

Determination of genetic structure in *Barbus tauricus* Kessler, 1877 populations inhabiting a few streams along the Black Sea Region (Turkey) inferred from mtDNA Cytochrome *b* gene sequence analysis

Karadeniz Bölgesi (Türkiye) boyunca bazı akarsularda yaşayan *Barbus tauricus* Kessler, 1877 populasyonlarının mtDNA Sitokrom *b* sekans analizleri ile genetik yapısının belirlenmesi

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Abstract: This study was carried out in order to determine the intra-specific variations in *Barbus tauricus* populations sampled from different localities in the Black Sea Region. A total of 250 *B. tauricus* samples were collected from inland waters (Akçay Stream (Samsun), Engiz Stream (Samsun), Terme Stream (Samsun) Karadere Stream (Trabzon) and Değirmenağzı Stream (Düzce)) of Black Sea Region. Intra-species variations in the species of *B. tauricus* were determined using mitochondrial DNA *cyt b* sequencing. The population genetic structure was revealed by analyzing the molecular variance (AMOVA). For this study, a member of the related fish group, *Cyprinus carpio* L., 1758 (DQ868874.1) (as an outgroup) and other *Barbus* species from the GENBANK database was used in comparative analyses. Therefore, total sequence of 1141 bp of the *cyt b* gene region was obtained and 11 haplotypes were determined. The molecular data obtained by AMOVA revealed that there were intraspecific variations of *B. tauricus* and that 26.61% of the variation were among the populations. The F_{ST} value estimated for all populations and loci confirms genetic heterogeneity in *B. tauricus* populations ($F_{ST} = 0.59310$). Genetic analyses revealed that *B. tauricus* populations are geographically structured.

Keywords: Crimean barbel, genetic structure, *cyt b*, Black Sea, Turkey

Öz: Bu çalışma Karadeniz Bölgesi'ndeki farklı alanlardan örneklenen *Barbus tauricus* populasyonları arasındaki tür içi varyasyonları ortaya çıkarmak için gerçekleştirilmiştir. Karadeniz Bölgesi'ndeki farklı içsulardan (Akçay (Samsun), Engiz Çayı (Samsun), Terme Çayı (Samsun) Karadere (Trabzon) and Değirmenağzı Deresi (Düzce)) toplamda 250 örnek yakalanmıştır. Tür içi varyasyonlar mtDNA *cyt b* sekansı kullanılarak saptanmıştır. Popülasyonların genetik yapıları moleküler varyans analizi (AMOVA) ile belirlenmiştir. Bu çalışmada GENBANK veri tabanından alınan *Cyprinus carpio* L., 1758 (DQ868874.1) (dış grup) ve diğer *Barbus* türleri karşılaştırmalı analizlerde kullanılmıştır. *Cyt b* gen bölgesinin 1141 baz çiftlik toplam sekansı ve 11 haplotip elde edilmiştir. AMOVA analizinden elde edilen moleküler veriler *B. tauricus* tür içi varyasyonun varlığını ve varyasyonun % 26.1 oranında gruplar arasında olduğunu ortaya çıkarmıştır. Tüm populasyonlardan elde edilen F_{ST} değeri populasyonlardaki genetik heterojeniteyi doğrulamaktadır ($F_{ST} = 0.59310$). Genetik analizler, *B. tauricus* populasyonlarının coğrafik olarak farklılıklar gösterdiğini ortaya çıkarmıştır.

Anahtar kelimeler: Kırm bıyıklı balığı, genetik yapı, *cyt b*, Karadeniz, Türkiye

INTRODUCTION

The taxonomic classification of organisms, and understanding the diversity of biological life, were usually based on descriptions of morphological forms (Adams et al., 2004). However, when there were phenotypically very similar species, there could be some confusion in the studies. In terms of fisheries management and biology, it is important to determine the phenotypic variation caused by environmental and genetic factors. In this way, a more accurate stock management plan can be created. The use of genetic techniques in fisheries science has revealed that many phenotypically different populations or genera are not genetically different. Sometimes it is quite difficult to explain the reasons of morphological differences between populations (Cadrin, 2000), but it is assumed that these differences may be genetically related or might be associated with phenotypic plasticity in response to different environmental factors (Murta, 2000). Population genetic bottleneck, geographic isolation, living environment, gene flow, and natural selection have large effects on the genetic construction of populations (Li et al., 2007). It has been found that biogeography and long-term climate changes first influence the genetic diversity and morphological variations in the species with so many studies to date (Stiling, 1992; Raven and Johnson, 1995; Cabral et al., 2003; Pinheiro et al., 2005; Mahe et al., 2014).

