AQUATIC RESEARCH

E-ISSN 2618-6365

Aquat Res 4(4), 313-320 (2021) • https://doi.org/10.3153/AR21026 *Research Article*

Determination of genetic variations by using mitochondrial DNA cyt b sequences in populations of *Carasobarbus luteus* (Cyprinidae)

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Cite this article as:
Parmaksız, A. (2021). Determination of genetic variations by using mitochondrial DNA cyt b sequences in populations of Carasobarbus luteus (Cyprinidae). *Aquatic Research*, 4(4), 313-320. **https://doi.org/10.3153/AR21026**

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Submitted: 01.02.2021
Revision requested: 06.03.2021
Last revision received: 16.03.2021
Accepted: 16.03.2021
Published online: 19.05.2021

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Available online at
http://aquatres.scientificwebjournals.com

ABSTRACT

As the human population increases, freshwater fish has become an important alternative source of protein to meet the need of protein. Especially numerous fish species from the family Cyprinidae are consumed by the people. Among these fish, *Carasobarbus luteus* (Heckel, 1843) is also one of the most preferred species thanks to its edible flesh as well as its low price. Since it is economically important, there has been the pressure of overfishing and invasive species on the populations of this species, resulting in decrease of the sources day by day. Management and conservation of the species have importance therefore it is need to know its genetic variations in the first place. The present study analyzed sequences of mtDNA cyt b locus and established the genetic variability following the collection of 65 individuals from five different localities in diverse river systems where *C. luteus* populations naturally inhabit. Sequence analysis revealed 13 polymorphic sites and 5 haplotypes. Birecik was the locality with the highest value in terms of both haplotype diversity and nucleotide diversity, Diyarbakır was the one with the lowest value. Tajima's D and Fu's Fs values were found to be statistically insignificant for all the localities. Population genetic diversity of this fish species was found to be low in terms of mtDNA cyt b marker. It is recommended to take measures to stop the loss of genetic diversity and to start conservation studies.

Keywords: *Carasobarbus luteus*, cyt b haplotypes, Genetic diversity, Euphrates River, Tigris River

Introduction

Development of urbanization and agriculture have a gradually increasing impact on both natural habitats and species, decrease the quantity of habitats which are appropriate for the wildlife species (Goudie, 2018; Zhang et. al., 2020). Destruction or change of habitats may lead to decline in the diversity of species and even to extinction of certain species. It has been estimated that genetic diversity has been decreasing faster than diversity of species under increasing number of threats, however its spatial distribution has not been adequately documented on a global scale yet (Manel et. al.,2020). Genetic diversity directly reflects the ability of species or population to adapt in environmental factors of alien habitats (Frankham et al., 2002; Spielman et al., 2004).

Populations in aquatic habitats are frequently under threat because of human pressure such as pollution, harvesting, fishing, alien species, tourism, and urban development (Cognetti and Maltagliati, 2000). *Carasobarbus luteus* which is the subject of our study and a species exposed to those threats is called Bizir or Common carp by the local people. *C. luteus* is a species from Cyprinidae family which is widely distributed in Euphrates, Tigris rivers, natural and artificial lakes in Mesopotamia (Kuru, 1979; Ünlü, 1991; Gökçek and Akyurt, 2008; Coad, 2010). Different studies have been done for this species, some of which are; investigation of reproductive organs and tissues (Rahemo and Al-Shatter, 2012), spermatologic characteristics (Aral et al., 2014), content of digestive system (Çelik and Saler, 2016), reproductive biology (Bilici et al., 2017), parasite studies (Mansoor et al., 2020).

Particularly fishing and dominant status of invasive species are the factors threatening this species most, and lead to decrease in the number of individuals in populations day by day. Decreased individuals in natural populations may result in the extinction of unique genotypes that are not found anywhere else (Parmaksız, 2020). When a genetic data is lost, it is almost impossible to bring it back (Parmaksız, 2020). Therefore, measurements are needed to stop loss of genetic data and to protect future of this species should be taken. For an effective conservation program, firstly there should be reliable genetic data. Analysis of population genetics is a useful tool to acquire knowledge about a species in order to protect it (Ryman, 1991; Ward, 2000). Mitochondrial DNA markers have been utilized in genetic studies on various species (Xia et al., 2016). As compared to nuclear DNA markers, mitochondrial DNA markers are preferred thanks to their unique characteristics such as maternal inheritance, lacking of fast evaluation and recombination. Diverse mtDNA gene sequences can be used to determine the variation in fish species (Saraswat et. al.,2014). Cyt b gene which is an effective molecular marker and has been used to analyze genetic data of several species (Maltagliati et al., 2010; Li et al., 2013; Deng et al., 2014). Variation in mtDNA cyt b gene is utilized for studies for genetic analysis of fish populations from order Cypriniformes (Fayazi et al., 2006). mtDNA cyt-b gene locus has been appeared to be a multipotent genetic marker that can be especially used for genetic variation analysis of fish (Saraswat et. al., 2014).

