GENETIC VARIATION IN *CYPRINION MACROSTOMUS* HECKEL, 1843 POPULATIONS AS REVEALED BY PARTIAL COI SEQUENCES OF MITOCHONDRIAL DNA

PARMAKSIZ, A.

Department of Biology, Faculty of Science-Literature, Harran University, Şanlıurfa, Turkey (e-mail: aprmksz@gmail.com; phone: +90-414-318-3562; fax: +90-414-318-3541)

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Abstract. *Cyprinion macrostomus* Heckel, 1843 naturally thriving in river systems of Euphrates and Tigris is a species with economic importance. In this study genetic diversity of *C. macrostomus* populations was determined based on gene sequencing analysis of mitochondrial DNA cytochrome c oxidase subunit I (mtDNA COI) locus. Seven polymorphic sites and eight haplotypes were detected taking 41 samples from two populations. Mean haplotype diversity (h) and nucleotide diversity (π) were calculated to be $h = 0.529$ and $\pi = 0.00158$; respectively. All values obtained from two populations after neutrality tests were calculated to be negative and were statistically insignificant ($p > 0.05$). Results obtained with this research are the data noted for the first time for *C. macrostomus* species thriving in Turkey. Certain haplotypes (H3, H5, H6, H7 and H8) determined for mtDNA COI locus are the new results to the literature and created a novel data set for genetic diversity of this species.
Keywords: *Cyprinidae, genetic diversity, polymorphism, Euphrates River, Tigris River*

Introduction

Euphrates and Tigris River Systems have a great deal of important capacity and potential for fish biodiversity and fishery for Turkey. Euphrates and Tigris Rivers undergo considerable change as the result of human activities. Numerous dams were built on these rivers in order to provide water for production of energy, agricultural fields and neighboring cities. Thus, differences occurring in riverbed have led to significant variation in physical, chemical, and biological composition of the river. Furthermore, environmental factors such as industrial factors, intensive fishery, and destruction of habitats will lead to extinction of several species or decrease of populations (Kuru, 1986; Ünlü et al., 1997).

Consumption of freshwater fish which are an alternative to meet protein needs of the population that has been elevating especially because Syrian immigrants have been moving to Southeastern Anatolia in recent years, has increased. The fish species preferred most by people with economically low level of income was identified to be *C. macrostomus*. Body of this fish, that is maximum 20 cm long, is covered by scales and laterally flattened. The mouth is large, flat, and located ventrally. Caudal fin is forked, free edges of dorsal and anal fins are concave. This omnivorous species feeds on phytoplanktons and zooplanktons (Bilici, 2009).

C. macrostomus species belonging to Cyprinidae family has distribution in Iraq, Iran, Syria, and Turkey (Kelle, 1978; Kuru, 1980). This species inhabits in Euphrates and Tigris River Systems and Orontes (Asi) River in Turkey (Kuru, 1975; Kelle, 1978; Balık, 1988; Timur et al., 1983; Taysı, 2014).

Some studies conducted on this species include; karyotype analysis in Malatya Karakaya Dam Lake by Gaffaroğlu and Yüksel (2004); hematology in Sivas Topardıç Stream and Balıklı Çermik localities by Duman and Şahan (2014); phylogenetic and phylogeographical relationship of *C. macrostomus, C. kais, Carasobarbus chantrei* in Euphrates and Tigris

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Rivers studied by Durna et al. (2012) via nuclear DNA-ISSR (microsatellite) and mtDNA (PZR-RFLP) methods; histological characteristics of pancreas, liver, intestines in Sivas Balıklı Hot spring by Taysı (2014); morphological differences between *C. macrostomus* populations in Euphrates River by Bilici et al. (2015).

