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Genetic variability and population structure of *Capoeta gracilis* (Keyserling 1861) populations in the southern basin rivers of the Caspian Sea as revealed by the mtDNA sequences

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ABSTRACT

This study investigated the genetic structure and diversity of C*apoeta gracilis* (Keyserling 1861) from the southern basin of the Caspian Sea employing cytochrome *b* gene sequence analysis. For this purpose, a total of 83 specimens of this species were sampled and analysed from four rivers *viz*., CheshmeKile, Siahrood, Tajan and ZarrinGol. Nucleotide diversity ranged from 0.00030 to 0.00715 and haplotype diversity from 0.27895 to 0.68421 for the populations studied. A total of 14 haplotypes were obtained and haplotype no. 2 was shared by all. Pairwise F_{ST} analysis showed that there was a significant genetic difference between the populations studied, with the exception of the populations living in the Siahrood and Tajan rivers. Analysis of molecular variance (AMOVA) also confirmed the genetic differentiation among populations showing 76.03% of the genetic variation within populations and 23.97% among populations ($p<0.01$). A selective neutrality test showed that populations of *C. gracilis* were probably in equilibrium and were not experiencing a population expansion. In general, it can be said that distinct populations of this species are living in the rivers of northern Iran, though further studies using other genes and molecular markers are needed to confirm the findings.

Keywords: C*apoeta gracilis* Cytochrome *b* gene, Genetic diversity, Iran, Khramulia, Mitochondrial DNA

Introduction

In recent decades, genetic studies have increasingly been used to investigate the structure of fish populations and species, population dynamics and the understanding of evolutionary processes. Such studies form the basis for the development and implementation of management plans for the protection of wildlife populations and species (Hrbek *et al.,* 2005; Nazarizadeh *et al.,* 2017). Existence of genetic variability seems to be necessary for the survival of a species in its environment. Loss of genetic variation in species can lead to reduction or even total loss of populations and species and biodiversity loss can occur at a higher and faster rate in smaller and isolated populations (Barasa *et al.,* 2016). Therefore, population genetic or species molecular surveys are essential for the conservation programs on different species (Wang *et al.,* 2007; Diz and Presa, 2009).

The use of gene fragments, especially of mitochondrial genes (mtDNA), has increasingly been used in population genetics, species identification, phylogenetics and phylogeography studies. The use of mtDNA as a molecular marker has several advantages such as, ease of working due to the small size and uniformity in the arrangement of genes; high mutation rate; no recombination; single-parent and haploid mtDNA genes (Freeland, 2005; Gandolfi *et al.,* 2017). Therefore, mtDNA is an appropriate marker for distinguishing groups that may have been isolated for 100 to 10,000 years. Also, rapid evolution of mtDNA compared to nuclear DNA has led to its increased use for evolutionary studies (Nazarizadeh *et al.,* 2017).

The genus *Capoeta* includes 20 species and has a wide distribution in south-west Asia, with 7 species occurring in Iran (Coad, 2014), one of which is *Caproeta gracilis* (Keyserling, 1861) locally known as *Khramulia*. (Abdoli and Naderi, 2009; Froese and Pauly, 2019). This fish is widely distributed in all rivers of the southern basin of the Caspian Sea and Lake Urmia. The maximum length of this species is 350 mm (even in some lakes upto 550 mm). They are able to live in water with a temperature range of 5 to 25°C and a pH range of 7 to 9 and feed on aquatic insects

such as chironomidae and algae, including diatoms. From conservation point of view, it is listed as least concern (LC) in the IUCN Red List (Abdoli and Naderi, 2009; Anvarifar *et al.,* 2012). The fish live and spawn in lakes to a depth of 35 m and in rivers with rubble or sand beds and aquatic plants. The high frequency, wide distribution and high biomass of this species in the rivers of the southern basin of the Caspian Sea, such as Sardabrud and Chalus [more than 33% of the total fish landings belonged to this species (Abdoli and Naderi, 2009)], shows its importance in aquatic ecosystems. In addition, this species is important in terms of aquaculture, fisheries, sport fishing, economics and phylogeography studies. Therefore, the species is ecologically and economically important and knowledge on its biological characteristics are essential in delineating optimum nutritional and reproductive conditions (Abdoli and Naderi, 2009; Anvarifar *et al.,* 2012; Malvandi *et al.,* 2014).