The goal of the present survey is to identify genetic variation in populations of *C. luteus* naturally inhabiting in river systems of Euphrates and Tigris via gene sequence analysis of mtDNA cyt b locus.

Material and Methods

65 fish samples used as the material of the present study were collected from 5 localities belonging to 2 different river systems. The map of these localities is given in Figure 1. Fish samples were included in the study by random sampling from fishes caught by fishermen at different times. Previous field surveys were utilized for selection of the localities where fish samples would be collected. Accordingly; localities on the river systems of Euphrates and Tigris were concluded as appropriate considering the number of samples in populations, convenience of the land conditions, presence of fishermen in adequate number, and closer distance to city center.

These samples which were purchased from fishermen were kept inside an ice bucket to transfer Zoology Laboratory of Harran University, Faculty of Science-Literature, Department of Biology. Following the identification of species, muscle tissue dissected from the samples was transferred into microcentrifuge tubes with a content of 90% of ethanol and kept at -20 C until DNA was extracted.

Total DNA was isolated from muscle tissue using GeneJET Genomic DNA Purification Kit (Thermo Scientific). To check the presence of DNA after the protocol, DNA samples from all individuals were run placing in the wells of 1% of agarose gel with addition of SYBR Green, monitorized under UV light device (Smart View Pro Imager System, Major Science).

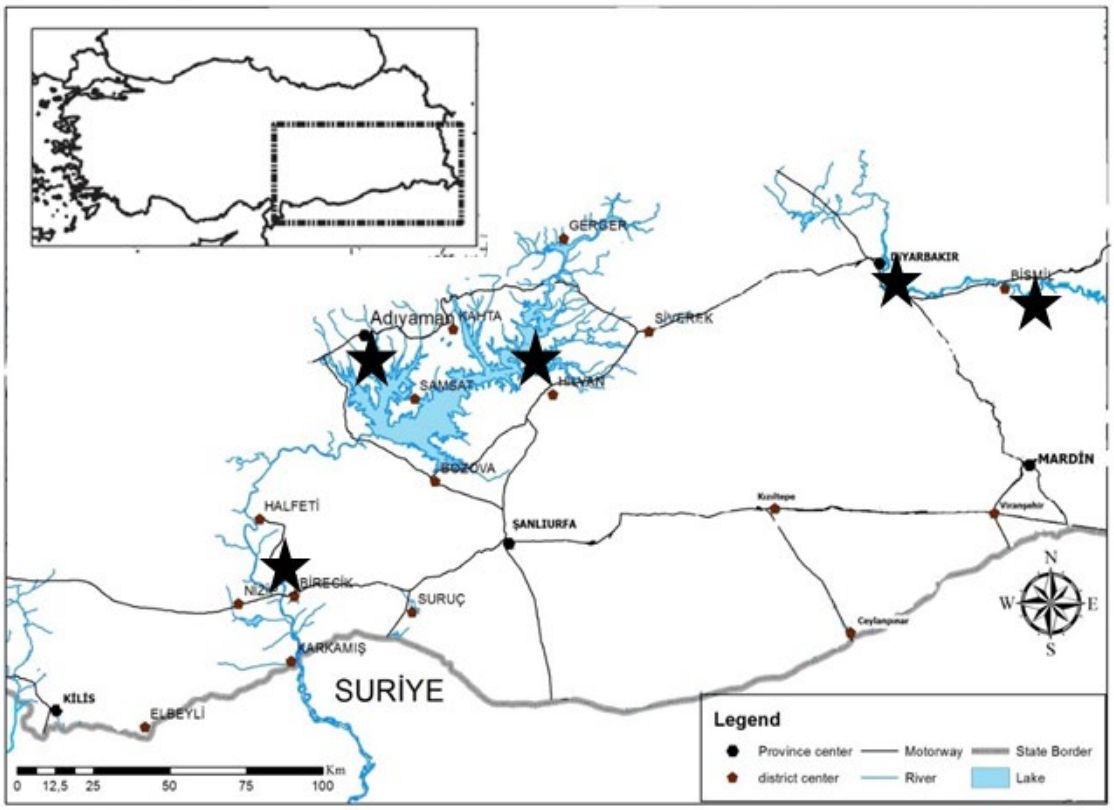

Figure 1. The map indicating the localities where *C. luteus* samples were collected

