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Genetic Diversity of *Cyprinion macrostomus* Heckel, 1843 (Teleostei: Cyprinidae) in Anatolia

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Abstract

In this study, the phylogenetic and phylogeographic relationships among *Cyprinion macrostomus* HECKEL, 1843, *Cyprinion kais* HECKEL, 1843 and *Carasobarbus chantrei* SAUVAGE, 1882 samples from Anatolia was analysed. While doing this, mitochondrial DNA polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and Nuclear DNA inter-simple sequence repeat (ISSR) were used. PCR amplified the complete mtDNA NADH ¾ dehydrogenase (ND-3/4) gene. Amplified fragment was digested by 14 restriction enzymes that produced 14 composite haplotypes for these populations. All identified mtDNA haplotypes differed from each other having a specific genetic profile. The genetic diversity among the mtDNA haplotypes of *C. macrostomus*, *C. kais*, and *C. chantrei* populations from drainage basins of the Mediterranean and those from the drainage basin of the Persian Gulf was purported by the extrapolation of mtDNA PCR-RFLP data. This result is also supported by the data explaining the geological history of Anatolia. Broadly, the data from mtDNA and from nDNA was consistent.

Keywords: mtDNA, RFLP, ISSR, *ND*-3/4 gene, *Cyprinion*, phylogeography.

Anadolu *Cyprinion macrostomus* Heckel, 1843 (Teleostei: Cyprinidae) Türünün Genetik Çeşitliliği

Özet

Bu çalışmada Anadolu' daki *Cyprinion macrostomus* HECKEL, 1843, *Cyprinion kais* HECKEL, 1843 ve *Carasobarbus chantrei* SAUVAGE, 1882 örneklerinin filogenetik ve filocoğrafik akrabalıkları, mitokondriyal DNA Polimeraz Zincir Reaksiyonu-Restriksiyon Fragment Uzunluk Polimorfizmi (PCR-RFLP) yöntemi ve Nüklear DNA-ISSR tekniği kullanılarak araştırıldı. Mitokondriyal DNA'nın PCR yöntemiyle çoğaltılan NADH ¾ dehidrojenaz (ND-3/4) gen bölgesi, 14 farklı restriksiyon enzimiyle kesildi ve bunun sonucunda çalışmamızdaki tüm populasyonlar için 14 farklı kompozit haplotip ortaya çıktı. Buradaki tüm tanımlanmış mtDNA haplotipleri sahip oldukları spesifik genetik profilleriyle birbirlerinden ayrılmaktadır. Ve PCR-RFLP verilerine göre, Akdeniz havzasından toplanan balık populasyonlarında ortaya çıkan mtDNA haplotipleri arasındaki genetik çeşitlilik, örneklerin toplandığı su havzalarının jeolojik tarihiyle de benzerlik göstermektedir. Nitekim Fırat-Dicle nehir sisteminin Asi nehrinden ve Akdeniz havzasındaki diğer nehirlerden daha erken dönemlerde izole olduğunu göstermektedir. Genel olarak mtDNA ve nDNA verileri birbiriyle uyumlu gözükmektedir.

Anahtar Kelimeler: mtDNA, RFLP, ISSR, *ND-3/4 geni*, *Cyprinion*, filocoğrafya

Introduction

The *Cyprinion macrostomus*, a member of Cyprinidae family adapted to the thermal springs, feeds on the scar tissues in the skin and is utilised to cure neurodermitis (Arkhipchuk, 1999), thus it is called doctor fish in Turkey. Living in the thermal waters of Sivas as a Northeast habitat, this fish has a medical purpose in curing psoriasis (Kuru, 1999) and is able to tolerate high salinities. At the generic level, the members, which crossed the Persian Gulf during the Pleistocene, are evidently related to Southern Iranian species and are parts of an extended Tigris-Euphrates basin (Coad, 1996).

