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Genetic structure of the *Capoeta aculeata* populations inferred from microsatellite DNA loci

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Abstract. *Gandomkar H, Shekarabi SPH, Abdolhay HA, Nazari S, Mehrjan MS. 2020. Genetic structure of the Capoeta aculeata populations inferred from microsatellite DNA loci. Biodiversitas 21: 4565-4570.* The present study aimed to investigate the genetic variation of *Capoeta aculeata* on the basis of DNA microsatellite loci from three rivers (Beshar, Khersan, Maroun) in Kohgiluyeh and Boyer-Ahmad Province in Iran. DNA from fin clips of 120 specimens extracted and was examined with eight microsatellite markers. Genetic differences between the populations were discerned by pairwise comparison based on allelic distribution. 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 to 0.579, implying that a moderate level of genetic variation. Among three populations, Maroun River population displayed the highest level of variability in terms of heterozygosity. Tests of Hardy-Weinberg showed that the microsatellite loci deviated significantly in the populations. The results indicate that some of the populations were significantly differentiated from one another based on pairwise F_{ST} estimates. Genetic distance-based measures supported the clustering of Maroun, Beshar, and Khersan rivers. The neighbor-joining dendrogram topology constructed on the basis of genetic distances among populations supported observed division between the populations. The non-significant differentiation between *C. aculeata* samples from Beshar and Khersan can be explained by a relative disconnection of these two populations and/or small amounts of gene flow.

Keywords: *Capoeta aculeata*, microsatellites, genetic structure, conservation genetics

Abbreviations: n: Number of samples used, Na: Number of alleles per loci, Ne: Effective number of alleles per loci, He: Expected heterozygosity, Ho: Observed heterozygosity

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 fishes in Iran. This genus is a potamodromous cyprinid fish, with about seven species reported from 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 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). The previous phylogenetic and phylogeographic studies found that populations of *C. aculeata* are different from the others (Zareian et al. 2016; Khaefi et al. 2018).

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 used mitochondrial DNA (mtDNA) markers (Alwan et al. 2016; Ghanavi et al. 2016). 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 in fact are clearly distinct species.

Genetic diversity and population structure are highly important for the sustainability of many species (Khoshkholgh and Nazari 2019). 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 (Khoshkholgh and Nazari 2020). The genetic variation and population structure of *C. aculeata* have not been carefully characterized until now, which has posed a serious obstacle to conservation and management of this species. 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 in-depth understanding of the genetic diversity and population structure. Conservation genetic and optimum lasting authority rely on knowing the distribution, characteristics of all stock segments and maintaining their diversity (Levin et al. 2017), therefore in the present study, microsatellite DNA loci in *C. aculeata* were utilized to delineate the level of genetic diversity among collections of *C. aculeata.*

MATERIALS AND METHODS

Study area

A total of 120 *C. aculeata* samples from three sampling areas (Table1, Figure 1) were obtained. Sample collection of adult *C. aculeata* carried out from September through late November of 2018 commercial fishing seasons.

Procedures

DNA extraction and microsatellite genotyping

Tissue samples, obtained from caudal fins of the *C. aculeata*, were immediately maintained either in 96% ethanol, and then stored at -20 °C freezer, until being processed for DNA extraction. The tissues of ethanol-preserved were incubated in lysis buffer with proteinase K at 37ºC overnight for 14 hours. Total genomic DNA was extracted according to the standardized procedure described by Nazari et al. (2016), and then stored at-20 °C. DNA Extraction was examined for concentration using spectrophotometer (Nanodrop ND1000) and standardized to a specific concentration (for example, 50 ng/µl for Polymerase Chain Reaction (PCR)). The quality DNA specimens were checked optically on a 0.8% agarose gel. Cyprinid-specific dinucleotide microsatellite loci were exploited in this study (Samaee et al. 2006).

Table 1. *Capoeta aculeata* samples collected for population genetic analysis. Sampling localities, numbers (see Fig. 1), and number of individuals sampled (*n*)