Target mtDNA cyt b gene locus was amplified by Polymerase Chain Reaction (PCR), the primers were referenced form the study by Briolay et. al., 1998 (L15267 F:5'-GTT TGA TCC CGT TTC GTG TA-3'; H15891 R:5'-AAT GAC TTG AAG AAC CAC CGT-3; Gene bank Accession number: AY026411). Using Thermal Cycler device (BIO-RAD T100^{TM}), target mtDNA cyt b gene site was amplified by optimizing the PCR conditions, concentrations of the chemicals, and annealing temperatures of the primers according to the study of Parmaksız and Şeker (2018). 2% agarose gel was used in order to check the products occurring after PCR process. Agarose gel with addition of SYBR Green was placed in the tank with a content of 0.5x TBE solution, 4 µL of PCR products and 4 µL stain were loaded in the wells together, then were run at 120 V electricity currency for 25 minutes, and monitorized under UV light device. PCR products of target site were sent to a commercial company for sequence analysis with 3500 XL Genetic Analyzer (Thermo Fisher Scientific) device.

Raw data of mtDNA sequences were firstly converted in to FASTA format by evaluating with Chromas Pro v 2.0.1 (Technelysium Pty Ltd) and resulting sequences of all the samples were ranked 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 statistics for the populations were identified by using DNA SP5.10.01 program (Rozas et al., 2003). The phylogenetic relationship between haplotypes was identified via Network version 5.0 program.

Results and Discussion

Genetic Variation

From river systems of Euphrates and Tigris, variable sites and haplotypes were identified using sequence analysis of 586 bp locus on mtDNA cyt b (Figure 2) for a total number of 65 *C. luteus* samples. Variability of nucleotides by haplotypes from this gene site can be seen on Table 1.

Mean nucleotide, Cytosine (C), Timin (T), Adenine (A), and Guanin (G), content for all sequences was calculated as follows; 27.7%, 29%, 28.5%, and 14.8%, respectively.

13 polymorphic sites and 5 haplotypes were identified in a total number of 65 *C. luteus* samples collected from five diverse localities, variations of nucleotides by haplotype are shown on Table 1. Haplotype H1 was found in 54 samples and the most prevalent one. Haplotypes H3, H4, and H5 were identified in only one sample.