Molecular studies have been of increasing interest to researchers in recent decades and many studies, using different techniques, have been carried out on animal species, especially fish. Zareian *et al.* (2016) studied 5 samples of *C. gracilis* in the Sefidrud River of Iran using cytochrome *b* gene. Other studies on the genus *Capoeta,* include that of Ghanavi *et al.* (2016) in Iran using the sequence of cyt *b* gene; Samaee *et al.* (2006) on the biodiversity of *C. capoeta* in Shirud, Haraz and Sefidrud rivers using RAPD. In another study, Anvarifar *et al.* (2012) investigated the relationship between morphological traits and RAPD marker in this species from two stations in the Tajan River (Anvarifar *et al.,* 2012). Levin *et al.* (2012) also examined the relationship between the polymorphism of *Capoeta* species based on sequencing of cyt *b* gene. In many studies, the genetic diversity of different populations has been evaluated using sequencing of mitochondrial genes. The cytochrome b gene has also been used for the study of European anchovy (Borrell *et al.,* 2012), silver carp, bighead carp, grass carp, black carp (Lu *et al.,* 1997) and knife fish species (Ma *et al.,* 2010).

Although studies have been done on the genetic structure of the genus *Capoeta,* so far, no study has been conducted on genetic variation between and within the populations of *C. gracilis.* This study evaluated for the first time, the genetic structure, genetic diversity and differentiation of the population of this species in the rivers of the southern Caspian Sea (CheshmeKile, Siahrood, Tajan and ZarrinGol) using cyt *b* sequence analysis.

Materials and methods

Sampling

Four rivers, CheshmeKile, Siahrood, Tajan and ZarrinGol, located in the northern provinces of Iran were selected for the study, which are separated from each other and connected only through the Caspian Sea. Specimens of *C. gracilis* were sampled in 2013 (Fig. 1, Table 1) and muscle tissue was collected from each fish sample and then stabilised in 96% ethanol.

DNA extraction, amplification and sequencing

Extraction of genomic DNA was done using salting out method (Aliabadian *et al.,* 2012). Tissue samples were placed in sterile vials with extraction buffer containing

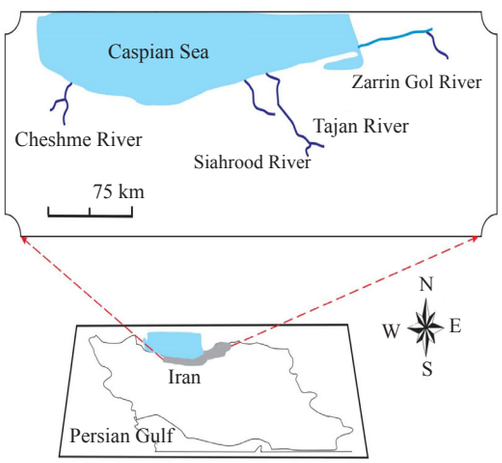

Fig. 1. Map showing the sampling locations of *C. gracilis* population

Table 1. Details of sampling sites and the number of samples in the study areas

River	Site no.	No. of samples	Longitude	Latitude
ZarrinGol	1	10	54° 57’ 16″	36° 52’ 25″
(n=20)*	2	10	54° 54’ 18″	37° 00’ 08″
Tajan	1	9	53° 19’ 32″	36° 11’ 25″
(n=29)	2	10	53° 04’ 57″	36° 30’ 15″
	3	10	53° 05’ 10″	36° 33’ 52″
Siahrood	1	11	53° 00’ 35″	36° 26’ 54″
(n=15)	2	3	52° 53’ 20″	36° 28’ 43″
CheshmeKile	1	10	50° 50’ 30″	36° 44’ 30″
(n=19)	2	10	50° 52’ 43″	36° 49’ 04″

