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INVESTIGATION OF PHYLOGENETIC RELATIONSHIP AMONG TWO BARBUS SPECIES (CYPRINIDAE) POPULATIONS WITH MITOCHONDRIAL DNA USING PCR-SEQUENCING

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ABSTRACT

This study was conducted to Investigate cytochrome oxidase I (COI) region from *Barbus* sharpeyi and Barbus xanthopterus mitochondrial DNA (mtDNA), In Other to study Genetic and phylogenetic Relationships. A total number of 31 Individuals of Fishery farms, Jrahi river in Shadegan and Karkhe river in Hovaizeh in B.sharpeyi specie and 23 Individuals of Karoon river in Ahvaz, Fishery farms, Karoon river in Shushtar and Karkhe river in Hovaizeh in B. xanthopterus in southwest of Iran were sampled. After DNA extraction COI Region from mtDNA (aprox. 610 bp) was amplified using COI – 625R and COI -625F primers. A total number of 41 amplified PCR products were sequenced. Then sequenced samples was Investigated using BioEdit 5.0.9, MEGA5 and Arlequin 3.5 softwares. The average nucleotide diversity and average of Heterozygosity in B. sharpeyi specie was 0.046, 0.053 and in B. xanthopterus was 0.015 and 0.013 respectively. This result showed a low nocleutide diversity within populations in these species.The UPMGA dandrogram which was constructed based on the distance matrix between groups with MEGA5 program that showed there was genetic distance between B. sharpeyi and B. xanthopterus. The smallest part of genetic variance (1%) in AMOVA table was in among populations within species that show populations within species have high similarity and about part of within populations variance was 4.5% that show indinividuals in populations have high similarity. These results suggested

for maintanance of genetic diversity within species and within populations at appropriate levels, should be used new and a large number reproductive stoks.

Keywords: Barbus, Cytochrome Oxidase I, mtDNA, Sequencing

INTRODUCTION

The study of genetic structure of populations in marine fisheries is important for taxonomic comparisons and species protection and also management of genetic resources and designing protection programs of the marine ecology **[1]**. The reduction of genetic diversity in each population is harmful and has effects the level of performance. Therefore, for optimal utilization of various species of aquatics must be sufficient information from the species and their performance **[1]**. To use of mtDNA in molecular phylogenetic studies was very useful because this molecule shows evolutionary relationships between new segregated groups include populations, species and even individuals **[2, 3, 4]**. All of proteins are coded by mitochondrial-enzymic complexes or metabolic routes and other members of complexes or routes are made by nuclear Genome **[5, 6, 7]**. mtDNA is transported to offsprings from maternal line, means all of offsprings inherite maternal mtDNA haploied genotype **[2, 8]**. Cytochrome oxidase I (COI) gene has the most phylogenetic signs and there are suitable primers for COI (COX1) gene that extension possibility of 5´ head is provided **[9]**.

Barbus genus is from Cyprinidae family with 800 species and is the dominant species of Cyprinidae that have been throughout the Europe, Africa and Asia **[10]**. The Barbus genus is from Barbinae sub-family **[11]**. The Cyprinidae are the largest group of fish populations European - Asian breeds in rivers **[12]**. Many studies have been conducted on genetic structure of the family **[13]**. Phylogenetic relationships and taxonomic identification of Barbus fish is much debated **[10]**. Purposes of this study, is to determine sequencing from genes based in mtDNA molecules in populations of Barbus sharpeyi and Barbus xanthopterus species, determining the genetic structure of this species in major habitats and drawing phylogenetic tree (population structure) of examined species, and also showing how to communicate of populations in different habitats. Given the importance of Barbus fishes such as Barbus sharpeyi and Barbus xanthopterus species in fisheries economics of Khuzestan province, we have tried to paved the way for management to protect this species by investigate the genetic structure of this population and found strategies to minimize of genetic diversity. These species are native species in south Tigris and Karoon river area in southwest of Iran that have economic value **[14]**.