The largest and most important river system between the Nile and the Indus, the Tigris-Euphrates basin is the main drainage system of Southwest Asia. Gathering freshwater from all over the Middle East and linking the Black and Caspian Seas before the Pliocene orogenesis, the proto-Euphrates renders the Mesopotamian Basin. It is an important centre for inland water fauna, thus facilitating a mixture of

African and Asian species for a short period (Por, 1989), and leading many to assume the Middle East as a major biogeographical crossroad with elements from the Palaearctic, the Mediterranean and the Oriental regions (Banarescue, 1977; Por and Dimentman, 1985, 1989). The Middle East may have been an important intersection for ichthyofauna or a centre of speciation as a phylogenetic study of Cyprinae and Leuciscinae species from the Middle East and neighbouring biogeographical regions demonstrated (Durand *et al*., 2000; Durand *et al*., 2002; Bardakci *et al*., 2006).

To examine the genetic variety in cyprinidis across a broad range of geographic regions, various molecular markers, in particular allozymes, mitochondrial DNA (mtDNA) (Durna *et al*., 2010; Durand *et al*., 2002, 2003; Gross *et al*., 2002; Briolay *et al*., 1998; Zardoya *et al*., 1999) and nuclear DNA have been widely used together with the developments in molecular techniques that have elucidated molecular variation in organisms. In the study of phylogeographic patterns in freshwater and anadromous fishes, mtDNA polymorphisms have been reported to be an effective means (Avise, 1994; Bernatchez and Wilson, 1998). Maternal inheritance and a lack of recombination render mtDNA, which acts as a molecular clock, functional to identify maternal lineages and their split times from common ancestors (Gross *et al*., 2002; Avise, 1994).

This study aimed at determining genetic diversity and dispersal patterns among *Cyprinion macrostomus*, *Cyprinion kais* and *Carasobarbus chantrei* populations splitted in Anatolia was completed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays in the segment of mitochondrial NADH ¾ in addition to nuclear DNA ISSR markers. *C. macrostomus* species is adapted to the thermal springs and feeds on the skin tissues and cure neurodermitis. So it is important to know the differences among the fish populations adapted different habitats.

Materials and Methods

Sample Collection

A total of 76 *Cyprinion macrostomus* samples were picked from 7 localities representing the drainages of the Euphrates and the Tigris. In addition, four *Cyprinion kais* samples from the Tigris River were also included in the analysis to cover all species of the *Cyprinion genus* inhabiting this region. 9 *Carasobarbus chantrei* samples picked from the Orontes River were compared as an out group (Table 1 and Figure 1). *Carasobarbus chantrei* is also a member of Cyprinidae family belonging to the same sub-family with *Cyprinion macrostomus* making it possible for us to use it as an out group. Most of the samples were caught by electro-fishing and then preserved in 95% (v/v) ethanol.

Laboratory Protocol

Total genomic DNA was extracted either from the muscle tissue at the bottom of dorsal fin or alternatively from the pectoral fin tissues of specimens. Apart from the restrictions enzymes (*Hinf1, Hin61, Mbol1, Xba1, Taq1, Hpa2, Alu1, Eco471, Cau2, Tfi1, Cai1, Pvu2, Mbo2, Rsa1*) the whole protocol applied in this research including DNA isolation, PCR-RFLP technique, electrophoretic application and the remaining applications were the same as described by Durna *et al*. (2010) and Gross *et al*. (2002).

In terms of nuclear DNA, five ISSR primers sequences, PCR conditions and ISSR products