’n’ represents the total number of samples

sodium dodecyl sulfate (SDS) and proteinase K and then placed in a water bath at 37°C for 12 h. The extraction process was completed by adding salt (NaCl), cold isopropanol and ethanol to the samples and centrifuged. At the end of the process, 50 ml of sterile nuclease free water was added to the extracted DNA.The amplification of cytochrome *b* gene was performed with GluDG.L 5'-TGACTTGAARAACCAYCGTTG-3 and H16460 5'-CGAYCTTCGGATTAACAAGACCG-3 primers (Perdices and Doadrio, 2001). Polymerase chain reaction was performed by initial denaturaation for 2 min at 94°C, then 30 cycles of: denaturation at 94°C (45 s), annealing at 46°C (60 s), extension at 72°C (90 s) followed by a final extension at 72°C for 5 min. After determining the quality the PCR products, were sequenced at MACROGEN Sequencing Co., South Korea.

Data analyses

The errors in nucleotide sequences were first checked using CLC Main Workbench Ver. 5 and then sequences were aligned using Bioedit Ver. 7.0.4.1. The number of haplotypes, haplotype diversity (h) and nucleotide diversity (π) were estimated with DnaSP Ver. 4.0. Genetic distance (D) within and between populations was calculated using Mega Ver. 6.0 and the isolation-by-distance effects among populations based on pairwise FST values were analysed using Arlequin Ver. 3.5. Hierarchical analyses of molecular variance (AMOVA), Tajima's D and Fu's Fs tests and Simple Mantel tests were performed using Arlequin Ver. 3.5. AMOVA determined the genetic differentiation among populations. For neutrality testing, Tajima's D and Fu's Fs tests were used to determine whether populations deviated from neutrality. The correlation between genetic variation and geographical distance was evaluated by Simple Mantel tests (McMillan *et al.,* 2006; Fratini *et al.,* 2008; Cheng *et al.,* 2011; Borrell *et al.,* 2012; Zhao *et al.,* 2016).

Results

A fragment of 919 bp of the mtDNA cytochrome *b* gene in 83 samples of *C. gracilis* from each of the sampled rivers was amplified and sequenced. Bases of this gene in the total samples used in the present study comprised 25.96% A, 18.56% C, 23.98% G and 31.47% T. Among the 40 polymorphic sites, 6 were related to singleton variable sites and 34 were related to parsimony-informative sites. Haplotype diversity ranged from 0.27895 to 0.68421 and nucleotide diversity from 0.00030 to 0.00715 among populations. The highest and lowest haplotype diversity was observed in Tajan River and ZarrinGol River, respectively. The nucleotide diversity level was different among four populations studied. As with haplotype diversity, the highest and lowest genetic diversity was observed in Tajan River and ZarrinGol River, respectively (Table 2).

A total of 14 haplotypes was identified in all samples and the CheshmeKile, Siahrood, Tajan and Zarrin-Gol rivers had 5, 2, 9 and 3 haplotypes respectively (Table 2). Among the haplotypes obtained, the haplotype no. 2 occurred in all rivers, haplotype no. 1 was shared between CheshmeKile and Siahrood rivers and haplotype no. 3 was shared between the CheshmeKile and Tajan rivers. Other haplotypes were unique to each river and the highest number of unique haplotypes was observed in the Tajan River.

Genetic distance values (D) between and within the groups based on the Kimura-2 parameter for each of the rivers are shown in Table 3. The range of molecular variations between groups ranged from 0.0008 to 0.0062 and within a group ranged from 0.0003 to 0.0073. The highest level of between-groups genetic distance was observed between the Tajan River and CheshmeKile River and the lowest level was observed among Siahrood and ZarrinGol Rivers. The highest within-group genetic distance was found in the Tajan River and the lowest in the ZarrinGol River.