In recent years, many molecular techniques have been developed and Mitochondrial DNA (mtDNA) sequencing is one of the these techniques. mtDNA is the most studied part of the animal genome because of many reasons (Avice, 1986). Mitochondria are semi-autonomous organelles possessing their own genome and the machinery for its replication, transcription and for protein synthesis (Saccone et al., 2000). Mitochondrial DNA requires fewer individuals and find the source of the various haplotypes among geographical regions faster than any nuclear markers (Maes et al., 2003) and one of the most studied parts of the genome in population studies (Tsigenopoulos et al., 2002; Kotlík et al., 2008; Myoung and Kim, 2014; Im et al., 2017). Mitochondrial DNA of animals consists of 37 coding genes (22 tRNA, 2 rRNA, 13 proteins) and one noncoding region. It has been reported that *cyt b* genes differ in population level. Consequently, this region was preferred for a lot of studies (Gilles et al., 1998; Aboim et al., 2005; Sevilla et al., 2007; Rylková et al., 2013; Kartavtsev et al., 2017; Kumar et al., 2017).

Cyprinidae is the most preferred fish family with systematic and phylogeny studies. The world's largest freshwater fish fauna (with highest number of species) belongs to the Cyprinidae, with over 200 genus and over 2100 species (He et al., 2008). *Barbus tauricus* Kessler, 1877 is a widely distributed fish species in Blacksea

watersheds (Kottelat and Freyhof 2007). *Tauricus*, the genus epithelium, comes from Tauris and Taurida, the names of the Crimean Peninsula in ancient Greek and Latin (Bănărescu and Bogutskaya, 2003).

The aim of this study was to examine the intra-specific variation in *B. tauricus* populations sampled from different localities in the Black Sea Region by using mtDNA *cyt b* region.

MATERIAL AND METHODS

Sampling

A total of 250 *B. tauricus* samples were collected from inlandwaters (Akçay Stream (Samsun), Engiz Stream (Samsun), Terme Stream (Samsun) Karadere Stream (Trabzon) and Değirmenağzı Stream (Düzce)) of Black Sea Region with electroshocker (Table 1-Figure 1).

Table 1. Coordinates of sampling locations

Locality	Coordinates	Sampling Time
Karadere Stream	40°51'54.43"N 40° 1'10.06"E	August 2015
Değirmenağzı Stream	41°05' 07.21" N 31°06' 06.50" E	July 2016
Akçay Stream	41°05' 30.99" N 37°07' 20.89" E	April 2016
Terme Stream	41°09' 34.03" N 36°53' 28.48" E	June 2015
Engiz Stream	41°28'55.48"N 36°02'49.58"E	August 2016

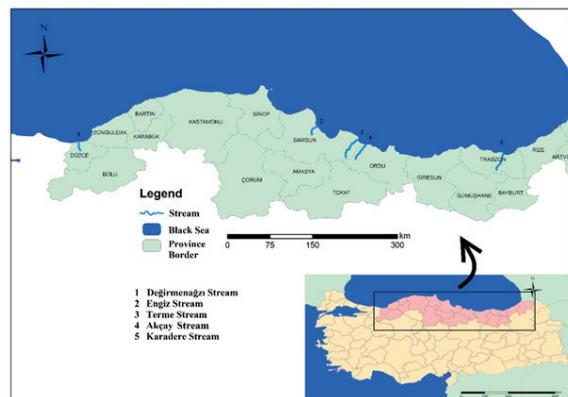


Figure 1. The map of study

50 samples from each locality were investigated for systematic position of *Crimean barbel*. Identification of the specimens was conducted following relevant literature (Geldiay and Balık, 2007; Kottelat and Freyhof, 2007; Polat and Uğurlu, 2011). A total of 49 samples for genetic studies were utilized in the laboratory.