Genetic studies based on protein electrophoresis is highly effective to determine the differences and determine the relationship between such groups of fish **[2]**. In addition to protein and nucleus DNA-based markers, different mtDNA gene sequences have been reported to be useful to determine the amount of variation among species and within species **[15]**. The rapid evolution of mtDNA (about 5-10 times more than the nucleus genes) and their inheritance through the dam, make them useful for species studies **[15]**. The aim of this study, to sequence COI gene of mtDNA molecule in order to phylogenetical study of Barbus populations.

MATERIAL AND METHODS

Sample collection

The samples were taken from Fishery farms, Jrahi River in Shadegan, Karkhe River in Hovaizeh, A number of 16, 10 and 5 fish were sampled for B. sharpeyi and taken from Karoon river in Ahvaz, Fishery farms, Karoon river in Shushtar, Karkhe river in Hovaizeh A number of 4, 2, 3, 14 fish were sampled for B. xanthopterus Species **(Figure 1)**.

DNA extraction and Primer design

A piece of 1-2 cm2 taken from the soft tissue of dorsal fin of each fish and stored in 95% ethanol. The amplification of the mtDNA gene of COI with 650 bp performed with a pair of primers that were designed using the Primer 3.0. software package. The sequences of which were as following:

COI-625 F: 5-TCAACCAACCACAAAGACATTGGCAC -3 and COI-625 R: 5 -GACTTCTGGGTGGCCAAAGAATCA -3.

PCR amplification

The PCR (Polymerase Chain Reaction) amplification conditions were as follows: one preliminiary denaturation step at 95 ° C for 5 min followed by 35 PCR cycles. Denaturation was at 95°C for 4 sec, annealing at 62 ° C for 45 sec and primer extension at 72°C for 40 sec. A final extension at 72 ° C for 5 min was performed. PCR Reactions were conducted in a total volume of 25 mL with 50 ng DNA solution, 5 IU Taq DNA Polymerase, 0.2 mM dNTPs, 1 mM $MgCl_2$, 0.5 µM From each primer, 2.5 µL to 25 µL of buffer 1x and distilled water (ddH_2O) was added until 25 µL. In order to verify amplification of the exact region of the gene,the PCR products were then runed on 1.5 % agarose gels of electrophoresis and stained with ethidium bromide and visualized under UV light .

DNA sequencing

The PCR products of 14, 8 and 3 samples of B.sharpeyi specie from Fishery farms, Jrahi river in Shadegan, Karkhe river in Hovaizeh, respectively and a number of 2, 3 , 9 and 1 samples of B. xanthopterus specie from Karoon river in Ahvaz, Fishery farms, Karoon river in Shushtar and Karkhe river in Hovaizeh respectively, were used for gene sequencing with ABI 3130 device and sequencing method. For studying of phylogenetic relationships, the Nei's genetic distance matrix **[16]** and dandrogram were made with UPGMA method, using MEGA5 **[17]** software. The nucleotide diversity of the species determind with Arlequin 3.5sofware **[18]**. The genetic structure of species compared with Analysis of Molecular Variance (AMOVA) **[19]**.

RESULTS AND DISCUSSION

The Spectroscopy results show the quality and quantity of extracted DNA was desirable **(Figure 2)**.

Electrophoresis of PCR products on 1.5% Agarose gels **(Figure 3)** showed the fragment for cytochrome oxidase I with 610 bp length (proximately) amplified well to compare with obtained sequences, firstly, all sequences in software 5.0.9 BioEdit **[20]** were converted to Fasta format, then were aligned with MEGA5 software. To draw dendrogram, the genetic distance matrixes were used using Distance between groups method **[17]**. Dendrogram were plotted from Cytochrome oxidase I gene sequence analysis using UPGMA method and MEGA 5 software **[17]**.

As shown in **Figure 4 and Table 1**, the closely relationship is shown between Karoon river in Ahvaz and Karoon river in Shushtar and Karkhe river in Hovaizeh compared to Karoon river in Ahvaz with Jrahi river in Sadegan.