Table 3. Genetic distance (D) within (in the diagonal) and between (below the diagonal) in sampling populations of *C. gracilis* from the four study locations

Rivers	CheshmeKile	Siahrood	Tajan	ZarrinGol
CheshmeKile	0.0017			
Siahrood	0.0028	0.0011		
Tajan	0.0062	0.0044	0.0073	
ZarrinGol	0.0033	0.0008	0.0041	0.0003

Table 2. Descriptive statistics of genetic diversity indices for *C. gracilis*

Sampling location	Region code	n[a]	H[b]	h[c]	π[d]
CheshmeKile	C	19	5	0.68421	0.00168
Siahrood	S	15	2	0.34286	0.00107
Tajan	T	29	9	0.57389	0.00715
ZarrinGol	Z	20	3	0.27895	0.00030
Total	T	83	14	0.60212	0.00387

[a]Sample size, [b]No. of haplotypes, [c]Haplotype diversity, [d]Nucleotide diversity

Genetic differences were also evaluated using the F_{ST} index for the sampled populations from the four rivers studied. Table 4 shows a significant difference between the F_{ST} values of all rivers (with the exception of F_{ST} values between the Siahrood and Tajan rivers) ($p<0.05$). The most significant differences were observed between the rivers Siahrood-CheshmeKile and Siahrood-Zarin-Gol.

AMOVA test (Table 5) showed a high genetic diversity (76.03%) within the populations as well as a high and significant genetic diversity (23.97%) among the populations. Statistically, the results of analysis of molecular variance showed a significant genetic differentiation among populations ($p<0.01$).

The selective neutrality test is often used to determine a populations' history. Results of the Tajima's D and Fu's Fs tests for the populations of each river are shown in Table 6. Values of Tajima's D test in the Tajan and ZarrinGol rivers were negative, while values in the CheshmeKile and Siahrood rivers were positive. Also the Fu's Fs values were positive in all rivers studied. Results of these two tests showed that the populations of these rivers did not significantly depart from neutrality ($p<0.05$).

Discussion

Understanding the population genetic structure of natural populations of wildlife species provides essential information for the development of conservation and management strategies (Nakagawa *et al.,* 2016; Stepien *et al.,* 2017). The survival of a species or population depends on its ability to adapt to environmental changes by its genetic variation and thus genetic variation is essential for long-term survival and evolution (Bataillon *et al.,* 1996). Cytochrome *b* gene has been used to analyse genetic diversity and genetic structure of various populations and species, especially fish but so far, no study has been done on populations of *C. gracilis* other than by Zareian *et al.* (2016) in the Sefidrud River. However, due to the lack of calculation of genetic diversity and genetic indices (such as haplotype diversity, nucleotide diversity, F_{ST}, AMOVA and selective neutrality tests) and the small number of samples (only 5), it is not possible to compare the data from the above study with that from the current study. Hence, results of the present study were compared with the published results from other species.

Table 4. Population pairwise F_{ST} analysis in *C. gracilis* population. pairwise F_{ST} below diagonal and F_{ST} p value above diagonal

Rivers	CheshmeKile	Siahrood	Tajan	ZarrinGol
CheshmeKile		0.00013	0.00000	0.01110
Siahrood	0.49027		0.42351	0.00020
Tajan	0.24484	0.01518		0.00000
ZarrinGol	0.14610	0.53307	0.24959	

Table 6. Statistical tests of neutrality for *C. gracilis* populations

Rivers	Neutrality test			
	Tajima's D		Fu's Fs	
	D	p	Fs	p
CheshmeKile	0.39884	0.68827	0.03604	0.52363
Siahrood	0.34155	0.69337	2.70961	0.89103
Tajan	-1.07434	0.11833	2.40689	0.85407
ZarrinGol	-0.68305	0.27353	1.44081	0.79827
Mean	-0.25425	0.44218	1.44081	0.79443

The current study, based on analysis of the cytochrome *b* gene, showed that in most *C. gracilis* populations there were relatively high haplotype diversities. This may be due to the large population size, high mutation rate, life-history characteristics and the environmental heterogeneity of the rivers studied (Cheng *et al.,* 2011; Sun *et al.,* 2013). This conclusion about the Tajan River is the most convincing, as the river is longer than the other three rivers and therefore has more populations.

