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Research Article

Genetic structure of *Capoeta aculeata* populations in the Zagros river basin using mitochondrial DNA sequences and nuclear DNA markers

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Abstract

The genetic population structure of *Capoeta aculeata* from the Zagros Basin including three rivers (Beshar, Khersan, and Maroun) was investigated using the partial mitochondrial DNA (mtDNA) control region (D-loop) sequences (n=90) and eight microsatellite markers (n = 120). To address this issue, genetic structure differences between the three populations were evaluated by haplotype diversity and pairwise comparison based on allelic distribution. Both methods *used* were concordant in that they showed low to moderate levels of genetic variation and suggested that *C. aculeata* populations were partly diversified. For the mtDNA method, the average haplotype diversity (*h*) and nucleotide diversity (π) were 0.846±0.027 and 0.0037±0.005, respectively. For the microsatellite markers approach, the average number of alleles per locus ranged from 4 to 14, while the average observed heterozygosity (*Ho*) at various loci varied between 0.212 and 0.579. The Hardy-Weinberg test showed that the microsatellite loci deviated significantly in the populations indicating a deficit of heterozygotes. Besides, the results of pairwise F_{ST} estimated and analyzed molecular variance (AMOVA) alongside neighbor-joining and structure analyses showed that most of the variations occurred between samples and finally significant genetic differentiation between populations for both mtDNA and microsatellite markers. At least two genetically distinct management units were observed using the two methods among the sampling sites. The non-significant differentiation between *C. aculeata* samples from the Khersan and Beshar rivers can be explained by a relatively recent disconnection of these two populations and/or small amounts of contemporary gene flow between the two gene pools. The analyses of both mtDNA and microsatellite markers provided potential markers for identifying probable populations and characterizing the conservation genetics of *C. aculeata*.

Keywords: *Capoeta aculeata*; Mitochondrial DNA; Genetic variation, Microsatellites

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Introduction

The genus *Capoeta* in Iran is a freshwater cyprinid species and highly diversified with 14 species and is one of the most important freshwater cyprinid fish in Iran. This genus is a potamodromous cyprinid fish, with about seven species reported from the interior water of Iran, occurring in both lotic and lentic water bodies (Samaee *et al*., 2006). Kohgiluyeh and Boyer-Ahmad province in the southwest part of Iran is a region with a high number of endemism in some freshwater fish species including *Capoeta aculeata* (Valenciennes, 1844). This species is widely distributed within Kavir and Namak basins. There is no sexual dimorphism in this species and both sexes have similar morphometric characteristics (Esmaeili *et al*., 2018).

However, researchers recently clarified the phylogeny and taxonomy of *C. aculeata*, and found that this species is distributed in the Zagros basin (Zareian *et al*., 2016; Khaefi *et al*., 2018). Given its importance in fisheries, previous studies on *Capoeta* species have mainly focused on its taxonomy and mitochondrial genome barcoding, but the genetic variation and population structure of *C. aculeata* have not been carefully understood. Contrary to inland cyprinid fish species, the genetic structure of *Capoeta* in the Zagros basin has scarcely been addressed and most of them have been studied morphologically. Many studies have been done to describe the genetic variation within and among populations of freshwater cyprinid fish using various molecular markers, which is the basic goal of population genetics (Samaee *et al*., 2006; Chen *et al*., 2015; Parmaksız and Eksi, 2017). Previous studies examining molecular phylogeny of *Capoeta* species have primarily utilized mitochondrial DNA (mtDNA) markers (Alwan *et al*., 2016; Ghanavi *et al*., 2016). Some studies based on the control region in cyprinid species have shown its usefulness and the control region has been recommended for assessing intraspecific genetic variation in cyprinids as well as in population studies in other aquatic species (Dai *et al*., 2010; Khoshkholgh *et al*., 2011; Wang *et al*., 2021). They found most of the genetic diversity of cyprinid fish was considerable in its sampled area of distribution. Furthermore, evidence of significant genetic differentiation in microsatellite allelic frequencies among cyprinid fish populations suggests they are reproductively isolated. Bektaş *et al*. (2017) have been genetically defined Anatolian *Capoeta* species with extensive molecular research using cyt *b* gene sequences. A comparison of the different subspecies shows that several of them are clearly distinct species.

In general, genetic variation is the basis of the ecosystem and species diversity, and any species has its unique gene pool or genetic background form (Chen *et al*., 2015; Khoshkholgh and Nazari, 2019). Population genetic tools suggest an opportunity to depict the genetic level, patterns of dispersal (connectivity), and histories (past and present), to better understand species responses' to ecological changes and anthropogenic impacts (Dong *et al*., 2016; Chen *et al*., 2019). Thus, the multi-

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locus genetic approach is increasingly favored due to the high success rate in delineating the contemporary and historical events for the targeted species (Xue *et al*. 2014; Levin *et al*. 2017; Zhang *et al*. 2020). To better clarify the population genetics of this important species, identification of *C. aculeata* genetic stock structure in Iran is essential. For the management and conservation of fish species with economic importance, it is important to have a depth understanding of the genetic diversity and population structure.