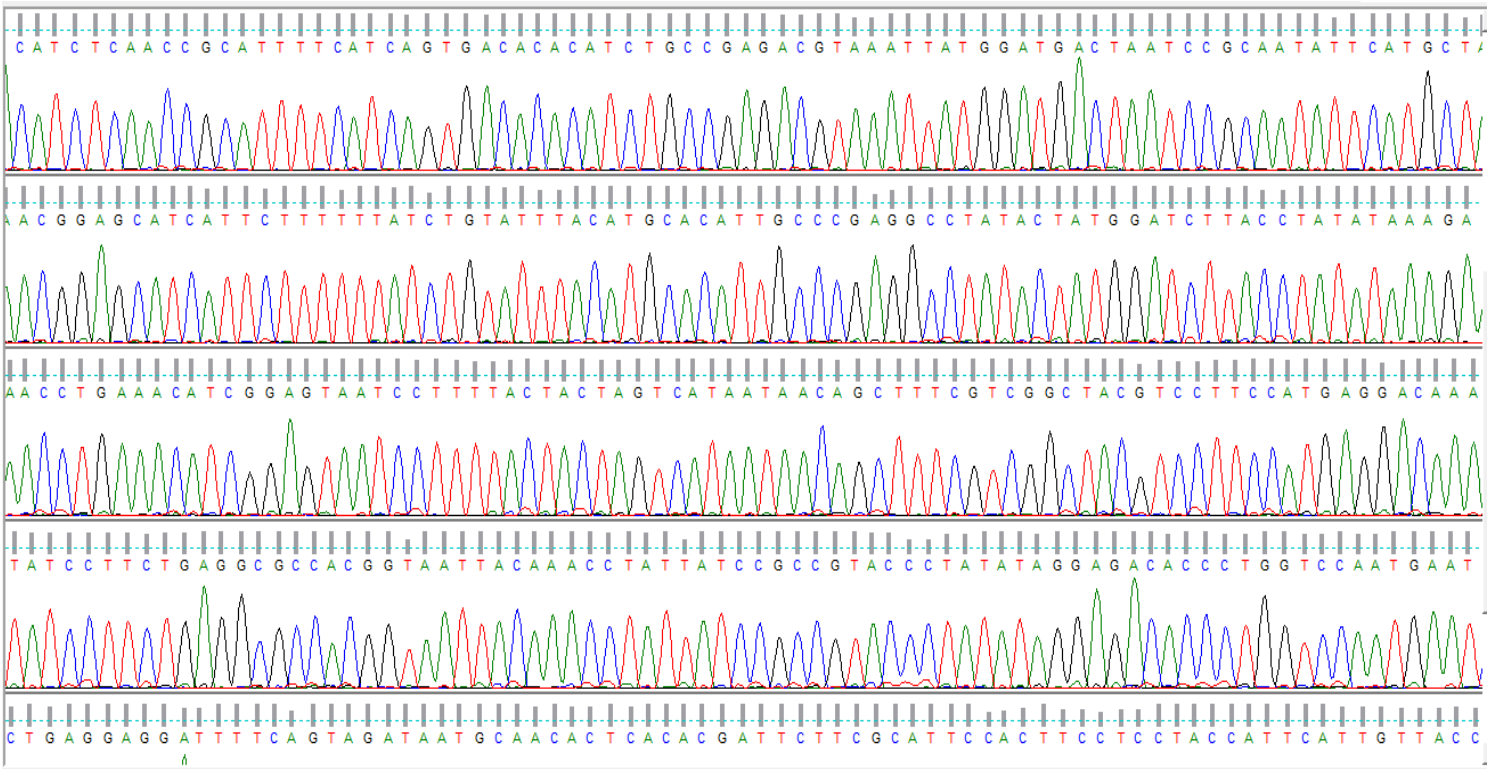

Figure 2. Chromatogram image of a sequence analysis from mtDNA cyt b site

Table 1. Nucleotide diversity and haplotypes of cyt b locus

Haplotype	Polymorphic Sites													Accession number
	117	174	303	324	345	408	424	444	456	474	493	537	541	
H1 (54 samples)	T	G	T	A	C	G	T	A	G	A	G	C	G	MW725236
H2 (8 samples)	C	A	C	G	T	.	.	G	A	G	.	T	A	MW725237
H3 (1 sample)	C	A	C	G	T	A	.	G	A	G	.	T	A	MW725238
H4 (1 sample)	.	.	.	.	.	.	C	.	.	.	.	.	.	MW725239
H5 (1 sample)	C	A	C	G	T	.	.	.	.	G	C	.	.	MW725240

Table 2. Genetic diversity and neutrality tests of localities (N: number of samples, Nh: number of haplotypes, Hd: haplotype diversity, π: nucleotide diversity)

River System	Locality	N	Nh and Haplotype distribution	Hd	π	Tajima's D	Fu's Fs
Euphrates	Adıyaman	16	4 (H1: 13) (H2: 1) (H3: 1) (H5: 1)	0,350	0,00536	-0,51025	2,810
Euphrates	Hilvan	13	2 (H1: 11) (H2: 2)	0,282	0,00481	-0,49831	5,847
Euphrates	Birecik	10	2 (H1: 7) (H2: 3)	0,467	0,00796	1,41919	7,272
Tigris	Diyarbakır	5	1 (H1: 5)	0000	000000	0000000	0000
Tigris	Bismil	21	3 (H1: 18) (H2: 2) (H4: 1)	0,267	0,00325	-1,32016	3,128

As seen on Table 2, haplotype H1 was the only one which was seen in all localities. Haplotype H2, on the other hand, was commonly found in all other localities, except Diyarbakır. H3 and H5 were identified only in Adıyaman, H4 only in Bismil locality. In Adıyaman, Hilvan, Birecik, Diyarbakır, and Bismil localities had; four, two, two and three different haplotypes respectively. The locality with the highest number in terms of both haplotype diversity and nucleotide diversity was Birecik, whereas Diyarbakır was the one with the lowest number. The results of Tajima's D and Fu's Fs neutrality tests were presented on Table 2. Neutrality tests are used to determine whether populations have been selected in the past. The Tajima D test is mostly used to determine natural selection from DNA polymorphism. Neutrality tests are used to determine whether populations have been selected in the past. The Tajima D test is mostly used to detect natural selection from DNA polymorphism and the Fu's Fs test is used to determine population expansion. Tajima's D and Fu's Fs values were determined to be statistically insignificant ($p>0.05$) for all localities.

