, marine fish generally represent greater genetic diversity than freshwater fish (Rossi et al., 2004).

The Fs_T was calculated at 0.499 between the Gilan and Golestan Provinces ($p \leq .05$). So there is a relatively high differentiation in *Liza aurata* populations leading to the formation of two different populations of this species in the southern coasts of the Caspian Sea. In Gold and Richardson (1991) study, Fs_T values of the four heterogeneous mtDNA haplotypes ranged from 0.019 to 0.137. A similar conclusion was reached by Naderi, 2011 based on this study Fs_T values of *Liza saliens* ranged from 0.017 in Gomishan and Miankaleh to 0.120 in Behshahr and Babolsar. In contrast to the present study in Ghodsi et al., 2011 investigation the Fs_T of *Liza aurata* in the coasts of the Golestan province calculated 0.016.

The genetic difference between Gilan and Golestan Provinces was calculated to be 0.259 indicating that the difference between two populations is high. Based on the Nei (1980) model, the rate of gene flow was 0.310.

According to Li (2007), if Nm > 1, the main factor in genetic differentiation is gene flow. In this study, the gene flow rate among regions was low (Nm< 1) which indicated the low rates of immigration among regions. Hence the reproductive isolation was occurred which is a factor for different populations.

The genetic differences are created by accumulation of individuals in a specific area. A unique gene pool is produced by inbreeding of populations within a species and reproductive characteristics are the main elements for population differentiation (Turan et al., 2005).

If the rates of gene flow among regions are high, the genetic differences will be less (Nei, 1972). In Ghaneh et al., 2011 investigation, high level of gene flow (5.05) observed among the *Liza saliens* samples. In Ghodsi et al. (2011) study, the gene flow rates of the *Liza aurata* ranged from 5.153 to 39.264. Based on Ghaneh (2011) and Ghodsi (2011) high rates of gene flow, low genetic difference and non-discrimination of populations consider in the studied regions.

In this study the observed genetic diversity for golden mullet was 1.000 which is in agreement with marine species such as *Sciaenops ocellatus* (Gold and Richardson, 1991), *Clupea harengus* and *Brevoorita tyrannus* (Kornfield and Boydanowicz, 1987; Avise et al., 1989) but in contrast with *Anguilla rostrata*, *Arius felis* and *Cynoscion nebulosus* (Avise et al., 1989).

Although Identification of acceptable factors for reason of genetic diversity is not easy but based on experiments conducted on *Pagrus auratus* (Hauser et al., 2002), *Gadus morhua* (Hutchinson et al., 2003) and *Sebastes crameri* (Gomez-Uchida and Banks, 2006), overfishing is the main factor for reduction of genetic diversity. However in studies conducted on *Colossoma macropomum*, *Lutjanus campechanus* and *Sciaenops ocellatus*, despite the significant

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decline in stocks, reduction in mtDNA diversity was not observed. In fact increase in genetic diversity occurred due to human activities resulting in high pressure on fish stocks in the region (Cheng et al., 2008).

All the effective factors on population size should be identified to create sustainable populations and provide sustainable development. Based on results obtained from this study, there are two different populations of golden mullet, *Liza aurata* in the southern coasts of the Caspian Sea in Gilan and Golestan Provinces which preserved its structure in Gilan region.

There are different ecological conditions in sampling regions (Anzali and Gomishan) that usually in the beginning of spawning season, temperature, salinity and dissolved oxygen in southeast (28.2°C, 12.28 ppt, 6.62 mg/l) are higher than southweas (23.3°C, 11.97 ppt, 6.53 mg/l) costs of the Caspian Sea (Lalouei et al., 2011). Therefore, the ecological conditions of the Gilan Province can be considered as barriers compared to those in the Golestan Province that can makes different spawning time and finally develop different populations of *Liza aurata* in southeast and southweast coasts of the Caspian Sea.

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مطالعه ژنتیک جمعیت ماهی کفال طلایی(Risso, 1810) *Liza aurata* در سواحل جنوب شرقی و غربی دریای خزر با استفاده از روش D-Loop sequencing

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چکیده

تنوع ژنتیکی به عنوان یکی از شاخص های مهم وضعیت اکولوژیک اکوسیستم های آبی می تواند به عنوان ابزاری منحصر به فرد و توانمند برای ارزیابی و مدیریت جوامع زیستی مطرح باشد. به منظور بررسی امکان تنوع ژنتیکی بین گونه های کفال طلایی *Liza aurata)* موجود در سواحل جنوب شرقی و غربی دریای خزر با استفاده از روش تعیین توالی ژن D-Loop تعداد ۲۳ نمونه باله ماهی کفال طلایی از استانهای گیلان (انزلی) و گلستان (گمیشان) استحصال گردید سال انجام آزمایش. DNA نمونه ها با استفاده از روش استات آمونیوم استخراج و کمیت و کیفیت آنها با استفاده از روش اسپکتروفتومتری و الکتروفورز ارزیابی گردید. نمونه های DNA تأیید شده ، PCR و سپس توالی یابی شدند. توالی ناحیه D-Loop در DNA میتوکندریایی کفال طلایی پس از ویرایش شامل ۹۰۰ جفت باز (bp) بود. با استفاده از نرم افزار MEGA version 5.05 درجه خویشاوندی با تست Tajima 1993 بین کفال ماهیان طلایی محاسبه گردید که نتایج حاکی از اختلاف ژنتیکی بالا در میان مناطق گیلان و گلستان بود. برای محاسبه فاصله ی ژنتیکی از آنالیز Kimura 2-parameter استفاده شد که فاصله ی ژنتیکی مشاهده شده بین استانهای گیلان و گلستان (۰/۲۵۹) بود. میزان بالایی از Fst بین مناطق گیلان و گلستان وجود داشته که این امر بیان کننده ی تمایز بین جمعیت های موجود می باشد. طبق نتایج حاصل از این بررسی در جنوب دریای خزر ، در محدوده ی استانهای گلستان و گیلان دو جمعیت متفاوت از ماهی کفال طلایی (*Liza aurata*) وجود دارد.

کلمات کلیدی: تنوع ژنتیکی ، کفال طلایی ، دریای خزر ، mtDNA ، فاصله ژنتیکی

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