Table 1. Localities and drainages of samples included in this study

Population	Abbreviation	N	Locality	Altitude (m a.s.l.)	Coordinate	River System and Drainage
Topardic Creek	TC	26	Kangal, Sivas	1425	N 39°14'17" E 37°23'15"	Euphrates, Persian Gulf
Kalkim Creek	KC	15	Kalkim Village, Kangal, Sivas	1573	N 39°13'01" E 37°37' 60"	Euphrates, Persian Gulf
Kangal Thermal Spring	KTS	13	Kangal, Sivas	1425	N 39°14'17" E 37°23'15"	Euphrates, Persian Gulf
Karakaya Bridge	KB	4	Cungus-Diyarbakir	939	N 38°26'34" E 38°49'05"	Euphrates, Persian Gulf
Tigris River 1	TR1	9	University Bridge, Diyarbakir	630	N 37°55'08" E 40°14'57"	Tigris, Persian Gulf
Devegecidi Bridge	DB	5	Diyarbakir	675	N 38°03'29" E 38°59'03"	Tigris, Persian Gulf
Tigris River 2	TR2	4	Central Diyarbakir	630	N 37°53'54" E 40°15'12"	Tigris, Persian Gulf
Orontes River *C. chantrei*	OR	9	Sinanli-Hatay	16	N 36°06' 04" E 36°05'18"	Orontes, Mediterranean
Karakaya Bridge *C. kais*	CK	4	Cungus-Diyarbakir	939	N 38°26'34" E 38°49'05"	Euphrates, Persian Gulf

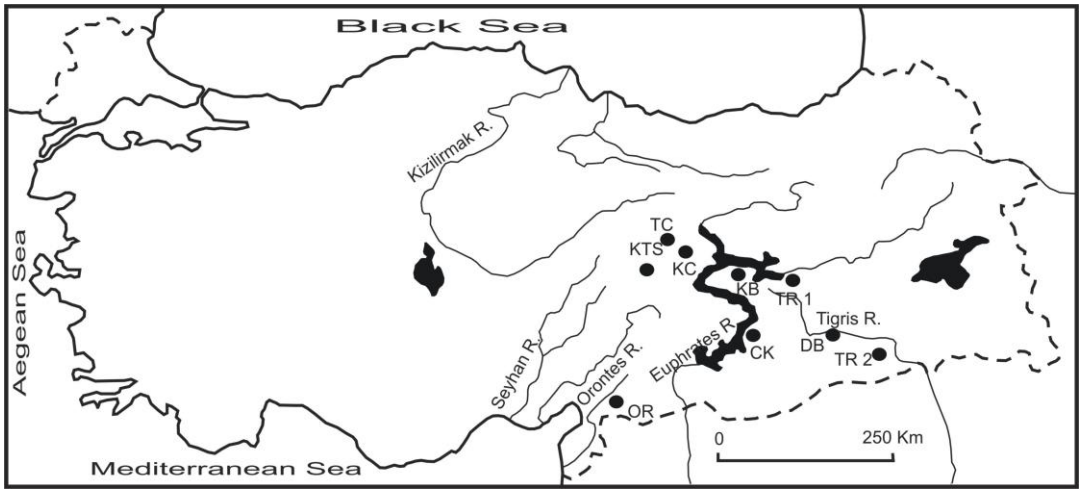

Figure 1. Sampling localities for the specimens in Anatolia.

development were also performed in accordance with this depicted study's protocol (Durna *et al*., 2010).

Data Analyses

Each distinct restriction enzyme fragment pattern was designed according to the study of *Garra* sp., by Durna *et al*. (2010). The composite haplotypes for each fish were designated by a multi-letter code representing the fragment patern for each restriction enzyme. All the details of both PCR-RFLP and ISSR output are shown in Table 2 for the species of *C. macrostomus*, *C. kais* and out group population analysis. Haplotype and nucleotide diversity values were computed with restriction fragment data using program DA in the REAP package (McElroy *et al*., 1991). Also REAP GENERATE, PHYLIP SEQBOOT, RESTDIST and PHYLIP DOLLOP programmes (Nei and Li, 1979; Nei, 1987) were used for constructing the Dollo parsimony and UPGMA trees by using PHYLIP version 3.6a3 as previously published by Durna *et al*., 2010.

The ISSR paterns in this study were compared within and between populations. Data were analyzed using Tools for population genetic analyses (TFPGA) 1.3 software (Miller, 1997) for constructing a UPGMA tree based on Nei's (1978) unbiased distance.