These genetic distances can be caused by variation of species in Shushtar. Also differences of climates in Northern of Khuzestan (Shushtar area) with other areas and being less of water pollution in this area, is suitable habitat for various species of fishes.the relationship between Jrahi river in Shadegan and Karoon river in to investigate genetic diversity of Kuzestan Barbus fishes by using mtDNA and PCR-RFLP method, is shown by Fayazi et al., 2006 **[21]**.

The genetic distance between B.sharpeyi and B. xanthopterus populations is shown(Figure5). This result is shown by Fayazi *et al.*, 2006 **[21]**.

It shows that there is genetic distance between *B.sharpeyi* and *B. xanthopterus* species **(Table1)**.

As shown in **Table 3**, low heterozygosity and nucleotide diversity within B. sharpeyi and B. xanthopterus populations is showed that it’s can be a serious alarm to avoid of this situation.

Sample graph of COI gene barcode of B.sharpeyi specie **(Figure 6)** and B. xanthopterus specie **(Figure 7)** using chromas lite 2.1 software is shown.it's a way for identifying this specie using COI sequence.

Table of analysis of molecular variance (AMOVA) indicated that The major part of genetic variance between species is 94.5 %. Means extent of, the species to distance together in many years. The smallest part of genetic variation (1%) is between populations within species and within population has small part of total variation (4.5 %) **(Table 2)**.

The part of variation between populations within species (variation of populations within one specie in several rivers) is related to relationship and gene exchange between rivers and sampling distances **(Table 3)**. This result shows that There is relationship and gene flow between Karoon, Karkhe and Jrahi rivers.

Whereas, low variation within populations or high similarity within populations in specie. This result is shown by calculating of Nucleotide diversity and heterozygosity within populations using Arlequin 3.5 software, too.The mean nucleotide diversity within four sub- populations of B. xanthopterus is 0.004,0.006, 0.003 , 0.009 and is within three sub-populations of B.sharpeyi 0.028,0.079 and 0.007 **(Table 3)**.

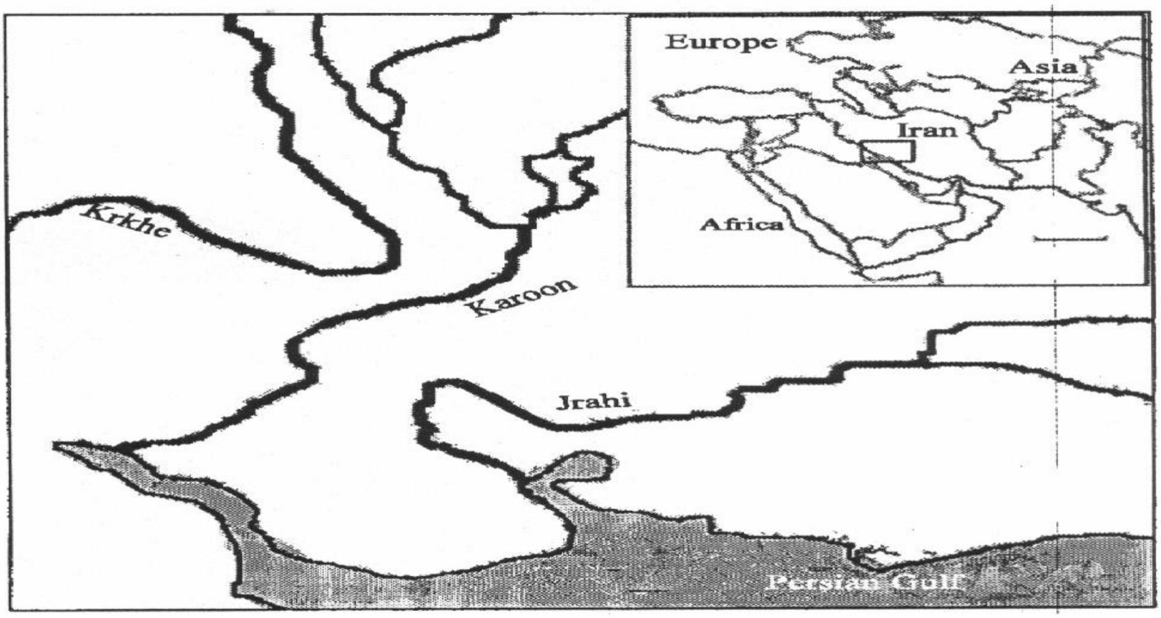

Figure 1: Map of sampled geographic areas of two Barbus species populations (In Karoon river in Ahvaz and Shushtar, Jrahi in Shadegan and Karkhe in Hovaizeh)