C. macrostomus is caught by fishermen and has economic importance because it is consumed by local people. Genetic diversity and population structure of that species need to be known in order to manage and protect the species possessing economic importance. There is only one study (Durna et al., 2012) about genetic diversity of this fish species amongst the studies which have been carried out on *C. macrostomus* up to the present time, that study did not included any DNA sequencing analysis. The goal of this research is to determine genetic diversity of *C. macrostomus* populations in Euphrates and Tigris Rivers by practicing sequence analysis for mtDNA COI locus. mtDNA is generally considered to be an ideal indicator for studies of population genetics because it is maternally inherited and has rapid mutation rate (Avise, 1987). Besides its use for distinguishing similar species, mtDNA COI locus is one of the most used molecular markers for determination of the differences between populations of the same species (Croos and Palsson, 2010; Keskin and Atar, 2012).

Materials and methods

Study area, sample collection, DNA extraction and amplification of mtDNA COI

The localities of Euphrates River (Adiyaman) and Tigris River (Diyarbakır) System were found to be appropriate for sampling because of the number of populations, the availability of land conditions, the availability of sufficient number of fishermen and the proximity to the city center. 18 individuals from the Adıyaman, 23 individuals from Diyarbakır (*Fig. 1*), 41 in total, were caught by fishing method, transferred in to laboratory alive preserving within chambers with ice. 2 g specimen were dissected from muscle tissues at the base of pectoral or dorsal fins of samples caught, kept in refrigerator at +4 °C until DNA isolation process by placing inside 1.5 ml centrifuge tube containing 95% ethanol.

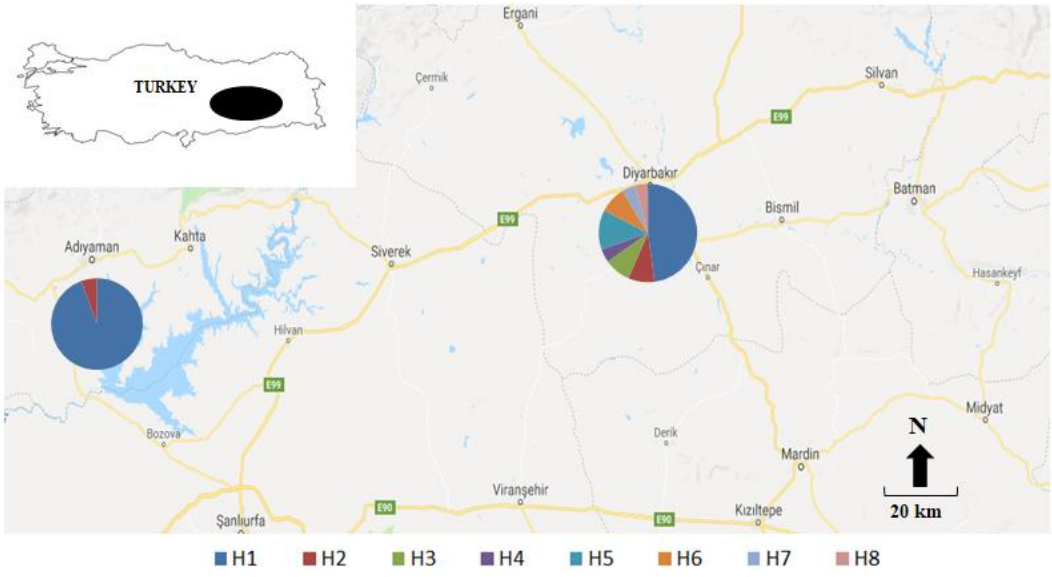

Figure 1. *Location of study area and geographic distribution of haplotypes*

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In this study, total DNA isolation from muscle tissue was practiced using GeneJET Genomic DNA Purification Kit (Thermo Scientific). Total DNAs were obtained by applying the protocol for this kit. In order to control the existence of DNA, 2 µl was taken from DNA samples of each individual, placed in to tank including 0.8% agarose gel, 0.5xTBE (Tris/Boric acid/EDTA Buffer) solution with the addition of 2 µl stain (3x Loading dye) and SYBR Green, run in electrophoresis at 120 V for 30 min, then viewed in device giving off ultraviolet (UV) light. The 5-end of the mtDNA COI region was amplified using the following primers: COI-625F (5'-CAACCAACCACAAAGACATTGGCAC-3'), COI-625R (5'-GACTTCTGGGTGGCCAAAGAATCA-3') (Darabi et al., 2014).