DNA extraction

Caudal fin tissue of each samples were preserved in 98% ethanol and stored at -20°C until DNA extraction. Total genomic DNA was extracted from 15-20 mg of tissue by the INVITROGEN, PureLink™ Genomic DNA Tissue Kit using the following the manufacturer's protocol.

PCR amplifications and sequencing of *cyt b*

Fragment of the mtDNA, *cyt b* gene (1141 bp) was amplified by PCR using the following primers: the entire mitochondrial *cyt b* gene was amplified with primers located within the Glu tRNA (5'-GAAGAACCACCGTTGTTATTC-3') and Thr tRNA genes (5'-GATCTTCGGATTACAAGACC-3') PCR was performed at 95 °C for 4 min, followed by 35 cycles at 95 °C for 30 s, 50 °C for 30 s, 72°C for 1 min 30 s, and a final extension at 72 °C for 5 min. PCR products were visualized in 1.5% agarose low-melting-point gels stained with ethidium bromide for band characterization via ultraviolet transillumination.

Sequencing was performed directly using the corresponding both forward and reverse PCR primers. Sequence analysis was carried out at the Macrogen sequencing service (Macrogen Inc., Amsterdam, Netherlands).

Sequence alignment and genetic analysis

Nucleotide sequences were initially aligned using Clustal X (Thompson et al., 1994) and the final alignment was done manually with BioEdit (Hall, 1999). mtDNA was analyzed to assess levels of pairwise nucleotide variation and determine the nucleotide composition for each population using MEGA 5 (Tamura et al., 2011). The number of haplotypes (H), haplotype diversity (h), nucleotide diversity (π), and the average number of nucleotide substitutions (K) were calculated using DnaSP v.5.10 (Librado and Rozas, 2009). Intra and inter-population genetic diversity was measured by the maximum likelihood estimation of the average number of nucleotide substitutions per site within and between populations (Nei, 1987). jModelTest v.0.1 (Posada, 2008) indicated that the best-fitting model of nucleotide substitution by Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC). Neighbor-Joining (NJ: Saitou and Nei, 1987), Maximum Parsimony (MP) and Maximum Likelihood (ML) algorithms are used to determine the phylogenetic relationships between populations. Relationships between the haplotypes and concordance with geographic sampling locations were assessed by constructing a statistically parsimony network at the 95% confidence interval using TCS 1.21 (Clement et al., 2000).

Maximum-likelihood phylogenetic analyses were performed using the BEST approach implemented

in PhyML, version 3.0.1, which combines NNI (nearest neighbor interchanges) and SPR (subtree pruning and regrafting) algorithms to maximize tree likelihood (Guindon and Gascuel 2003), and using the TIM1+I+G models with parameters estimated by jModelTest (Posada 2008): the base frequencies A=0.3068, C=0.3029, G=0.1286, T=0.2617, and the gamma shape parameter equal to 2.2500, -lnL= 4433.6746 for *cyt b*.

Phylogenetic relationships were inferred using different analytical approaches, performed with PAUP version 4.0b10 (Swofford, 2003) and PHYLIP v.3.68 (Felsenstein, 1993). Maximum parsimony (MP) analysis was conducted assuming equal weightings for all characters.

The population genetic structure was assessed by analyzing the molecular variance (AMOVA) (Excoffier et al., 1992) in ARLEQUIN v.2.0 (Schneider et al., 2000). SplitsTree (Huson and Bryant, 2006) were used to determine the relationships between haplotypes as another approach. *Barbus* and *Luciobarbus* species from GenBank database (AF090781, AF090780, KC465948, AF095605, AF145935, AF090784, AF090782, AF090791, AF090789, AF090786, AF095603 and AF090779) and also *Cyprinus carpio* (DQ868874.1-outgroup) were used in analyses