Microsatellites as highly variable molecular genetic markers provide a strong means for investigating genetic variation owing to their extreme degree of polymorphism and neutrality (Khoshkholgh *et al*. 2013; Wang *et al*. 2019). Among the many procedures available for population study, microsatellite DNA loci are one of the most effective because they are simple, highly variable and permit the assay of large numbers of samples (Khoshkholgh *et al*. 2013; Gandomkar *et al*., 2021).

Mitochondrial DNA (mtDNA) has been successfully utilized in many aquatic animal populations for the determination of intra-specific population structure, helping to define conservation units in many endangered species (Nazari *et al*., 2019), or to identify fisheries stocks in commercial species (Khoshkholgh and Nazari, 2020). Its application is supported by both practical and biological reasons; mtDNA is structurally and functionally simpler than nuclear DNA, has a high mutation rate, lack of recombination and maternal inheritance, making it ideal to detect lineages produced on relatively short time periods (Avise, 1994; Nisar *et al*., 2019). Traditionally coding genes as 16s and cytochrome *b* are more extensively used for phylogenetic studies (Liua and Cordes, 2004; Brown, 2008) because they are conservative sequences, whereas the control region has been more used for micro-evolutionary processes at the population level because it is generally the most rapidly evolving region (Chakmehdouz Ghasemi *et al*., 2011, Kartavtsev *et al*., 2017; Kumar *et al*., 2017).

Therefore inferring that both paternal and maternal DNA markers are essential in providing a better understanding of evolutionary history. Conservation management plans with no prior knowledge of the genetic background could result in disturbance to the population structure with adverse effects on the gene pools of wild populations (Grunwald *et al*. 2008; Khoshkholgh and Nazari, 2020). Based on the results of previous studies and on the need to use high-variable genetic markers, in the present work, the population genetic structure of *C. aculeata* was evaluated based on mitochondrial DNA (mtDNA) control region (D-loop) sequences and nuclear DNA markers. The main goals of this study were to assess the genetic variation and structure of *C. aculeata* and examine the geographical pattern of its haplotypes. Genetic variation and gene flow were also assessed, and factors influencing genetic variation were investigated.

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Materials and methods

Sampling and DNA etractions

A total of 120 *Capoeta aculeata*, were collected from three different rivers (Beshar, Khersan, Maroun) in the Zagros Basin. The geographic locations and sample sizes are shown in Table 1, respectively. All samples were obtained during spawning seasons by the fisherman. Collected tissue samples (3-5 g dorsal fin tissue), were immediately preserved in 96% ethanol, and then stored at -20˚C. The tissues of ethanol-preserved were incubated in lysis buffer with proteinase K at 37℃ overnight for 14 h. Total genomic DNA was extracted according to the standardized protocol described by Nazari *et al*. (2016), then stored at -20˚C. The quality and quantity of DNA specimens were checked on a 0.8% agarose gel and by Nanodrop ND 1000, respectively.

Table 1: DNA samples of *Capoeta aculeata* used to DNA markers analyses.

Region	*n* (microsatellites)	*n* (mtDNA)
Beshar River	40	30
Khersan River	40	30
Maroun River	40	30
Total	**120**	**90**

Amplification of mtDNA markers and sequencing

A primer pair was designed to amplify the 5' and the 3' halves of the variable regions for the mitochondrial control region. Amplification of the mitochondrial control region was performed using the oligonucleotide primers**:** *D loop* F (5´-CGTCATCTCTCTAAGCATAG-3´) and *D loop* R (5´-TCGTCTGAGCGAGCATATGTG-3´). These primer pairs were used to genotype the remaining 90 individuals. The PCR for the primer was performed in a total volume of 25 µL, including 2.5 µL 10×PCR buffer (Fermentase), 5 mM $MgCl_2$ (1.5 µL),1 µL *D loop* F (0.4 mM), 1 µL *D loop* R (0.4 mM), 0.25 U *Taq* DNA Polymerase (Invitrogen™) and 1 µL deoxynucleotide triphosphate (dNTPs) (2 mM) and 50–100 ng genomic DNA. The standard or touchdown PCR regimes were applied. Standard conditions were as follows: 94°C (3 min), then 35 cycles at 94°C (30 s)/Tm (30 s)/72°C (60 s). Touch-down conditions were as follows: 94°C (3 min), then 15 cycles at 94°C (30 s)/ 58.4°C (decreasing by 0.5°C/cycle, 30s)/72°C (80 s) followed by 20 cycles at 94°C (30s)/53°C (30s)/72°C (80 s). A final extension at 72°C was applied for all reactions for 8 minutes. Obtained PCR products were visualized and recorded with the method of horizontal gel-electrophoresis in 0.8% agarose in the presence of ethidium bromide in 0.5× TBE buffer. Amplicons were submitted for sequencing in both forward and reverse directions using the same primers utilized for amplification. The samples were sequenced from both 5' and 3' ends using ABI auto-sequencing machine (MegaBACE^{Tm}, Applied Biosystems; www.appliedbiosystems.com) using a DYEnamic^{Tm} ET dye terminator cycle sequencing kit (MegaBACE^{Tm}) following the manufacturer's instructions by BGI Inc. (China).