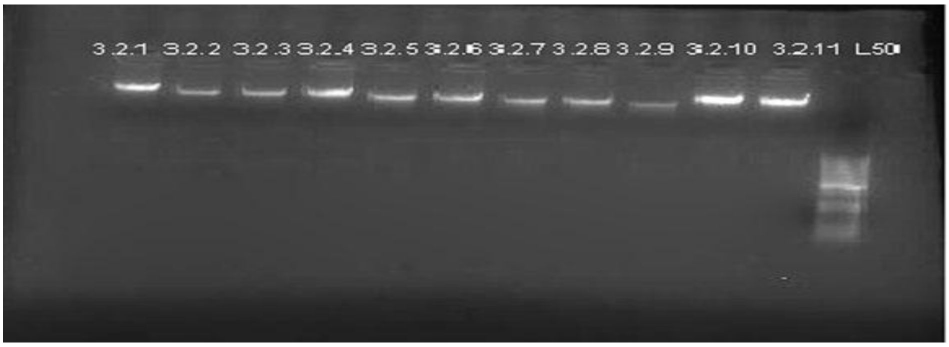

Figure 2: Extracted DNA on agarose gel

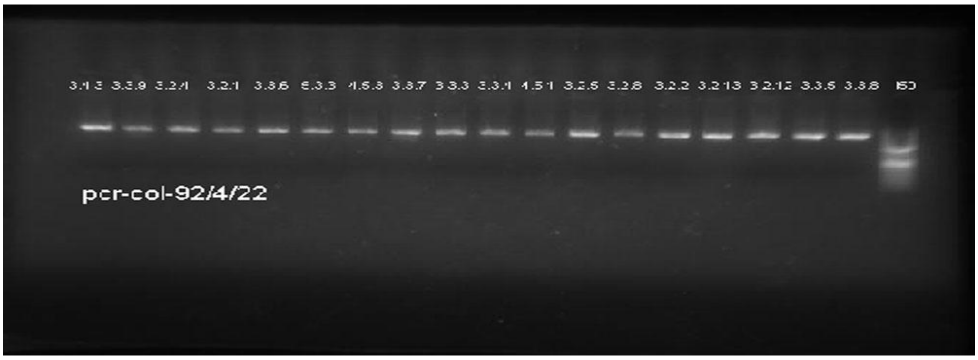

Figure 3: PCR product on agarose gel

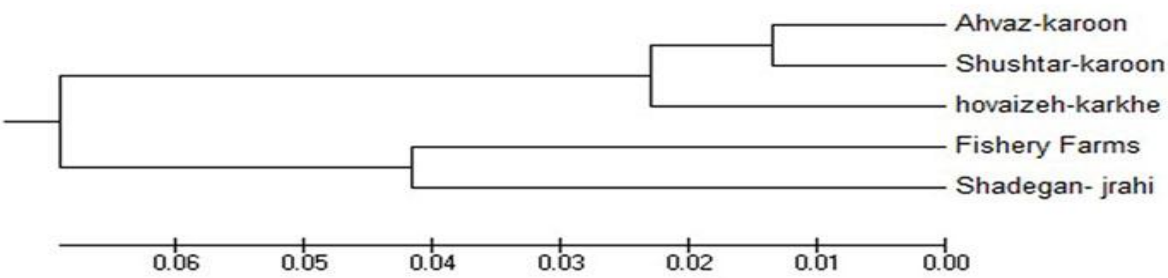

Figure 4: obtained dendroram from comparison of the nucleotide sequences of cytochrome oxidase I gene of investigated populations of B. xanthopterus and B. sharpeyi species via UPGMA method by using MEGA 5 software