Results

PCR-RFLP mtDNA Haplotype Polymorphism

The size of amplified ND-3/4 segment of mtDNA was 2.4 kb in length. This segment of mtDNA taken from 89 individuals was digested by using 14 (10 polymorphic and 4 monomorphic restriction enzymes) restriction enzymes. The restriction digestion analysis produced 61 cut sites that represented an estimated 275 nucleotides. Analysis of RFLP results showed a total of 14 composite mtDNA haplotypes in populations studied. The obtained composite haplotypes and their occurrences in each population are summarized in Table 2.

Phylogenetic Relationship among Haplotypes

Based on pairwise comparison, the divergence rates among 14 haplotypes of fish populations from each river basin ranged from 0.023 to 7.35 % (Table 3). Haplotype 13 (only in the Orontes population) and haplotype 4 (only one sample from the Karakaya river) were ascertained to be the most distinct haplotypes regarding the values of pairwise nucleotide differences between haplotypes. The study also clearly puts forward that the haplotype 13 (belongs to *C. chantrei*) was the most distinct haplotype. The nucleotide divergence values of haplotypes are summarised in the form of a UPGMA tree (Figure 2). An unrooted Dollo parsimony majority-rule consensus tree based on the +/- of restriction fragment data was also constituted (Figure 3). All of *Carasobarbus chantrei* specimens which are used as outgroup, had been grouped in just one haplotype (type 13) and called as the Orontes river lineage (Figure 3, Table 2). Haplotype 13 is very different from the other lineages' haplotypes in regard to the restriction enzymes apart from *Xba I* restriction enzyme. The common haplotypes found in the samples from the Topardic river, Kalkim river, Havuz, Karakaya Bridge populations were detected in the Euphrates lineages and also the common haplotypes from the Devegeçidi Bridge, University Bridge, and Tigris river populations were detected in the Tigris lineages (Table 2; Figure 2 and 3).

MtDNA Variation within and among Populations

Majority of the studied locations exhibited more than one mtDNA haplotypes excluding *C. chantrei* samples from the Orontes river. Average haplotype

Table 2. Frequency distribution of 14 composite haplotypes among 9 populations of *C. macrostomus, C. kais and C. chantrei* species based on *14* restriction enzymes. Composite haplotypes denoted by capital letters are given in the following order: NADH 3/4: *Hinf1, Hin61, Mbo1, Xba1, Taq1, Hpa2, Alu1, Eco471, Cau2, Tfi1, Cai1, Pvu2, Mbo2, Rsa1.*

Haplotype	Composite Haplotypes	Population									
		Euphrates				Tigris			Med.	Euph	
		TC	KC	KTS	KB	DB	TR1	TR2	OR	CK	Total
Type 1	AAAAAAAABAAAAA	26	15	13	1					1	56
Type 2	BAAAADAABAAAAA				1						1
Type 3	AAAAADAABAAAAA				1						1
Type 4	AAAAACABCAAAAA				1						1
Type 5	BAAAAAAABBAAAA					3		2			5
Type 6	BAABAAAABBAAAA					2	1				3
Type 7	BAABACAABAAAAA						1				1
Type 8	BAABBAAABAAAAA						1				1
Type 9	BAABAAAABAAAAA						2				2
Type 10	BAAAAAAABAAAAA						4			1	5
Type 11	BBAAAAAABBAAAA							1			1
Type 12	BAAAAAABBBAAAA							1			1
Type 13	CCBABBBCACAAAA								9		9
Type 14	AAAAAAABBAAAAA									2	2
Number of Individuals		26	15	13	4	5	9	4	9	4	89

Table 3. Nucleotide diversity within (main diagonal) and net nucleotide divergence (below main diagonal) among the 2 lineages of studied species and the geographic regions where they were found

Lineages	Euphrates	Tigris	Orontes River *(C. chantrei)*	Karakaya Bridge *(C. kais)*
Euphrates	0.0000000			
Tigris	0.3248716	0.0000000		
(Orontes River) *C. chantrei*	7.3527786	7.2384817	0.0000000	
(Karakaya Bridge) *C. kais*	0.0235376	0.2649473	7.2596845	0.0000000