In a study by Ma *et al.* (2010) on knife fish populations, the haplotype diversity, based on the cytochrome *b* gene, ranged from 0.6333 to 0.9517 and nucleotide diversity ranged from 0.0016 to 0.126. Borrell *et al.* (2012), found haplotype diversity ranging from 0.7260 to 0.9800 with an average of 0.9330 and nucleotide diversity in the range of 0.1194 to 0.01638, with an average of 0.01542 in. a study based on the cytochrome *b* gene in European anchovy. In a study based on same gene carried out in carp species, Lu *et al.* (1997) found haplotype diversity to be 0.681, 0.884, 0.271 and 0.890 and nucleotide diversity to be 0.018, 0.008, 0.002 and 0.11 in silver carp, bighead carp, grass carp and black carp respectively. Haplotype diversity and genetic diversity found in *C. gracilis* in this study are lower than those mentioned in the above studies but closer to the reported values in carp species by Lu *et al.* (1997).

In the present study, one haplotype (no. 2) was shared in the samples of four rivers studied and haplotypes no. 1

Table 5. AMOVA of four populations of *C. gracilis* on mt DNA cyt*b* gene sequences

Source of variation	d.f.	Sum of squares	Variance components	% of variation	F Statistic	p value
Among populations	3	36.070	0.51160	23.97	0.23970	0.00000
Within populations	79	128.195	1.62273	76.03		
Total	82	164.265	2.13433			

and 3 were shared in two rivers. Other haplotypes were unique and the highest number of unique haplotypes was observed in the Tajan River. Cheng *et al.* (2011) suggested that unique haplotypes could be used as stock identification indicators.

Based on the results of the genetic differentiation index (F_{ST}), Freeland (2005) presented three interpretations: values between 0 and 0.05 represent little genetic differentiation, values between 0.05 and 0.25 represent moderate genetic differentiation and a value greater than 0.25 expresses a pronounced level of genetic differentiation. In the current study, the F_{ST} index showed a significant difference between *C. gracilis* populations living in the Tajan River and all the other rivers and the population located on the Siahrood River with the population living in the CheshmeKile River. In addition, the results obtained from the AMOVA analysis supported the results of F_{ST} index and showed significant difference between populations in the rivers studied ($p<0.01$). Such a genetic structure could be a reflection of the reduced gene flow between populations living in these rivers.

The pairwise F_{ST} values obtained from all rivers, with the exception of values between the Siahrrod and Tajan rivers, showed a significant difference ($p<0.05$). Perhaps the reason for the non-significant index in these two rivers is the lesser distance between them than between the other rivers. A simple Mantel Test was used to test this effect. The results showed a positive correlation but no significant difference between the F_{ST} values and the geographical distance ($p = 0.39$, $r = 0.177$). Also, simple Mantel Test showed a negative and no significant correlation between genetic distance factor (D) and geographical distance ($p = 0.747$, $r = -0.265$). Therefore, the model of isolation by distance is rejected. Consequently, effect of the geographical distance on the genetic structure observed in this study cannot be proven. In a study on *Glossogobius giuris* and *Glossogobius celebius* in the Philippines, Ardestani *et al.* (2014) found no significant correlation between geographic and genetic distances. On the other hand, Egger (2017) found a strong correlation between geographic and genetic distances in *Astatotilapia burtoni* in the shoreline of East African Lake Tanganyika and Rabone *et al*. (2015) in *Forsterygion lapillum* and *Forsterygion capito* in New Zealand.

When a population experiences a population expansion, the null hypothesis is rejected (Xiao *et al.,* 2012; Galvan-Tirado *et al.,* 2013). Tajima's D and Fu's Fs tests were used for the sequences of the gene selected in this study. In general, negative values for Tajima's D as well as Fu's Fs, indicate the expansion of a fish population size. Negative but non-significant values of Tajima's D for the Tajan and ZarrinGol rivers may indicate a possible population expansion of *C. gracilis* in these two rivers. In contrast to the positive values obtained from Tajima's D in the other two rivers studied, as well as the positive values of Fu's Fs from all four rivers, indicated that the populations of this species are in equilibrium.

In conclusion, a significant difference between the *C. gracilis* populations was observed in the northern Iranian rivers studied based on the sequence of cytochrome *b* gene. This could indicate the separation of populations from each other and the lack of gene exchange between them. This conclusion is consistent with the characteristics of *C. gracilis*, which is a riverine species not having high potential for migration and dispersal. The present study is of particular importance for conservation and management of this economically important fish species. However to strengthen the conclusions, studies using other genes and other molecular markers are suggested.

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