The PCR process was carried out by using BIO-RAD T100^{TM} Thermal Cycler device. Protocols of PCR were completed in totally 35 cycles including; initial denaturation at 95 °C for 3 min, denaturation for 30 s at 95 °C, 30 s at 61 °C for annealing, and 45 s at 72 °C for extension, and finally terminated keeping specimens at 72 °C for 5 min. PCR mixture used in order to amplify this locus is as follows; totally 25 µl consisting of 13.9 µl dH_2O, 2.5 µl 1x PCR buffer, 2 µl $MgCl_2$, 0.5 µl dNTPs, 1 µl primer (F + R), 0.1 µl Taq polimeraz and 50 ng template DNA. 2% agarose gel was used in order to check resulting products of PCR process. Sequence analysis was carried out in 3500 XL Genetic Analyzer (Thermo Fisher Scientific) by sending obtained PCR products to a commercial company.

Sequence analysis of mtDNA COI

Raw data of mtDNA sequences, which were delivered by commercial company, were evaluated and converted in to FASTA format by using ChromasPro v 2.0.1 (Technelysium Pty Ltd). Resulting sequences of all individuals in FASTA format were aligned utilizing BioEdit software version 7.2.5 program.

The number of polymorphic sites and haplotypes, diversity of haplotypes and nucleotides, Tajima D and Fu's Fs statistics were calculated for populations by using DnaSP5.10.01 program. The phylogenetic relationship between haplotypes was identified by Network version 5.0 software. Phylogenetic analyses were performed in MEGA 7 program with respect to Neighbor joining tree model using K2 parameter and phylogenetic tree was built. Bootstrap test (1000 repeats) was used for testing reliability of nodes (branches) on the tree. Sequences obtained from the present study were compared to sequences found in GenBank and phylogenetic tree was established.

Results

Genetic variation

Seven variable sites and eight haplotypes were identified by sequencing an average of 600 bp fragments of mtDNA COI 625 locus in totally 41 *C. macrostomus* samples from Euphrates and Tigris Rivers. Nucleotide variations of this region are shown in *Table 1*. Haplotype diversity (h), the nucleotide diversity (π) and the neutrality tests for each population were given in *Table 2*.

As seen in *Table 3*, totally 2 (H1-H2) haplotypes in Euphrates population and all of the 8 haplotypes in Tigris population were indentified. Haplotype which is common in both populations and represented with the highest number of individuals is H1 (*Fig. 2*).

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Table 1. *Nucleotide variations and haplotypes of mtDNA COI 625*

Haplotypes	190	316	334	421	436	508	550
H1	A	G	C	C	C	C	C
H2	.	A	.	.	.	.	.
H3	.	A	.	T	.	.	.
H4	.	.	.	.	.	T	.
H5	G	.	.	.	T	.	.
H6	.	.	.	.	T	.	.
H7	.	A	T	.	.	.	.
H8	.	A	.	T	.	.	T

Table 2. *Genetic diversity and neutrality tests of C. macrostomus populations (n = number of individuals, Nh: number of haplotypes, h: haplotype diversity, π: nucleotide diversity)*

Locality	n	Nh	h	π	Tajima's D	Fu's Fs
Euphrates River	18	2	0.111	0.00018	-1.16467	-0.794
Tigris River	23	8	0.759	0.00252	-0.67096	-2.925
Total	41	8	0.529	0.00158	-1.18176	-3.637

Mean haplotype diversity was calculated as (h = 0.529); mean nucleotide diversity as (π = 0.00158). Population of Tigris was the one to have both higher haplotype diversity (h = 0.759) and higher nucleotide diversity (π = 0.00252).