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Nuclear DNA markers

From the previously described microsatellite loci and for the capacity to identify polymorphism among genotypes, cyprinid-specific dinucleotide microsatellite loci were used in this study (Samaee *et al*., 2006). Detailed information on genomic-SSR loci including forward and reverse primers, cycling condition, repeat motifs, expected fragment size and observed fragment size range were reported by Gandomkar *et al*. (2020). Each primer set was tested by varying the PCR conditions and evaluating the PCR products on 1.5% agarose gels. All PCR amplifications were performed in 20µL reaction volume with template DNA (50 ng) using an Eppendorf 5331 thermocycler (Eppendorf, Germany). Experimental conditions tested included, $MgCl_2$ concentration (1-2.2 mM), deoxyribonucleoside triphosphate (dNTP) concentration (150-190 µM), 0.75-1.2 U of *Taq* DNA polymerase (Vio *Taq*^{Tm} VT1001, Fermentase) and 1µL each of forward and reverse primers (10-20 pmol). The PCR reaction comprised leading denaturing for 4 minutes at 94°C, followed by 25 cycles of 30 s at 94°C, 30 s at best annealing temperature, and 10 minutes for ending extension at 72°C, followed by 4°C hold. Products of PCR were separated by 6% non-denaturing polyacrylamide gel electrophoresis in 0.5× TBE buffer for 2 to 3 h at 250 V and subsequently checked by the silver staining method. The pictures acquired were examined by testing BioCapt software (version 2.0).

Data analysis

Microsatellite analysis

The numbers of Alleles, expected heterozygosity (H_E) and observed heterozygosity (H_O) (Nei, 1972) were analyzed for each locus by the Excel Microsatellite Toolkit (Liu *et al*., 2015). Deviations from the Hardy–Weinberg equilibrium (HWE) tests for each locus were estimated by GENEPOP version 3.2 software (Raymond and Rousset, 1995) with the Markov chain parameters. The frequencies of the null allele were assessed using the software MICRO-CHECKER (Wang *et al*., 2019). Genotype distributions between populations were inspected with the software GENEPOP 3.2 (Raymond and Rousset, 1995). All populations were estimated by the genetic differentiation index (F_{ST}; Collin and Fumagalli, 2015) using Arlequin software (Excoffier and Lischer, 2010). The program STRUCTURE v2.3.4 (Pritchard *et al*., 2000) was used to assign most likely number of distinct populations (K) and individuals were identified to genetic clusters and populations using the Bayesian assignment approach. Optical evaluations of the genetic connections between populations were created over the structure of a neighbor-joining tree according to the Cavalli-Sforza and Edwards (1967) chord distance implemented in PHYLIP software and the bootstrap amount was estimated to depend on 1000 repeats. Mantel tests were conducted based on genetic distance (F_{st} / (1 –F_{st})) and geographic distance (km) in GenAlEx version 6.5.

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MtDNA analysis

DNA sequence alignment was performed using Clustal X 2.1 multiple-alignment program (Thompson *et al.,* 1997) with subsequent refinement by means of the Chromas 2.6.6 program (available at http://www.technelysium.com.au).

Sequence polymorphisms and genetic distances within and between the populations were estimated. An UPGMA tree was constructed for all haplotypes according to the Kimura 2-parameter model (Kimura, 1980) using Mega Version 7.01 (Kumar *et al*., 2016). Molecular diversity indices such as haplotype (*h*), average number of pairwise nucleotide differences (*k*), and nucleotide diversity (π) were estimated using DnaSP 4.0 (Rozas *et al*., 2003). Population structure was evaluated using the analysis of molecular variance model (AMOVA) (Excoffier *et al*., 1992) by using ARLEQUIN Version 3.5 software package (Excoffier and Lischer, 2010). Fixation indices (F_{ST}), were also calculated to assess genetic differentiation within and between paired populations. The statistical significance of the total and pairwise fixation indices was estimated by comparing the observed distribution with a null distribution generated by using bootstrap analysis on 1000 replications. In addition, neighbour-joining (NJ) phylogenetic tree was constructed based on matrix of Kimura 2-parameter (K2P) distance implemented in MEGA 7.01 to evaluate the relationships among haplotypes of the species. The robustness of the inferred trees was evaluated and the sequential Bonferroni method was applied to correct the multiple tests for adjusted significance levels. Statistical significance was at p=0.05 (Fig. 1).