	Region	n
1	Beshar River	40
2	Khersan River	40
3	Maroun River	40
	Total	120

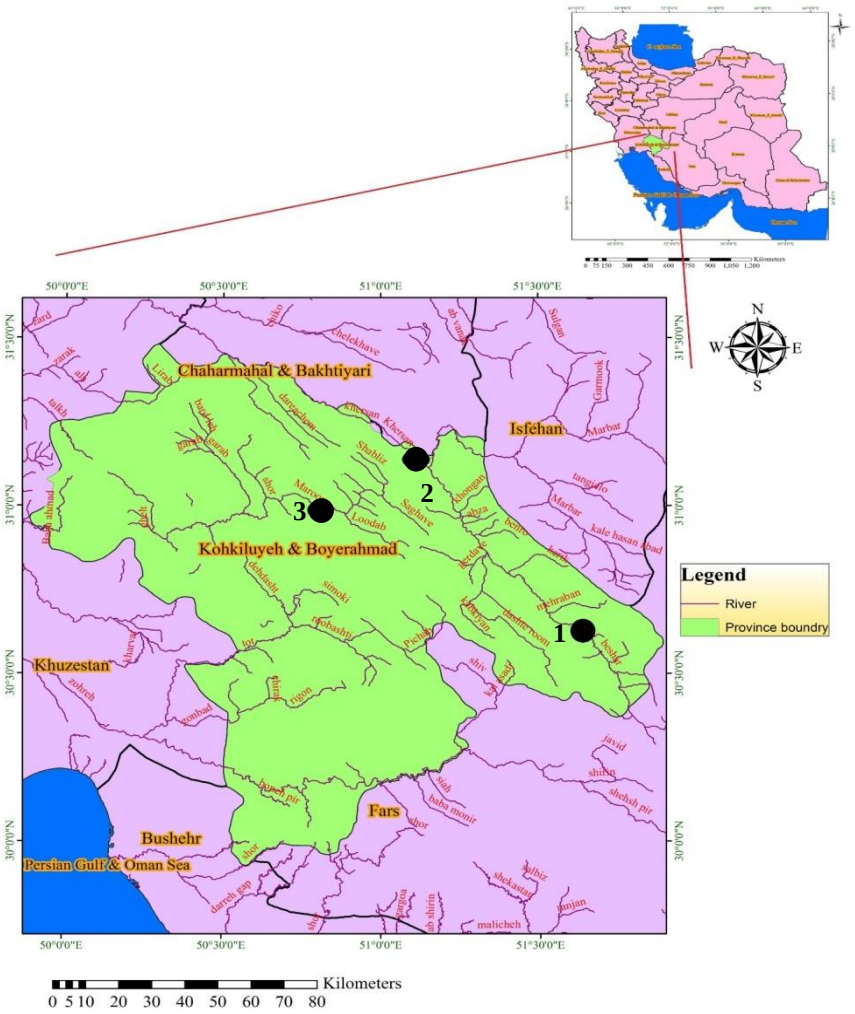

Figure 1. Map depicting the locations of the three populations of *Capoeta aculeata* used in the study, i.e. Kohgiluyeh and Boyer-Ahmad Province, Iran. 1. Beshar River, 2. Khersan River, 3. Maroun River. Detailed information about the sites can be found in Table 1.

Each primer set was tested by varying the PCR conditions and evaluating the PCR products on 1.5% agarose gels. Amplifications were performed in 20µl reaction volume with template DNA (50 ng) using an Eppendorf 5331 thermocycler (Eppendorf, Germany). Experimental condition tested included, MgCl2 concentration (1-2.2 mM), deoxyribonucleoside triphosphate (dNTP) concentration (150-190 µM), 0.75-1.2U 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 second at 94 °C, 30 second at best annealing temperature (Table 2), and 10 minutes for ending extension at 72 °C, followed by 4 °C hold. Products of PCR were separated by 6% non-denaturing polyacrylamide gels electrophoresis in 0.5× TBE buffer for 2 to 3 hours at 250 V and subsequently checked by silver staining method. The pictures acquired were examined by testing BioCapt software (version 2.0) (Table 2).

Data analysis

The number 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). 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 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 et al. 2010). The patterns of the population structure were assessed using the Bayesian clustering approach in STRUCTURE 2.3 (Pritchard et al. 2000). 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 depend on 1000 repeats.

RESULTS AND DISCUSSION

In the present study, the average of the allele numbers inspected at each locus varied between 4 for locus Rser10 to 14 for locus Z21908 (Table 3). 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. Most of overall similarities were significant subsequent sequential Bonferroni adjustment, after pooling rare alleles (Table 4). 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 and Maroun River and Kharsan 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 panmictic population. Pairwise F_{ST} values varied between 0.189 to 0.359 (Table 5).