Table 3. *The haplotypes distribution according to populations*

Haplotypes	Euphrates River	Tigris River	Total
H1	17	11	28
H2	1	2	3
H3	-	2	2
H4	-	1	1
H5	-	3	3
H6	-	2	2
H7	-	1	1
H8	-	1	1

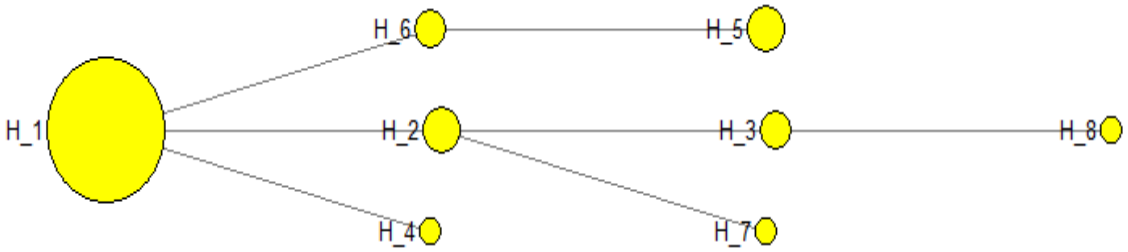

Figure 2. *Haplotype network of C. macrostomus haplotypes*

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Eight haplotypes in total were determined in Median-Joining Network of haplotypes created for 41 *C. macrostomus* samples analyzed, resulting network shows the existence of a haplotype (H1) indicating an evolutionary connection. This haplotype was detected to be the most abundant one in both of the populations (*Fig. 2*).

As seen in *Table 4*, similarities were compared by blasting H1 haplotype sequence, which was obtained in or study and is an ancestry one in GenBank (available at http:// blast.ncbi.nlm.nih.gov).

Table 4. *Information about sequences of H1 haplotype obtained in our study showing the maximum ident in GenBank*

Species	Country	Accession	Maximum ident %
Cyprinion sp.	Iranian	KM590431.1	100
Cyprinion sp.	Iranian	KM590430.1	99
Cyprinion macrostomus	Iranian	KM590433.1	99
Cyprinion sp.	Iranian	KM590432.1	99
Cyprinion sp.	Iranian	KM590429.1	98
Cyprinion watsoni	Iranian	KM590434.1	99
Cyprinion watsoni	Iranian	KM590435.1	99
Cyprinion semiplotum	Japan	AP011253.1	90
Cyprinion semiplotum	India	KF511536.1	90
Cyprinion semiplotum	India	KJ957768.1	90

Phylogenetic tree drawn based on 8 haplotypes of mtDNA COI locus from populations Euphrates and Tigris and sequences of species from genus Cyprinion in GenBank are seen in *Figure 3*.

Figure 3. *Neighbor-joining tree based on haplotypes of Cyprinion genus*

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Two major branches are seen on the tree in *Figure 3* and samples of this study and samples from Iran are placed on one branch, *Cyprinion semiplotum* species from India and Japan is on the other branch. Samples of Iran, Cyprinion sp (KM590430.1 and KM590431.1) have haplotype H1, *Cyprinion macrostomus* (KM590433.1) H4, Cyprinion sp. (KM590432.1) H2; these are similar with haplotypes in our research.

Neutrality tests

Neutrality tests are used broadly to reveal the past population history. In present study Tajima's D (1996) and Fu's Fs (1997) tests were applied for the deviation of populations from the standard neutral mode. Tajima's D value; was negative in both Euphrates (-1.16467) and Tigris (-0.67096) populations, and it was also negative in sum (-1.18176) which was found to be statistically insignificant ($p > 0.05$). Fu's Fs tests were negative in Euphrates (-0.794) and Tigris (-2.925) populations as well, it was also negative in sum (-3.637) and found to be statistically insignificant ($p > 0.05$).