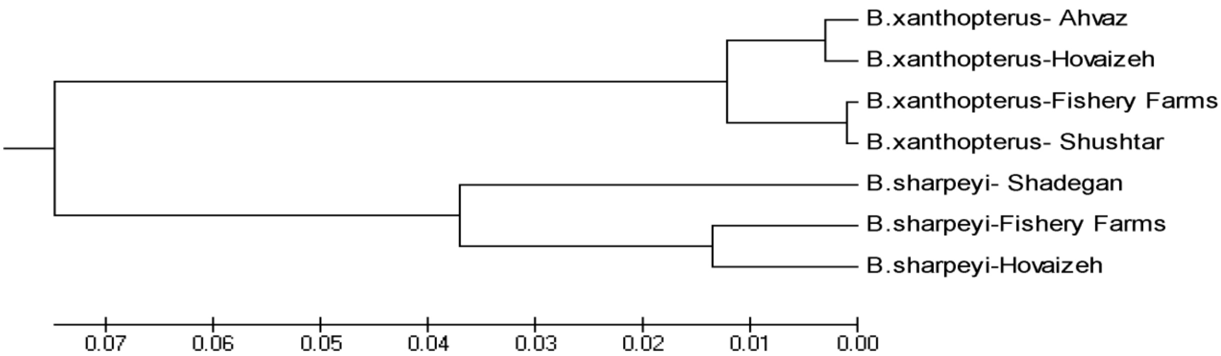

Figure 5: Drawn dendrogram based on nucleotide sequences of cytochrome oxidase I gene in populations of B. xanthopterus and B. sharpeyi species via UPGMA method based in maximum composite likelihood model in MEGA 5 Software

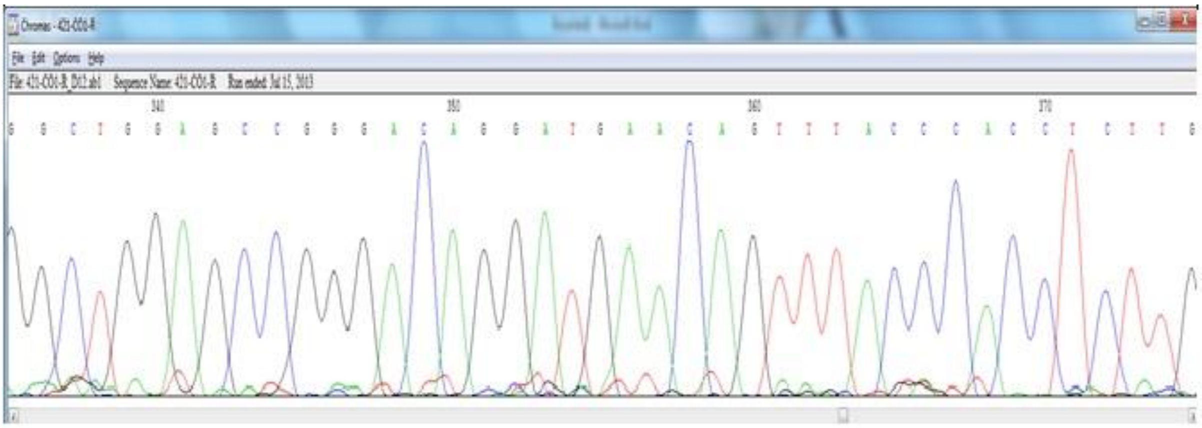

Figure 6: Sample graph of COI gene barcode of B.sharpeyi specie using chromas lite2.1