Table 2. PCR condition and reaction, locus, product sizes, and repeat motifs on *Capoeta aculeata*

Locus	Product size (bp)	Components	Cycling condition	Repeat motif	Number of alleles
MFW17	184-265	1.5mM $MgCl_2$, 170µ MdNTPs, 15 pmol each primer and 1.2 UTaq DNApolymeraz	95/4 min[94/30sec, 51/30 sec and 72/30sec]^{30},72/5min	CT(26)	8
MFW2	180-236	1.5mM $MgCl_2$, 165 µMdNTPs, 10 pmol each primer and 1.2 UTaq DNApolymerase	95/4 min[94/30sec, 60/30 sec and 72/30sec]^{30},72/5min	TA(22)	11
MFW26	172-225	1.5 mM $MgCl_2$, 190 µMdNTPs, 20 pmol each primer and 1.1 UTaq DNA polymerase	95/4 min[94/30sec, 52/30 sec and 72/30sec]^{30},72/5min	CT(24)	12
CypG3	168-296	1.6 mM $MgCl_2$, 175 µMdNTPs, 10 pmol each primer and 1.2 UTaq DNA polymerase	95/4 min [94/30sec, 52/30 sec and 72/30sec]^{30},72/5min	GT(27)	10
CypG24	295-387	1.5 mM $MgCl_2$, 180 MdNTPs, 20 pmol each primer and 0.90 UTaq DNA polymerase	95/3min[94/30sec, 48/30 sec and 72/30sec]^{30},72/5min	GT(27)	8
Z21908	124-172	2 mM $MgCl_2$, 165 µMdNTPs, 20 pmol each primer and 1.2UTaq DNA polymerase	95/4 min[94/30sec, 55/30 sec and 72/30sec]^{30},72/5min	TA(20)	12
Rser10	176-248	1.65 mM $MgCl_2$, 185 MdNTPs, 10 pmol each primer and 1.2 UTaq DNApolymerase	95/4 min[94/30sec, 58/30 sec and 72/30sec]^{30},72/5min	GT(29)	18
Lid1	121-196	1.75 mM $MgCl_2$, 160 µMdNTPs, 20pmol each primer and 1.1UTaq DNApolymerase	95/4 min[94/30sec, 59/30 sec and 72/30sec]^{30},72/5min	GT(24)	12

Table 3. Allelic variability at eight loci in the survey *Capoeta aculeata* populations

Locus	Maroun River	Kharsan River	Beshar River	Variable
MFW17	N	39	40	40
	N_a	5	6	8
	N_e	3.421	4.257	6.497
	He	0.748	0.788	0.796
	Ho	0.321	0.224	0.297
MFW2	N	40	40	40
	N_a	8	8	9
	N_e	5.396	4.345	4.532
	He	0.775	0.688	0.764
	Ho	0.419	0.463	0.212
MFW26	N	38	40	39
	N_a	9	9	11
	N_e	8.385	6.924	9.121
	He	0.748	0.821	0.796
	Ho	0.455	0.352	0.336
CypG3	N	40	40	40
	N_a	9	11	12
	N_e	7.174	5.855	7.019
	He	0.752	0.647	0.837
	Ho	0.436	0.396	0.579
CypG24	N	40	39	40
	N_a	11	9	8
	N_e	8.284	5.349	6.379
	He	0.864	0.728	0.837
	Ho	0.589	0.386	0.579
Z21908	N	40	40	39
	N_a	10	12	14
	N_e	7.984	11.014	12.141
	He	0.357	0.596	0.805
	Ho	0.269	0.411	0.484
Rser10	N	40	40	40
	N_a	4	6	6
	N_e	2.325	3.698	4.177
	He	0.567	0.716	0.863
	Ho	0.336	0.445	0.348
Lid1	N	40	40	40
	N_a	11	10	12
	N_e	8.437	8.119	9.586
	He	0.508	0.758	0.674
	Ho	0.402	0.469	0.296

For each population and locus: Number of samples used (n), Number of alleles per loci (Na), Effective number of alleles per loci (N_e), Expected heterozygosity (He) (Nei 1978), and Observed heterozygosity (Ho).

Population corresponding with non-significant F_{ST} measurements included Khersan River versus Beshar River. Mean F_{ST} for all seven populations was 0.271. Populations from the Maroun River were highly distinct from populations of the Khersan River and Beshar River (Table 5). The overall trial had a chi-square measure of endlessness and differences between allele frequencies among all populations were significant at all loci (P <0.0001).

The significant pairwise F_{ST} values with the neighbor-joining tree approach confirmed that the populations of *C. aculeata* from the Maroun River, Kharsan, and Beshar River separated with high bootstrap support and Maroun River also had the higher branch lengths (Figure 2). The neighbor-joining tree and Bayesian clustering results indicated that the Kharsan 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 location, indicating substantial population genetic structure in the province of Kohgiluyeh and Boyer-Ahmad.

Table 4. Exact *P*-value for Hardy Weinberg Equilibrium (HWE) estimation for the three different *Capoeta aculeata* samples after sequential Bonferroni adjustments

	Maroun River	Kharsan River	Beshar 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

Note: ***P ≤ 0.001, **P ≤ 0.01, *P≤0.05

Table 5. Pairwise estimates of F_{ST} between populations of *Capoeta aculeata*

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

Note: *P<0.05; ** P<0.01.