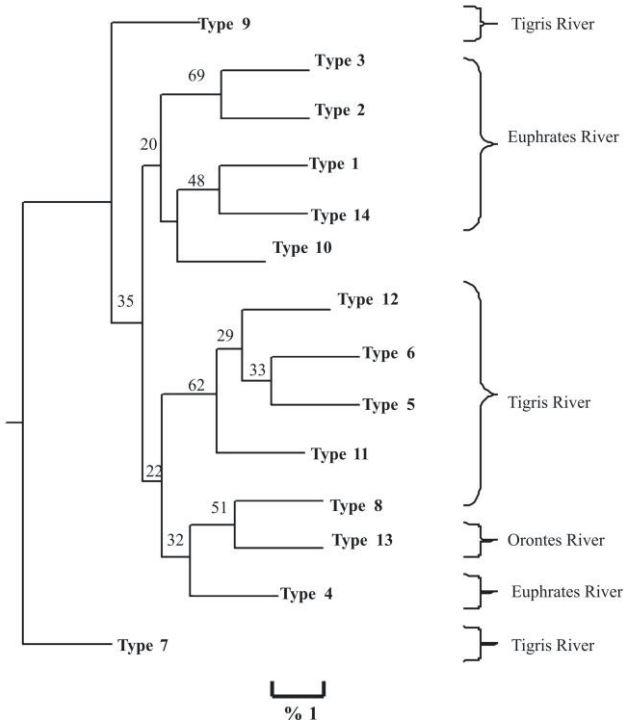

Figure 2. An unrooted UPGMA tree based on nucleotide divergence values between 14 mtDNA haplotypes defined by PCR-RFLP data of NADH-3/4 gene region. Numbers above the branches represent the bootstrap values obtained from 1000 replicates of the restriction fragment data between the haplotypes. Support values under 20% are not represented.

diversity was 0.4525 and average nucleotide diversity was 0.001765 within populations (Tables 3 and 4). Of these populations, Karakaya Bridge (KB) having the highest haplotype diversity was followed by the Tigris River (TR2) and *C. kais* (CK) populations in order. The mtDNA haplotypes and their distribution are summarized in Table 2. Four populations (TC, KTS, KB and KC) in the Euphrates drainage had 4 different haplotypes while three populations in (TR1, TR2, and DB) Tigris drainage basin had 8 different haplotypes. Besides, *C. kais* species had 3 different haplotypes. The Orontes and Tigris river (TR2) populations are the most distinct groups with 7.57 % nucleotide divergence value and in general *C. chantrei* species is the most distinct group from the others with regard to net nucleotide divergence value (Table 5). Genetic relationships among the studied populations were evaluated by constructing a UPGMA tree based on the percent nucleotide divergence values (Figure 4). Results showed three distinct groups of the populations reflecting the genetic relationships between mtDNA lineages belonging to the Tigris, Euphrates and *C. chantrei* which was in a different related group, and it was obvious that *C. chantrei* was a distinct group among all the samples (Figure 4). The aim of this work was to study the phylogenetic and phylogeographic distributions of *C. macrostomus* populations in Anatolia; and yet one *C. kais* population from the Euphrates river and one *C. chantrei* population from the Orontes river basin were also studied together with *C. macrostomus* populations to examine all the Cyprinion species prevailed in this area. RFLP data analysis showed a single fixed haplotype (Type 13) for *C. chantrei* which can be used as a genetic marker to classify this species. The restriction enzymes used in this study and produced unique banding patterns can be easily used in identifying the *C. chantrei* species (Table 2). One of the most impressive finding from these comparisons was probably the genetic divergence