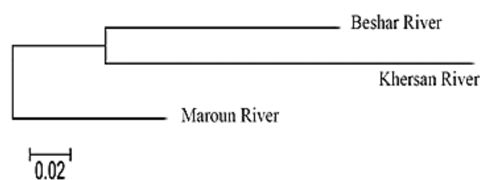

Figure 1: An UPGMA tree of the population genetic distances for the mtDNA control region sequence data from *C. aculeata* of three locations.

The historical expansion of populations was evaluated based on Tajima's D method (Tajima, 1989) and Fu's FS (Fu, 1997) statistics with 10000 permutations estimated in the software ARLEQUIN. Demographic history was also examined by mismatch distribution. Statistical significance was calculated based on the sum of squared deviation (SSD) and Raggedness index (R) (Rogers and Harpending 1992). Then, the expansion time in populations observed was calculated to have experienced expansion using the formula τ=2ut, in which τ was the expansion time expressed in the form of mutational units, u was the rate of mutation per generation, and t was the time measured in the generation since expansion (Cantatore *et al*., 1994).

Results

mtDNA

The aligned mtDNA sequence consisted of part of the control region containing 495 base pairs (bp). Thirty four variable sites (accounting for 5.0% of the total number of sites) were detected in the sequences and all substitutions were transitions, and no insertions or deletions

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were observed. Twenty-one different control-region haplotypes were observed among the sequences. The haplotypes tend to be restricted to separate populations and regions. All the individuals of *C. aculeata* in the rivers shared common haplotypes of A, B, and C. The haplotypes F1 and J were only seen in individuals from Beshar River. Two haplotypes including D and D2 were observed in the populations from Beshar and Maroun Rivers. The haplotypes E1 and F2 were observed in the populations from Khersan River (Table 2). The highest numbers of haplotypes were observed in Maroun River in which five haplotypes were observed in the populations from Maroun River that were absent for the other two rivers.

Table 2: Polymorphic sites of mitochondrial DNA control region of 21 haplotypes.

Number	**1 2 3 4 5 6 7 8 9 10**	**11 12 13 14 15 16 17 18 19**	**20 21 22 23 24 25 26 27 28 29 30 31 32 33 34**
Nucleotide site	**18 24 45 56 68 79 83 99 111 132**	**146 168 179 194 205 221 233 258 272**	**285 299 336 354 366 382 387 399 401 419 432 454 459 467 478**
Haplotype			
A	C C C T G T C T C C	T G T T A C T C A	T G C T G C T A G T A T C G T A
A1	. T A . . . . . T .	. A . . G . C G C	. C . C . T . . T . . . C . . .
B	. . . . G A .	. A . . G . C G G	. . . C . . A . T . . . C . . .
B1	. . A . . . . C . T	. G . . . . A A	. . . T . . . . T . . .
B2	. . A . . . . . . T	. . . . . . C G G	. A C C . . . . A C
C	T T A . . . T T . T	. C . . . . A . C	C T A C . . . . C G . .
C1	G . G . . . T C . .	. . . A C	. A . . . . . . A . . . C . . .
C2	T . A . . . . . . T	. G . . . . C G G	. A . C C . . . . G . . . . A C
D	A . T . . . . C . A	. . . T G	. A . . A . . . T . . . C . C .
D1	G . A . . . G G . .	C T . A G	C A . . . . . . T . . . .
D2	. T G	. . A . T	. T . . C . . G . . . . . T C .
E	. A . . T . . . G .	. . . . T . . G .	. T . . A . . . . C C .
E1	T . . . . T .	. . . . . C A . .	. . . . C A . . . .
D	T . . . . T .	. . . . . C A . .	. . . . C A . . . .
F	C . A . T . A G . .	C . . A G . . . C	C A . . . . G . T . . . . A T .
F1	A T G C	. . A . T . . T .	. T . . . . . G . . . . . T . .
F2	. T . . G . . C . .	. . A . C . . A .	. T . . . C . G . . . A . G . C
G	. A . . C . A . . .	C C A . . . A T .	. . . A . G G . . . . . . T A .
H	. C	. A . . C . C . .	A . . T . . G . C . . . . G .
I	. . T . . T	. . . . . G . . .	T . . A . C . . . G . . C . A .
J	C G . . . . . T . A	. A G	. T . . . . . C . T . G . . . T

In the present study, the haplotype analysis revealed that no Single Nucleotide Polymorphism (SNP) might discern the Khersan, Maroun and Beshar River collections, despite differences in the haplotype diversities between the collections populations. The mean haplotype diversity *(h)* and the nucleotide diversity (π) of the control region were 0.846±0.027 and 0.0137±0.005 respectively (Table 3). The average number of pairwise F_{ST} values were 342.33. Pairwise genetic differences ranged from 0.179 (between Maroun and Beshar Rivers) to 0.61 (between Khersan and Beshar Rivers). A significant

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difference between Khersan River and all other collections pairwise F_{ST} values and significant probabilities ($p \leq 0.0001$) based on 1000 replicates of haplotype frequencies after sequential Bonferroni correction was observed.