RESULTS

Sequence composition, nucleotide and haplotype diversity

In the present study, complete *cyt b* sequences (1141 bp) of *B. tauricus* from different localities were aligned. Among the 1141 nucleotide sites, 33 bp (2.89%) were variable, 31 bp (2.71%) were parsimony-informative across all samples. MtDNA *cyt b* sequences have already been used in inferring phylogenetic relationships among species (Brito et al., 1997; Machordom and Doadrio, 2001). Average nucleotide composition of adenine (A), thymine (T), cytosine (C), guanine (G) were examined as 28.11%, 27.19%, 29.26% and 15.37%, respectively. Sequence analysis of *cyt b* revealed 11 different haplotypes (Table 2). h and π were estimated as 0.7491 and 0.00474. The most common of the haplotypes identified was the Hap 4 (Table 3)

NJ, MP, ML trees were drawn using TIM1+I+G model (Figure 2, Figure 3, Figure 4). The *cyt b* sequences for 11 haplotypes were submitted in GENBANK under accession number MH013040-MH010350. Hap 6 (MH010345), Hap 7 (MH010346), Hap 10 (MH010349) were found to be at a different level from the other haplotypes (Figure 5). When the matrices of the pairwise relations of these three haplotypes were examined, it was observed that they were different from other haplotypes. The highest genetic distance was calculated between Hap 2 and Hap 7 (0.0117).

Table 2. Distribution of *cyt b* haplotypes determined for Crimean barbel sampled from the Black Sea Region (AS: Akçay Stream, ES: Engiz Stream, KS: Karadere Stream, TS: Terme Stream, DS: Değirmenağzı Stream)

Haplotypes	Base Position		Localities
	881111123344555666677888999991111		
	170125980607009444625349578890011		
	2131853951144056093461794702901		
		0243	
Hap 1	CTTAATCCACGAATAGAGTAACGCAAGGATCGA		AS
Hap 2	.C.....		AS
Hap 3	...G.....A.....		ES
Hap 4	...G.....A....G		ES, KS, TS
Hap 5	...G.....GA.....A....G		KS
Hap 6	T..GGCTTGAAGGCG..ACGGTAT.G.AGCTAG		TS
Hap 7	T..GGCTTGAAGGCGA.AC GG TAT.G.AGCTAG		TS
Hap 8	...G.....G....A....G		TS
Hap 9	...G.....G		TS
Hap 10	T..GGCTTGAAGGCG...CGGT.T.G.AGCTAG		TS
Hap 11	T.CG.....C....G.A....G		DS

Table 3. Distribution and frequency of *cyt b* haplotypes of *B. tauricus* populations (AS: Akçay Stream, ES: Engiz Stream, KS: Karadere Stream, TS: Terme Stream, DS: Değirmenağzı Stream)

Haplotypes	AS	ES	KS	TS	DS	Total
Hap 1	6	-	-	-	-	6
Hap 2	3	-	-	-	-	3
Hap 3	-	1	-	-	-	1
Hap 4	-	10	9	3	-	22
Hap 5	-	-	1	-	-	1
Hap 6	-	-	-	1	-	1
Hap 7	-	-	-	1	-	1
Hap 8	-	-	-	1	-	1
Hap 9	-	-	-	2	-	2
Hap 10	-	-	-	1	-	1
Hap 11	-	-	-	-	10	10
Total	9	11	10	9	10	49