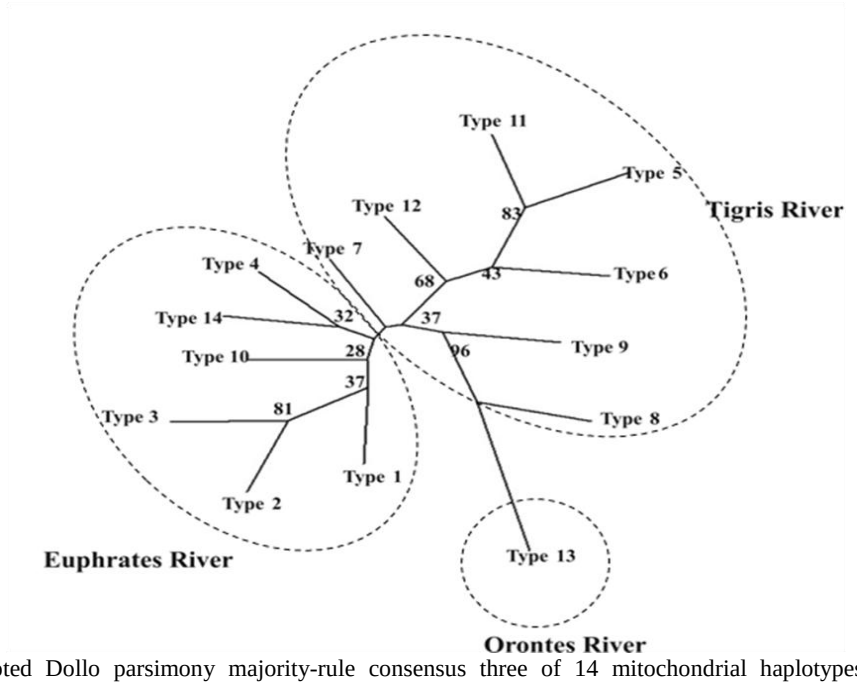

Figure 3. Unrooted Dollo parsimony majority-rule consensus three of 14 mitochondrial haplotypes determined by combining RFLP data of NADH-3/4 gene with values representing percent number of times at which gouping occurred in 1000 bootstrap replicates (only values above 20 % have been noted).

Table 4. The haplotype and nucleotide diversities of 9 populations belong to *C. macrostomus, C. kais*, and *C. chantrei*

Population	Haplotype Diversities	Nücleotide Diversities
Topardic Creek	0.0000 +/- 0.0000	0.000000
Kalkim Creek	0.0000 +/- 0.0000	0.000000
Kangal Thermal Spring	0.0000 +/- 0.0000	0.000000
Karakaya Bridge	1.0000 +/- 0.1767	0.006482
Devegecidi Bridge	0.6000 +/- 0.1752	0.001380
Tigris River 1	0.8056 +/- 0.1195	0.003884
Tigris River 2	0.8333 +/- 0.2224	0.001917
C. chantrei (Orontes River)	0.0000 +/- 0.0000	0.000000
C. kais (Karakaya Bridge)	0.8333 +/- 0.2224	0.002222
Average	0.4525 +/- 0.0216	0.001765 (0.0000005)

Table 5. % net nucleotide divergence among *C.macrostomus, C. kais and C. chantrei* populations collected from 3 drainages

Drainages		Euphrates				Tigris			*C.chantrei* Mediterranean	*C. kais* Euphrates
Population		TC	KC	KTS	KB	DB	TR1	TR2	OR	CK
Euphrates	TC	0.0000								
	KC	0.0000	0.0000							
	KTS	0.0000	0.0000	0.0000						
	KB	0.0591	0.0591	0.0591	0.0000					
Tigris	DB	0.5660	0.5660	0.5660	0.5403	0.0000				
	TR1	0.2513	0.2513	0.2513	0.2068	0.2396	0.0000			
	TR2	0.5578	0.5578	0.5578	0.5049	0.0225	0.3277	0.0000		
C. chantrei Mediterranean	OR	7.3582	7.3582	7.3582	7.3355	7.5489	7.0673	7.5741	0.0000	
C. kais (Euphrates)	CK	0.0279	0.0279	0.0279	0.0200	0.5171	0.1939	0.4482	7.2596	0.0000