Table 3: Levels of genetic diversity within the eight samples of *Capoeta aculeata* (*n* sample size; *h* haplotype diversity; *π* nucleotide diversity). Data shown as mean±standard error.

Location	*n*	Molecular diversity indices	
		π	*h*
Beshar River	30	0.0128±0.0056	0.845±0.028
Maroun River	30	0.0189±0.0017	0.914±0.041
Khersan River	30	0.0096±0.0079	0.781±0.014
All samples	90	0.846±0.027	0.0137±0.005

Pairwise comparison of Monte Carlo based chi-square values ranged from 56.37 (between Khersan and Beshar Rivers) to 94.18 (between Khersan and Maroun Rivers). Chi-square analysis supported the clustering of Khersan, Maroun and Beshar River populations indicating that a significant difference existed in the mtDNA haplotype frequencies between these populations. The Khersan River samples were highly differentiated from those in the Maroun and Beshar River populations ($p<0.0001$).

Due to some haplotypes being novel to the rivers, further genetic sampling across the Zagros Basin including important rivers and surrounding regions will lead to a much elucidation of the natal origin of *C. aculeata* population that displays novel haplotypes presented in our study. The results of the demographic statistics for mtDNA was shown in Table 4. Analysis of Tajima's D and Fu's F_S all showed significantly negative values in all populations, robustly reinforcing the population expansion model. Mismatch distribution analysis showed that the population expansion based on the consistent results of SSD and R statistics was significant (Table 4).

Table 4: Demographic statistics for mitochondrial DNA.

Group	Tajima's D		Fu's F_S		Mismatch Distribution				
	D	P	F_S	P	SSD	P_{SSD}	Raggedness	P_{RAG}	τ
Beshar River	-2.14	0.01	-23.246	0.00	0.00115	0.648	0.01652	0.827	4.013
Maroun River	-2.122	0.00	-23.078	0.00	0.00224	0.081	0.01891	0.951	5.207
Khersan River	-2.381	0.01	-23.465	0.00	0.00096	0.462	0.01848	0.463	6.805

Tajima's D and Fu's FS, corresponding to P value and SSD and Raggedness corresponding to P value were also indicated; τ: expansion time under mismatch distribution.

The AMOVA indicated that there were significant differences ($p<0.001$) among the three locations. The AMOVA also partitioned of total 26.39% genetic diversity among the populations and 59.42% of the total within the populations (Table 5), indicating that much of the variation was between the locations. The results from the AMOVA analysis were supported by the high and mostly significant F_{ST} values for overall population differences ($p<0.001$).

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Table 5: Analysis of molecular variance (AMOVA) of mitochondrial DNA composite haplotypes for the three Capoeta aculeata collections.

Source of variation	Percentage of variation	Fixation indices	*P*
Among populations	26.39	0.1428	<0.001
Among samples	14.19	0.05709	0.0396
Within populations Within samples	59.42	0.1980	<0.001

No significant differences between any of the other samples were detected, either before or after Bonferroni correction. The UPGMA dendrogram (Fig. 2), based on pair-wise simple matching dissimilarity index, reinforced the regional clustering of *C. aculeata* populations into Maroun and Beshar River. The most differentiated cluster included populations of the Maroun River and two other populations. The collections from Beshar and Khersan Rivers formed a clade and were not well supported by bootstrapping.

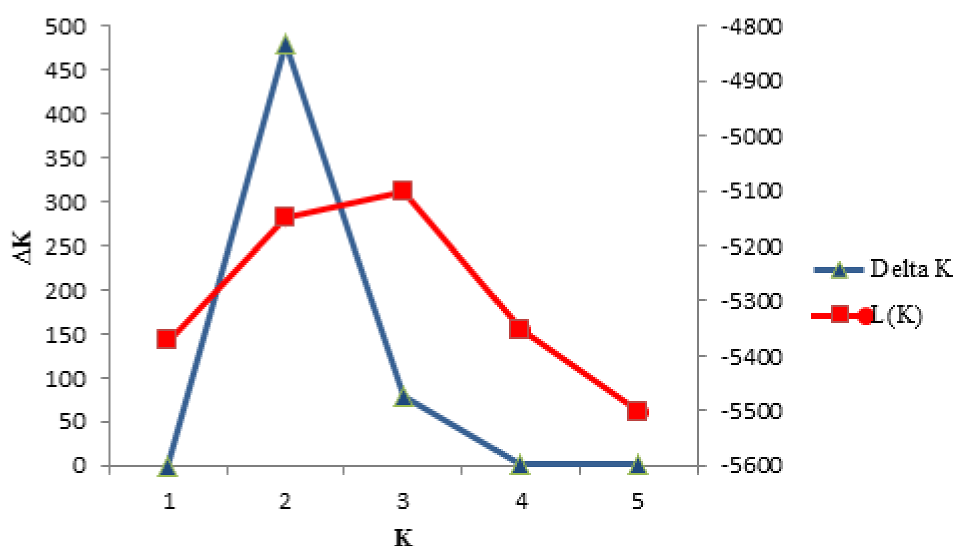

Figure 2: The log probability estimates according to the assumptions of K.