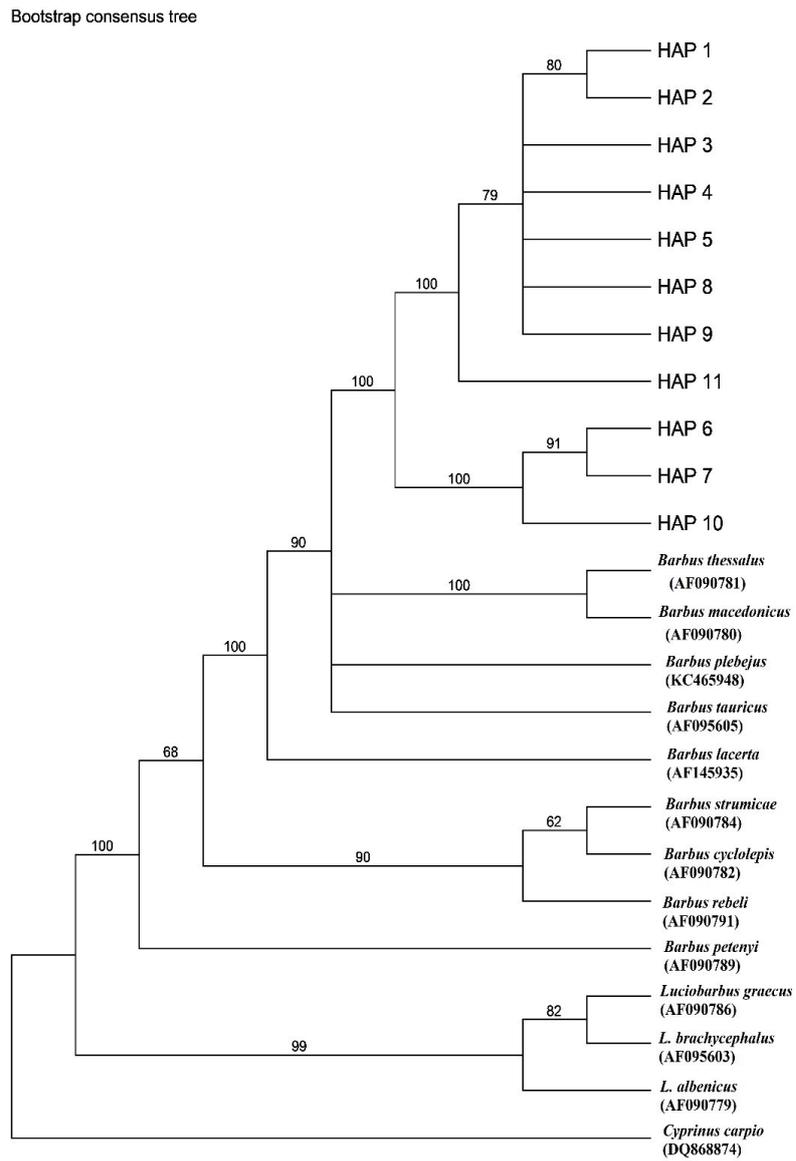


Figure 2. The NJ tree model was constructed for the *cyt b* gene region of the *B. tauricus* (1000 replicates)