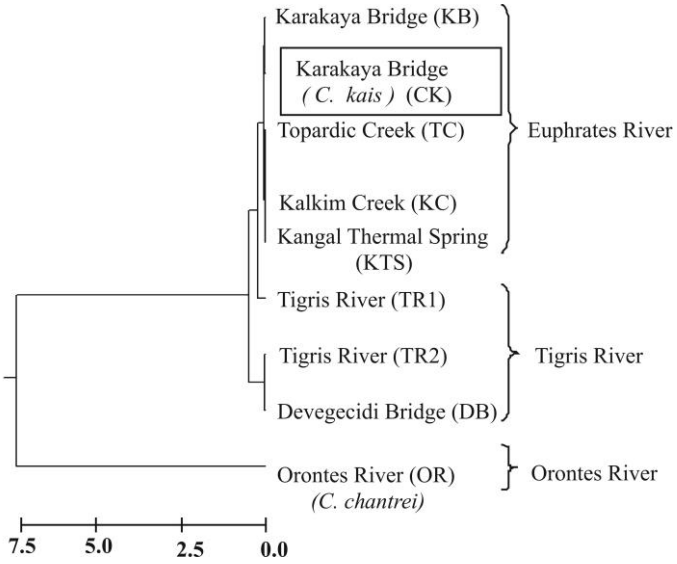

Figure 4. The UPGMA tree based on the nucleotide divergence values of NADH-3/4 gene across 9 populations of the *C. macrostomus, C. kais*, *C.chantrei* species.

values between *C. macrostomus* and and *C. kais* populations that were close and even lower than that found among some *C. macrostomus* populations from different drainage basins. Furthermore, *C. chantrei* population was quite away from the other populations (Table 3). As a result, a single fixed mtDNA haplotype of *C. chantrei* grouped in the Tigris along with other *C. macrostomus* haplotypes from the Tigris river basin (Figure 3). The work also showed that *C. chantrei* was located at the bottom of the population tree next to the Cyprinion samples (Figure 4).

Nuclear DNA Variation among Populations

To confirm the results acquired from the mtDNA data, nuclear DNA markers produced by ISSR analysis were used. In general, a resulting UPGMA tree based on Nei's (1978) genetic distance method, demonstrated that the ISSR markers were in line with mtDNA results (Figure 5). The nuclear DNA data proved relatively higher genetic divergence (0.406) between the *C. chantrei* populations from the Orontes and *C. macrostomus* populations from the Tigris river (TR2) drainage basins (Table 6). Higher genetic divergence between these species confirms the current taxonomic status of these species.

Discussion

The acquired results put forward the mtDNA divergence and variety within Anatolian *C. macrostomus*, *C. kais* and *C. chantrei*. The level of net divergence of mtDNA haplotypes of the tested populations from each river in the Euphrates, Tigris and Orontes drainage basins varies from 0.023% to 7. 574% that is in line with the level of intraspesific haplotype sequence divergence for freshwater fish reported to be as high as 10% (Billington and Hebert, 1991; Richardson and Gold, 1995; Imsiridou *et al*., 1998). The PCR-RFLP data from the mtDNA denoted

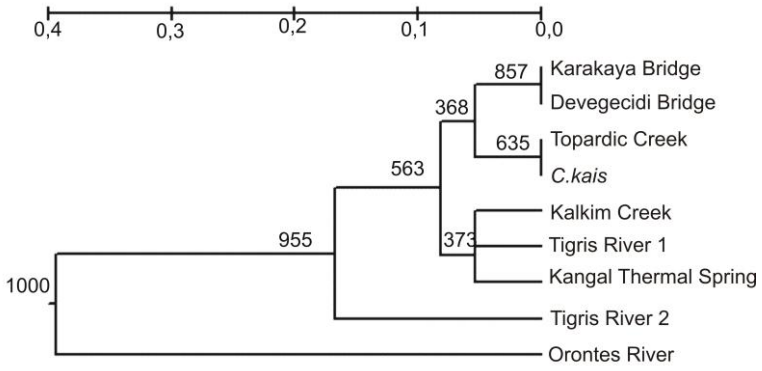

Figure 5. The UPGMA tree of populations based on Nei's (1978) unbiased distance calculated from ISSR markers.