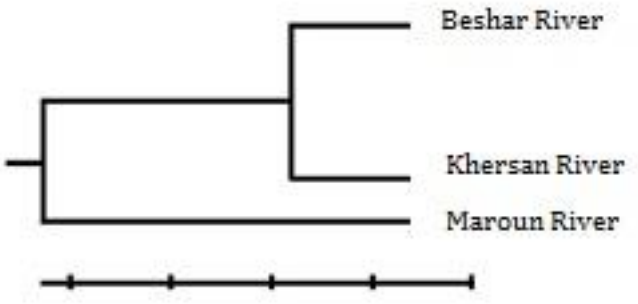

Figure 2. Unrooted neighbor-joining cluster analysis diagrams based on Cavalli-Sforza and Edwards' (1967) chord distance for the gene locus and microsatellite markers. The data were bootstrapped over loci, with replacement, for 1000 replicates; the numbers represent the percent support of the branch

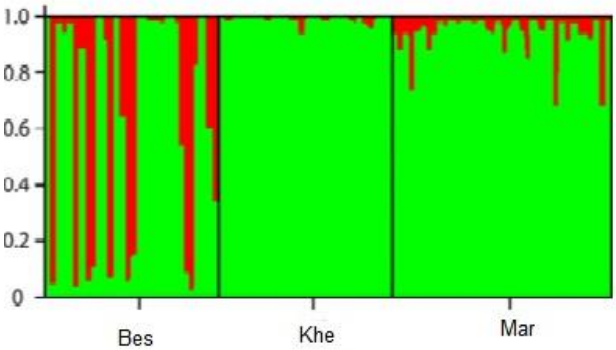

Figure 3. Clustering of individuals by structure at $K = 2$. Individuals are represented by vertical bars. Each vertical column represents one individual

Discussion

In the present study, amplification of eight microsatellite loci in *C. aculeata* was obtained after optimizing the experimental conditions. The results of the present study showed that majority of the pairwise estimates of inter-population variance in allele frequencies (F_{ST}) were 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 of microsatellite marker. The results of the genetic population identification showed that *C. aculeata* did not establish one panmictic population and structuring continued. In natural conditions, if migration between populations is low, it means association with high balanced of genetic distinction (Dorant et al. 2019; Cheng et al. 2020).

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 are 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 fishes ((Jouladeh-Roudbar et al. 2017). Our values are also comparable with those found for the common carp from southeastern part of Caspian Sea and for *Capoeta trutta* populations from two main watersheds in the Kurdistan province (Mirzaei et al. 2016).

In the current study, estimation of the genetic diversity showed that all the eight microsatellite markers are highly informative. The microsatellite markers are highly polymorphic, presenting a number of alleles between 4 to 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 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 with 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* populations and these findings agree with the patterns of microsatellite marker variation in other cyprinid fishes reported by Jahangiri et al. (2013), who observed significant differences in microsatellite DNA marker 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 location of origin (Maroun River, Kharsan and Beshar River (Fig. 2).

Our results, therefore, do not support the null hypothesis of a homogeneous gene pool for *C. aculeata* inhabiting in the three rivers. Bayesian analyses of population structure revealed a maximum ΔK value for genetic clusters. However little level of genetic distinctiveness in Kharsan 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 (Jouladeh-Roudbar et al. 2017; Bilici 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 Kharsan River population in comparison with Maroun River population might be correlated with effective population size owing to exploitation pattern in them (Corral-Lou et al. 2019; Behera et al. 2018). Factors like construction of dams, excessive fishing, and pollution which have played a major role in the destruction of the freshwater fish habitat, are thought to cause reduction of genetic diversity (Tibihika et al. 2018; Zhao et al. 2018). In congruent of this study, Mirzaei et al. (2016) in their study showed that environmental conditions impact to maintain genetic diversity of Capoeta trutta populations in two watersheds is moderate. 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 that were isolated (Souza et al. 2017; Khoshkholgh et al. 2020). Hence, it is possible that for C. aculeata movement models are recognizable between sites and the connection of these migratory models stays unexplored. The results showed that genetic structure between populations. However, other types of molecular markers like single nucleotide polymorphism (SNP) and mitochondrial DNA sequencing should be applied to complete a genetic population identification and quantify the potential subscription of distinct stock segments to mixed stocks found in the Zagros Basin. Furthermore, development of further microsatellite markers and sampling of more regions in different parts of the Zagros basin are helpful for describing sections for conservation and management.

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