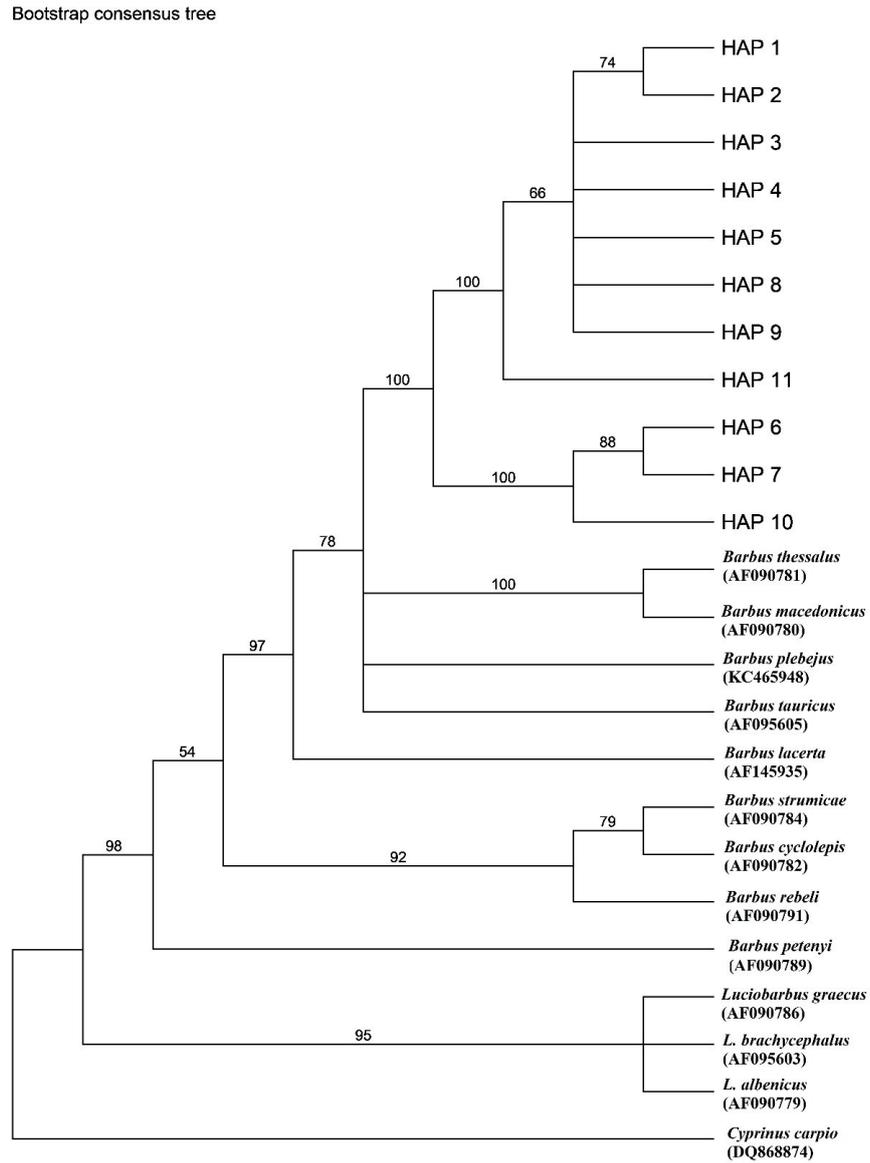


Figure 3. The MP tree model was constructed for the *cyt b* gene region of the *B. tauricus* (1000 replicates)



Figure 4. The ML tree model was constructed for the cyt b gene region of the *B. tauricus* (1000 replicates)

Molecular variance analysis (AMOVA)

AMOVA was performed using the ARLEQUIN for the analysis of the hierarchical distribution of the variance components. Assessment of the population structure

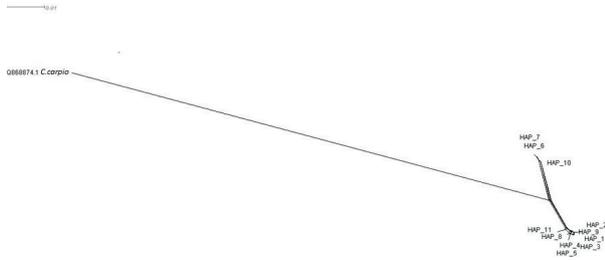


Figure 5. The tree model created using the SplitsTree program for the *cyt b* gene region

is an important part of population genetics. Variance components in groups, within groups, between populations, and within populations were tested against null distribution. It was found that 26.61% of the obtained findings were distributed among the groups, 32.70% among the populations within the groups and 40.69% within the populations for *cyt b* gene of 5 populations. The proportion of 'within the population' variance component is large (40.69%) and the variance components for the comparisons were statistically significant ($P < 0.001$) (Table 4). Multisample and multilocus F_{ST} values were found to be significantly higher than zero ($F_{ST} = 0.59310$), and the F_{ST} value calculated for all populations and loci confirms genetic heterogeneity in *B. tauricus* populations.

DISCUSSION

The Barbel is a complex polyphyletic group of Old World Cyprinidae that provides a fantastic model for

studying evolutionary phenomena in freshwater fish (Berrebi, 1995). The genus *Barbus* has been described by Myers (1961) as a 'monstrous aggregation'. At the present time, it is known that there are more than 1000 species of this genus together with their synonym names. The study of the speciation of the genus *Barbus* is an indirect means of understanding the evolution of the freshwater fish fauna and particularly that of the north Mediterranean basin (Berrebi, 1995). At the end of the Pleistocene, there was a giant freshwater lake in the Black Sea basin, which persisted for thousands of years and only became a sea in the Early Holocene (Ryan et al., 1997).

According to the phylogeographical evidence, this ancient lake system provided glacial refugia for fluviolacustrine barbels. Among these species included in genus *Barbus*, *B. tauricus* is one of the species with the widest distribution area. The main objective of this study was to detect genetic variability within and between the populations of Crimean barbel.