Table 6. Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

pop ID	TC	KTS	KC	OR	KB	DB	TR2	TR1	CK
TC	****	0.9608	0.9608	0.7843	0.9608	0.9608	0.8824	0.9608	1.000
KTS	0.0400	****	0.9608	0.7451	0.9216	0.9216	0.8431	0.9608	0.9608
KC	0.0400	0.0400	****	0.7451	0.9216	0.9216	0.8431	0.9608	0.9608
OR	0.2429	0.2942	0.2942	****	0.7451	0.7451	0.6667	0.7451	0.7843
KB	0.0400	0.0817	0.0817	0.2942	****	1.0000	0.9216	0.9216	0.9608
DB	0.0400	0.0817	0.0817	0.2942	0.0000	****	0.9216	0.9216	0.9608
TR2	0.1252	0.1706	0.1706	0.4055	0.0817	0.0817	****	0.8824	0.8824
TR1	0.0400	0.0400	0.0400	0.2942	0.0817	0.0817	0.1252	****	0.9608
CK	0.0000	0.0400	0.0400	0.2429	0.0400	0.0400	0.1252	0.0400	****

a geographic pattern in the dispersion of mtDNA haplotypes. All the populations from the Tigris, Euphrates and Orontes drainage basins show unique haplotypes (Table 2). None of the populations across these basins share any mtDNA haplotypes with each other. Genetic structuring among these populations evidenced an isolation of the basins they belong to. This study can be a preliminary for the next comprehensive research which takes more fish samples from the countries like Iraq, Iran, Israel and India. Yet because the sample sizes used in this study were not large enough to exactly estimate the populations' variation, the submitted values should only be regarded as indicators of the relative levels of variation. Also a low level of variability within and between the populations for species with a restricted dispersion complied with a general trend in the European cyprinids (Mesquita *et al*., 2001). The Cyprinid species was suggested to enter Anatolia during the Messinian "Lago Mare" phase (Krijgsman *et al*., 2004; Durand *et al*., 2002). As presented by Por and also reviewed by Heller (2007), the recent biogeographical evidence backs up the scenario of close faunal relationships originally existed between the Euphrates and the Orontes, making it one aquatic complex. As indicated by Heller (2007), today the Orontes and Euphrates rivers host closely related taxa among different freshwater living forms backing up the scenario of an early link between the Orontes and the Euphrates (Turan *et al*., 2004). A relatively lower level of genetic divergence value between *C. macrostomus* populations from the Euphrates-Tigris river basins purports a recent isolation of these two rivers from each other. The values of the genetic differences acquired by mtDNA-RFLP analysis in the mtDNA lineages of the Anatolian *C. macrostomus* species allude to a possibility that *C. macrostomus* species might have entered Anatolia relatively late and could not spread at a big scale due to the negative conditions of the glacial periods and spread into the upper Tigris and Euphrates river basins.

On the other hand, to predict the divergence time of lineages, doing a molecular clock study by using divergence values is possible. Nevertheless, the overall evaluation of the genetic divergence values reported in the previous studies indicates that mtDNA-RFLP based nucleotide divergence calculations may have been overestimated to some extent. In some fish lineages, the values acquired from the calibration and application of molecular clock to estimate divergence times may be highly variant (from 0.5 to 2.5% per million years) (Wilson *et al*., 1996; Smith, 1992; Martin and Palumbi, 1993; Bernatchez, 2001). This also could be the case for this study because the restriction enzymes we have used asserted to be informative for the *C. macrostomus* phylogeny. In order to acquire a higher resolution of phylogenetic and phylogeographic relationships of *C. macrostomus*, *C. kais* and *C. chantrei*, a thorough examination on the basis of the mtDNA and nuclear DNA string data would be of use.

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