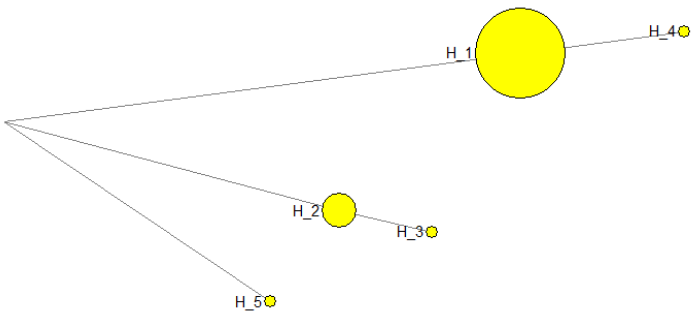

Figure 3. Median -Joining Network of haplotypes acquired after cyt b sequence analysis

Figure 3 shows totally five haplotypes joined on Median-Joining Network created for 65 *C. luteus* samples, resulting network indicates an evolutionary connection. It is also possible to speculate that haplotypes H2-H3 and H1-H4 were connected, haplotype H5 was different from these.

Euphrates and Tigris Rivers have been undergoing considerable change as the result of human activities. Several dams were constructed on these rivers in order to obtain energy and to provide irrigation to agricultural lands and surrounding cities. Thus changes occurring the river bed resulted in dramatic changes for physical, chemical, and biological combination of river. In addition, environmental factors such as industrial factors, intensive fishery, and destruction of habitats would lead to extinction of numerous species or reduction of their populations (Ünlü et al., 1997). Conservation of population size and genetic variability is needed for survival of species. Decrease in the population size results in reduced genetic variability and poses a threat regarding survival chance of population.

It is also known that *Carasius gibelio* and *C. auratus,* which are invasive species in the localities where the samples were collected, has become dominant and has had a negative impact on native species (Parmaksız et. al., 2017). As the result of observations, half of the fish caught in the nets of local fishermen were determined to be invasive species. This particularly puts a great pressure on the fish with economic importance. Because *C. luteus* is consumed by human, it is economically important, populations of this species are also influenced by this situation. Measurements should be taken in order to stop genetic loss of this species and to protect future of this species. To be able to apply an effective conservation program, there must be reliable genotypic data in the first place (Parmaksız and Altundağ, 2018).

Valuable data were provided by Parmaksız and Eskici (2018) using mtDNA COI to evaluate genetic variation of *C. luteus*, by Parmaksız (2020) using mtDNA D-loop sequences to enlighten genetic background of *C. luteus*. The use of multiple genetic marker systems increases the resolution power of genetic studies (Gruenthal et. al., 2007). The present study contributed to acquire further knowledge about genetic structure by analyzing cyt b sequences either. Some studies have been carried out with this marker (cyt b) in different fish species collected from similar localities and haplotypes have been determined (Parmaksız and Seker, 2018; Parmaksız and Altundag, 2018). For *Arabibarbus grypus* populations 5 polymorphic sites and 5 haplotypes were identified by Parmaksız and Seker (2018). For *Achantobrama marmid* populations 4 polymorphic sites and 5 haplotypes were identified Parmaksız and Altundag (2018).

In the present study 13 polymorphic sites and 5 haplotypes were identified following the assessment of cyt b sequences of 65 samples collected from five localities in two different river systems. While haplotypes H3, H4, and H5 were observed in one each sample, haplotype H1 was established as the common haplotype which was seen in 54 samples and all localities. Total haplotype diversity (Hd) was 0.299; Nucleotide diversity ($\boldsymbol{\pi}$) was 0,00453. Studies conducted in similar localities determined a total number of 7 haplotypes for D-loop locus, haplotype diversity (Hd) was 0.373; Nucleotide diversity ($\boldsymbol{\pi}$) was 0,00453 (Parmaksız, 2020); for haplotype were identified for COI locus haplotype diversity (Hd) was 0.534; Nucleotide diversity ($\boldsymbol{\pi}$) was 0,00367 (Parmaksız and Eskici, 2018). Results of the present study and other two surveys were in parallel. Haplotype and nucleotide diversity are im-

portant indicators of genetic variation, as higher values indicate higher genetic variation (Falush et. al., 2003; Liu, 2017). Higher levels of genetic diversity reflect strong adaptation and survival abilities of populations (Barrett and Schluter, 2008). As seen, haplotype and nucleotide diversity of this fish species were found to be lower with respect to mtDNA markers.

Conclusion

It is crucial to take measurements to stop loss of genetic diversity and to start conservation studies. Firstly, invasive species should be controlled and excessive fishing must be prevented. In case of failure to take measurements, the level of genetic diversity will decrease further, feeding, reproduction, competition, and adaptation abilities of populations will decline too and target organism will be under threat of extinction.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Ethics committee approval: Ethics committee approval is not required.

Funding disclosure: This study was funded by Harran University Research Fund (Project No: 17217).

Acknowledgments: -

Disclosure: -

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