Cyt b gene has been preferred for both in-species and interspecific variations in many studies (Berrebi, 1995; Callejas and Ochando, 2002; Kartavtsev and Lee, 2006; Sakai et al., 2009; Markovà et al., 2010; Zheng et al., 2016; Behera et al., 2017; Kumar et al., 2017). There are some studies about genetic structure of *Barbus* species from different regions performed on understanding the phylogeny among the species. Those studies were focused on sequence of only mtDNA data of a single or few specimens from each species (Tsigenopoulos and Berrebi, 2000; Machordom and Doadrio, 2001). However, there is no study that examines the variation among populations of *B. tauricus*. Tsigenopoulos et al (2002) investigated complete sequences of the *cyt b* in order to infer phylogenetic relationships between diploid, tetraploid and hexaploid 'Barbus' species is occurring in southern Africa. Kotlík et al (2004) reported sequence variation at the *cyt b* gene (1141

Table 4. Results of AMOVA analysis for *B. tauricus*

Source of Variation	df	Sum of Squares	Variance Components	Percentage of variation	F_{IS}	F_{ST}	F_{IT}	P value
Among groups	2	49.757	0.84961	26.61		0.5931*		< 0.001
Among populations within groups	2	22.926	1.04373	32.70	0.4455*			< 0.001
Within populations	44	57.154	1.29894	40.69			0.2662*	< 0.001
Total	48	129.839	3.19229					

bp) to assess the phylogeography of *Barbus* fishes in the Black Sea Region and indicate that the refugial *Barbus* populations remain as geographical isolates in the Black Sea region, continuing to diverge genetically from each other and from remaining populations. In this study, complete sequence (1141 bp) of *cyt b* was obtained, too. The results of present study also reveal the necessity of a genus-based revision because of phylogeographical lineages identified in the Black Sea barbels as suggested by Kotlík et al (2004). Zordoya and Doadrio (1999) were investigated evolutionary and biogeographical patterns of European Cyprinids, the results of that study provide a phylogenetic framework which turns out to be very useful in revising the systematics of some *Barbus* species. Tsigenopoulos and Berrebi (2000) were investigated molecular phylogeny of North Mediterranean freshwater *Barbus* species (21 different species except *B. tauricus*) inferred from *cyt b* sequences and observed important genetic divergence among species and subspecies of the genus *Barbus* and confirm the necessity for a taxonomic revision. The data obtained from the *cyt b* could help to identify new species. Antal et al. (2016) were discovered and described a new *Barbus* species, *B. biharicus* according to *cyt b* sequence data. Similarly, according to the results of this study, Hap 6, Hap 7, Hap 10 were different from the other haplotypes (Figure 5). That was interesting that these differences were not supported by morphological studies. The authors were planning more detailed studies on this subject.

AMOVA analysis was conducted to observe how genetic variation was distributed among populations and within populations. The results of AMOVA showed the presence of high (40.69%) within population variation which favored the haplotype differentiation.

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Livi et al (2013) evaluated the molecular and morphometric properties of the *Barbus* species sampled from Italy and reported 29.80% of *Barbus* spp. diversity within populations and 63.78% among groups. The results of the AMOVA analysis revealed that there were genetic differences among the populations ($P < 0.001$). The F_{ST} value is different from 0 and it is determined that this difference is statistically significant (Table 4).

Genetic differentiation is important for developing strategies for conservation and management of a fish species. The genetic variations present in different fish stocks of a species affect the stocking programs and other related propagation practices (Allendorf et al., 1987; Clarke et al., 2015; Behera et al., 2017).

This study was carried out to investigate the genetic diversity of *B. tauricus* living in some freshwater sources along the coast of Black Sea Region and to identify genetic differences using *cyt b* region. Cytochrome *b* sequences seem to provide a basis source of information that can improve taxonomic classification. Our genetic analyses revealed that *B. tauricus* populations are geographically structured, and this study will be useful in differentiating intra-specific genetic diversity in populations. Consequently, the results of the study should help planning the fisheries management and genetic conservation studies.

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