Microsatellite markers

In the present study, for the genomic-SSR loci, the average of the allele numbers inspected at each locus varied between 4 for locus Rser10 to 14 for locus Z21908. In total across the loci, the mean expected heterozygosity of each population varied between 0.357 (Beshar River) to 0.864 (Maroun River). Initially, 14 of 24 exact tests significantly departed from HWE at 0.05 across all loci. Most of the overall similarities were significant subsequent sequential Bonferroni adjustment, after pooling rare alleles. Entire loci had four significant deviations from HWE at least, with no locus out of HWE for more than two groups significantly and three populations including Beshar River, Maroun River and Khersan out of HWE for more than four loci at least and statistically significant. Examinations of genetic distinctiveness indicated that the *C. aculeata* collections did not show one

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panmictic population. Pairwise F_{ST} values varied between 0.189 and 0.359 (Table 6).

Table 6: Exact *P* value for Hardy Weinberg Equilibrium (HWE) estimation for the three different Capoeta aculeata samples after sequential Bonferroni adjustments.

	Beshar River	Khersan River	Maroun River
MFW17	0.000 ***	0.434	0.056
MFW2	0.057	0.015	0.012
MFW26	0.012*	0.352	0.000***
CypG3	0.000***	0.000***	0.062
CypG24	0.005**	0.027	0.000***
Z21908	0.016*	0.000***	0.000***
Rser10	0.000***	0.000***	0.000***
Lid1	0.078	0.165	0.113

*** $p<0.001$, ** $p<0.01$, * $P<0.05$

Population corresponding with non-significant F_{ST} measurements included Khersan River versus Beshar River. The mean F_{ST} for all seven populations was 0.271. Populations from the Maroun River were highly distinct from populations of the Khersan and Beshar Rivers (Table 7). The overall trial had a chi-square measure of endlessness and differences between allele frequencies among all populations was significant at all loci ($p<0.0001$).

Table 7: Pairwise estimates of F_{ST} between populations of Capoeta aculeata.

Location	Maroun River	Khersan River	Beshar River
Maroun River	-		
Khersan River	0.359**	-	
Beshar River	0.189*	0.267**	-

*$p<0.05$; ** $p<0.01$.

The significant pairwise F_{ST} values with the neighbour-joining tree approach confirmed that the populations of *C. aculeata* from the Maroun, Khersan, and Beshar Rivers separated with high bootstrap support and the Maroun River also had the higher branch lengths (Fig. 3). The neighbor-joining tree and Bayesian clustering results indicated that the Khersan and Beshar River regularly clustered composed. According to the tree, longer arm lengths isolated the *C. aculeata* populations of Maroun River from those of the other locations, indicating substantial population genetic structure in the province of Kohgiluyeh and Boyer-Ahmad.

The neighbor-joining dendrogram generated from microsatellite and mitochondrial data showed that Beshar River, Khersan River were clustered as one clade, while Maroun River was clustered as a separate clade. The graphic representing this analysis showed two groups among the samples, one including Beshar River, Khersan River and another formed by Khersan River.

The plots of ΔK according to the STRUCTURE analysis indicated that three is the most likely number of clusters that exist in the full dataset assigned to the different rivers as described by the simulation method of Evanno *et al*. (2005).

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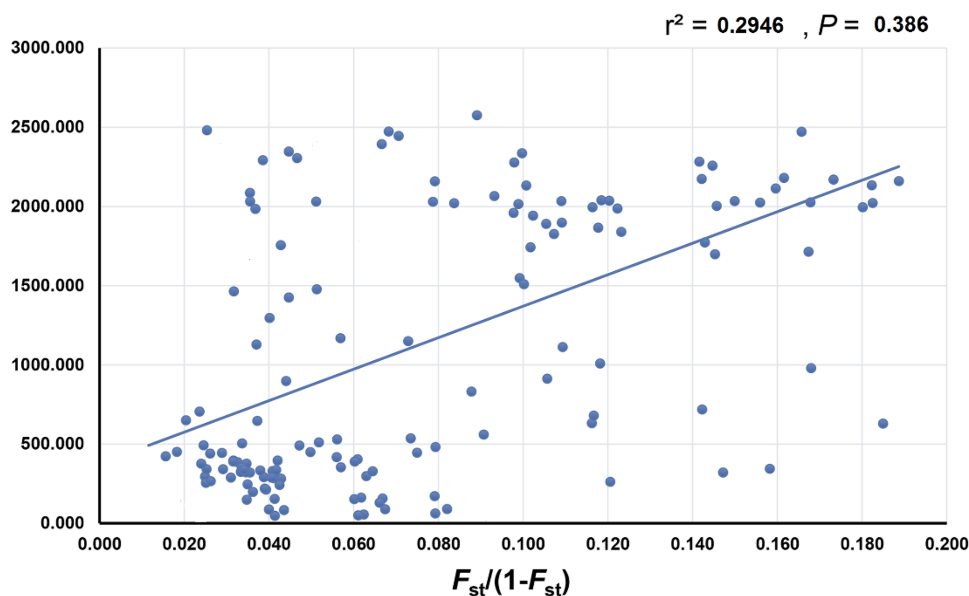

Figure 3: Relationships between pairwise Fst (genetic distances) and geographic distance for populations of *C. aculeata*.