Discussion

Genetic diversity of populations were researched in this study via sequencing mtDNA COI 625 of totally 41 *C. macrostomus* individuals including 18 samples from Adıyaman 23 individuals from Diyarbakır. Seven polymorphic and eight haplotypes were indentified on this locus. While only 2 haplotypes (H1-H2) were found in population of Adıyaman, all haplotypes were observed in population of Diyarbakır. Different haplotype diversity of both populations results from the fact that the habitat where samples were taken was either stream or dam lake. Because, all of the individuals representing Adıyaman population were obtained from Atatürk Dam Lake, all of the individuals representing Diyarbakır population were collected from river. Since rivers have richer habitats than lakes, it is expected for Diyarbakır population to have greater genetic diversity. In addition, it is also expected for geographical isolation resulting from dams built on these rivers to lead to genetic variations between populations of fish. Haplotype H1 was seen in totally 28 individuals and it is possible to declare that haplotype H1 is ancestry because it is the most abundant haplotype in both populations. It was estimated that occurrence of ancestral haplotype in both populations was associated with geological localization of both rivers in the past. Upper parts of Euphrates and Tigris in Pleistosen were flowing to fresh water lakes located behind the sea retreating in late Miyosen, lower parts were flowing to inland lake, then to Persian Gulf (Demirsoy, 1999).

With the same primer, Parmaksız and Ekşi (2017) determined 6 polymorphic sites and 7 haplotypes from populations of *Capoeta trutta* and Parmaksız et al. (2017) 2 polymorphic sites and 3 haplotypes from populations of *Barbus grypus*. The number of polymorphic sites and haplotypes in our study were higher compared to these two studies.

Data obtained as the result of analyzing mtDNA COI 625 sequences of certain fish species inhabiting in Euphrates and Tigris Rivers Systems in Turkey and data of this study were given in *Table 5*.

According to *Table 5*, both the number of haplotypes (8) and nucleotide diversity (0.00158) in this study are higher compared to other species. Nucleotide diversity is a precise method used for genetic analysis of populations (Nei and Li, 1979). Genetic diversity could be influenced by life period, characteristic of populations, environmental

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conditions and population size (Nei, 1987; Avise, 2000). *Barbus grypus* is the species with the lowest nucleotide variety. The reason for this is supposed to result from decreased number of individuals because it is the most fished species.

Table 5. *Data of fish species studied by using mtDNA COI 625 in Turkey*

Species	n	Nh	h	π	Tajima's D	Fu's Fs
Capoeta trutta	47	7	0.642	0.00138	-1.08945	-2.946
Barbus grypus	36	3	0.246	0.00045	-0.91306	-1.098
Cyprinion macrostomus	41	8	0.529	0.00158	-1.18176	-3.637

In Median joining network analysis, we can see that haplotype H1 is located in the center of network and dominant, also all haplotypes are consisted of haplotype H1 (*Fig. 2*). We can also report that haplotype H1 is connected with other haplotypes on Neighbor joining tree and therefore haplotype H1 is ancestry haplotype (*Fig. 3*). In addition, mtDNA COI 625 primer distinguishes species resplendently. It placed *Cyprinion watsoni* and *Cyprinion semiplotum* species on a branch separated from *Cyrinion macrostomus* species.

Tajima's D ve Fu's Fs values were negative in populations of Euphrates and Tigris, all of the resulting values were not statistically significant ($p > 0.05$). These values predict that populations are in neutral balance.

All of the results obtained by this study are the data extracted for the first time for *C. macrostomus* species thriving in Turkey. Despite the fact that haplotypes H1, H2 and H4 for mtDNA COI 625 locus were determined in studies conducted in Iran, haplotypes H3, H5, H6, H7 and H8 identified in this research are new results for the literature, created a new data set for genetic diversity of this species.

Conclusions

Since the number of individuals and populations used in the present study was low, it is likely for variations by chance to occur. Therefore, it will be useful for genetic diversity of this fish species to study on greater number of individuals and more populations in further research.

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