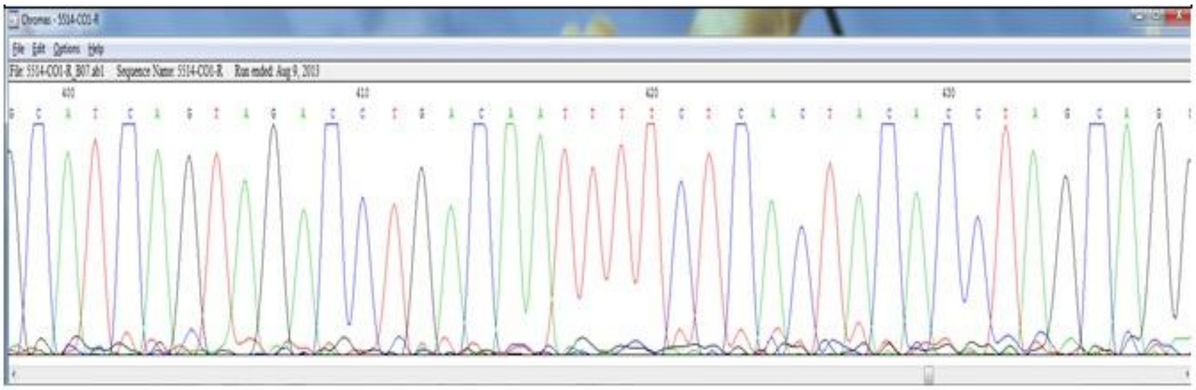

Figure 7: Sample graph of COI gene barcode of B. xanthopterus specie using chromas lite 2.1

Table 1: Matrix of genetic distance average from B.sharpeyi and B. xanthopterus species populations (using MEGA 5 program via Kimura 2-Parameter model)

Speice (sampling place)	1	2	3	4	5	6	7
B.sharpeyi(Fishery farms)	—						
B.sharpeyi(shadegan)	0.078	—					
B.sharpeyi(Hovaizeh)	0.027	0.070	—				
B.xanthopeterus(Ahvaz)	0.147	0.153	0.152	—			
B.xanthopterus(Fishery Farms)	0.142	0.154	0.149	0.025	—		
B.xanthopterus(Hovaizeh)	0.146	0.152	0.151	0.006	0.022	—	
B.xanthopterus(Shushtar)	0.143	0.155	0.150	0.027	0.002	0.023	—

Table 2: Analysis of genetic structure variance of 2 species of Barbus fish considering place of sampling using Arlequin software

Source of	Degrees of chaenges	The sum of freedom	Variance components squares	Percentage
Among species	1	3397.502	179.84260	94.48
Among Population	5	85.367	1.95234	1.03
Within species	33	282.006	8.54563	4.49
Within population Total	39	3764.875	190.34058	100

Fst index is equal to 0.9551, Fsc index is equal to 0.18597 and Fct index is equal to 0.94485

-With 1023 iterations (Permutation) of sample sequences (haplotypes) among population, significant differences were tested

Table 3: Nucleotide and Heterozygosity diversity within two species of Barbus fishes in Southwest of Iran and in sampling area (using the Arlequin 3.5 software)

Species	River/area	The average of diversity Heterozygosity	The average of nucleotide
B. sharpeyi	Fishery farms	0.03288	0.028615±0.015329
B. sharpeyi	Jrahi(Shadegan)	0.07957	0.078505±0.043549
B. sharpeyi	Karkhe(Hovaizeh)	0.00752	0.007220±0.006136
B. xanthopterus	Karoon(Ahvaz)	0.00261	0.003947±0.002993
B. xanthopterus	Fishery farms	0.00580	0.006354±0.004154
B. xanthopterus	Karoon(Shushtar)	0.00301	0.003515±0.003314
B. xanthopterus	Karkhe(Hovaizeh)	0.00829	0.009737±0.005785
Mean	0.00493		0.005889±0.004062

***Nucleotide diversity and Heterozygosity related to species and rivers /areas was calculated with Arlequin 3.5 software.The average of nucleotide diversity within species or river/area include the nucleotide diversity± SD (standard deviation)**

CONCLUSION

It's suggested to the Iranian Fishery organization to maintain variation within species and for decreasing negative effects of homozygosity, using a large number of male and female productive to replicate and regeneration.

This study is the first attempt to identify the genetic structure of B.sharpeyi and B. xanthopterus populations with COI marker in southwest of Iran. Although obtained results from used marker is desirable to identify the genetic structure of the species but it's cost is too high. It's recommended that D-LOOP gene was studied in this research to complementary investigate of genetic structure within species by using method.

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