Bayesian analyses of population structure revealed a maximum ΔK value for two genetic clusters with the highest likelihood. The Bayesian clustering analysis of samples from different populations showed that the measure Ln P(D) (the posterior probability) and Var [Ln P(D)] for K=2 (K, Number of homogeneous populations) were -5150.7 and 246.7, while for K=2 were -5109.4 and 389.4, respectively (Figs. 2 and 3). The estimates of log probability under assumption K were shown in Table 8.

Table 8: Descriptions of the structure output tables depicting the best K based on Evanno's methods. The highlighted rows show the largest value in Delta K corresponding to the best K.

K	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	-7041.92	1.261788	—	—	—
2	-7492.08	2.499532	335.9	192.25	78.3144
3	-7656.44	4.870134	142.65	427.19	87.9215
4	-7842.98	986.353	-286.54	947.33	0.81012
5	-7082.19	4.146873	660.79	789.98	190.501
6	-7311.38	54.32016	-129.19	169.93	3.12832
7	-7370.64	49.80047	39.74	141.36	2.85868

Discussion

Assessing the relative levels of genetic variation and also knowledge regarding the genetic variance and connectivity in the species *C. aculeata* is important given its usefulness as a fishery as well as food resource in the Zagros Basin. In the present study, amplifications of eight microsatellite loci in *C. aculeata* were obtained after optimizing the experimental conditions. Through a combination of mtDNA and microsatellite loci, the results of the present study showed that most of the pairwise estimates of inter-population variance in allele frequencies (F_{ST}) were

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also found to be statistically significant. Similar results were reported by Aliakbarian *et al*. (2014), who observed a cross-amplification of nine microsatellite loci in *Capoeta capoeta gracilis* in Madarsu and Gorganrud rivers by using microsatellite markers. The results of the genetic population identification and the STRUCTURE analysis showed that *C. aculeata* did not establish one panmictic population and structuring continued. In the natural condition, if migration between populations is low, it means an association with a high balance of genetic distinction (Dorant *et al*., 2019; Cheng *et al*., 2020).

Genetic distance-based measures supported the clustering of Maroun, Beshar and Khersan rivers may be genetically discrete from other *C. aculeata* populations. Despite having wide-ranging, the neighbor-joining dendrogram topology constructed based on genetic distances among populations supported observed division between the populations. Furthermore, the fact that Maroun River stock cluster *individually* indicates possible different independent original sources. The non-significant differentiation between *C. aculeata* samples from the Beshar and Khersan can be explained by a relative disconnection of these two populations and/or small amounts of gene flow.

The value of mean expected heterozygosity are lower compared to the population of *Capoeta capoeta gracilis* from Madarsu and Gorganrud rivers from north part of Iran (Aliakbarian *et al*., 2014), but it is higher than those found in rivers of Golestan Province of Iran for Spirlin (*Alburnoides bipunctatus*) (Jahangiri *et al*., 2013). Similar observations on heterozygosity range were reported for other cyprinid fish ((Jouladeh-Roudbar *et al*., 2017).

In the current study, the estimation of the genetic diversity showed that all eight microsatellite markers are highly informative. The microsatellite markers are highly polymorphic, presenting a number of alleles between 4 and 14. Considering that, the minimum number of alleles recommended for microsatellite loci is four; the markers used in this study are seen as appropriate for analysis of genetic variation in the populations of *C. aculeata*. Allelic diversity and genetic diversity of the population as a whole are also high, indicating their effective and appropriate use for conservation genetic programs. The number of effective alleles is lower than the observed number of alleles. This can be explained by the very low frequency of most alleles at each locus. Furthermore, most of the loci showed significant deviation from Hardy Weinberg equilibrium. This can be explained by the presence of null alleles, genetic drift and inbreeding (Jouladeh-Roudbar *et al*. 2015; Arthofer *et al.,* 2018). In Kohgiluyeh and Boyer-Ahmad Province in Iran. *C. aculeata* is an endemic freshwater fish species, Therefore, the population diversity and genetic diversity are influenced by many factors, such as habitation, anthropogenic activity, founder effects and bottleneck effects (Behera *et al*., 2018; Khoshkholgh and Nazari, 2019).

Our results indicate that there is a genetic structure in the *C. aculeata*

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populations and these findings agree with the patterns of microsatellite marker variation in other cyprinid fish reported by Jahangiri *et al*. (2013), who observed significant differences in microsatellite DNA markers among three populations of Spirlin (*Alburnoides bipunctatus*). Significant variance in microsatellite allele frequency provides evidence that *C. aculeata* populations are spatially genetically structured. Genetic affinities among populations revealed in the neighbor-joining tree showed high bootstrap and genetic distance support for three distinct population segments, generally corresponding to the location of origin (Maroun, Khersan, and Beshar Rivers).

Our results, therefore, do not support the null hypothesis of a homogeneous gene pool for *C. aculeata* inhabiting the three rivers. Bayesian analyses of population structure revealed a maximum ΔK value for genetic clusters. However little level of genetic distinctiveness in Khersan and Beshar River could be related high migration rate of this species. The species' life history also plays a role in influencing contemporary levels of spatial population structure (Bilici *et al*., 2017; Jouladeh-Roudbar *et al*., 2017). These data and limited information on *C. aculeata* suggest that *C. aculeata* adult habitat is distinctive within the river systems they use for spawning.

However, relatively lower genetic variability in Khersan River population in comparison with Maroun River population might be correlated with effective population size owing to the exploitation patterns (Behera *et al*., 2018; Corral-Lou *et al*., 2019). Factors like the construction of dams, reproductive philopatry, overfishing, and pollution which have played a major role in the destruction of the freshwater fish habitat are thought to cause a reduction of genetic diversity (Tibihika *et al*. 2018; Zhao *et al*., 2018). Low genetic variation among localities is an indicator of the fact that there is a high gene flow between populations or these populations were the last ones which were isolated (Souza *et al*. 2017; Khoshkholgh and Nazari, 2020). Hence, *C. aculeata* movement models may be recognizable between sites and the connection of these migratory models stays unexplored. The results showed genetic structure between populations. However, other types of molecular markers like single nucleotide polymorphism (SNP) should be applied to complete genetic population identification and quantify the potential subscription of distinct stock segments to mixed stocks found in the Zagros basin. However, further microsatellite markers and sampling stations in the Zagros basin are helpful for the conservation and management of *C. aculeata*.

Based on the results obtained, nucleotide sequences of the partial control region were determined to investigate the genetic relationships among local samples. The occurrence of 2 or more highly variable nucleotide regions (HVR) flanked by conserved (invariable) segments is a hallmark of the mtDNA control region in animals, including many fish species (Brown, 2008; Khoshkholgh *et al*., 2011). The

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location of the region of highest variability, however, differs among fish species (Gao *et al*., 2014). In *C. aculeata*, the DNA segment of greatest variability was located near the 3´ end of the control region and termed the hypervariable region. This may be due to the high mutation rate of HVR-1 which elevates within population diversity levels for this marker. Analysis of this region has proven adequate for resolving the relationships among closely related taxa, such as local races, sub-species, and sibling species (Xu *et al*., 2006; Zhang *et al*., 2017).

The F_{ST}-value of microsatellite markers in the three populations of the *C. aculeata* was higher than that estimated by mtDNA markers, indicating a high genetic differentiation. Besides, the overall F_{ST} of all collections inferred from the microsatellite markers was much higher than that from the mtDNA control region analysis, indicating a higher level of genetic differentiation at the microsatellite markers. Pairwise population comparisons of F_{ST}-values between the populations from Maroun River and other collections indicated strong population structure as well as those collections from Khersan River. Although the Beshar River was expected to be geographically and genetically isolated from those of the other locations, the haplotype composition as well as allele frequency of the Beshar River samples were not significantly different from those of other locations. The results inferred from mtDNA and simple sequence repeats (SSRs) SSR markers data should be integrated with those obtained from other types of markers such as SNP loci markers. Nevertheless, the estimates of the genetic differentiation slightly differed between the two molecular makers. The present study reinforced the finding that the genetic diversity of these native populations of *C. aculeata* was considerable and suggests that the genetic conservation of populations in the Maroun River is still possible despite many anthropogenic disturbances imposed on this river system. In the same manner, the genetic homogeneity revealed among Beshar River population suggests that this population could be designated as one unique unit for conservation. However, the use of other molecular markers such as microsatellites could further resolve the genetic structure of these geographically distinct populations.

In conclusion, the current mtDNA sequencing and microsatellite marker analyses provided some evidence for the existence of a significant genetic differentiation among some sample populations, suggesting that *C. aculeata* does not comprise a single panmictic population. Although geographical regions were limited in the present study, our data are consistent with the hypothesis of minimal, if any, geographic structuring of mtDNA diversity among *C. aculeata* in the Zagros Basin. This is important information for the conservation genetic program because there may be a lower risk of detrimental genetic impact when attempting to enhance a population of fish having limited genetic structure and high

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diversity. The high haplotype and nucleotide diversities along the sides of allele frequency observed here indicate that the hypervariable region and microsatellites might be useful as genetic monitoring tools in *C. aculeata* populations.

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