INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 5, No. 2, p. 41-46, 2014

RESEARCH PAPER OPEN ACCESS

Phylogenetic relationships of sub-populations of *Barbus sharpeyi* fish in southwest of Iran using mitochondrial DNA with PCR-sequencing method

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Key words: Barbus sharpeyi, cytochrome oxidase I, mtDNA, sequencing.

http://dx.doi.org/10.12692/ijb/5.2.41-46 Article published on July 17, 2014

Abstract

This study was conducted to Investigate cytochrome oxidase I (*COI*) Region from *Barbus sharpeyi* mitochondrial DNA (mtDNA), In Other to study genetic and phylogenetic relationships. A total number of 31 samples of fishery farms, jrahi river in shadegan and karkhe river in hovaizeh in southwest of Iran were studied. After DNA(Deoxyribo Nucleic Acid) extraction, *COI* Region from mtDNA (aprox. 610 bp) was amplified using COI – 625R and COI – 625F primers. A total number of 25 Amplified PCR(Polymerase Chain Reaction) products was sequenced. Then sequenced samples was Investigated using BioEdit 5.0.9, MEGA5 (Molecular Evolutionary Genetics Analysis Version 5.0.) and Arlequin 3.5 softwares. The average nucleotide Diversity was 0.0286, 0.0785 and 0.0072 respectively. This result showed a low genetic diversity within sub-populations in B.sharpeyi specie. The UPMGA(Unweighted Pair Group Method with Arithmetic Mean) dandrogram which was constructed based on the distance matrix between groups with MEGA5 program. The most part of genetic variance (prox. %85.5) in B. sharpeyi was within populations. The obtained results at the present study showed a gene flow between Jrahi and Karoon river was happened. This results suggested that for maintaining of genetic diversity of B. sharpeyi sub-populations at appropriate levels , the exchange and transfer of barbus stocking between Jrahi and other rivers should be avoided and should be used new reproductive stocks.

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Introduction

Study of Population genetic structure of marine fisheries is not only so important and interesting from taxonomic review and conservation aspect, but also because of these creatures important in supplying protein reserves, in reserves management and designing protection plans from marine environment is very important (Zhu *et al.*, 2002). Reduction in genetic diversity for different populations is harmful and has effect on their withdrawal amount. Exploiting the various species reserves, requires sufficient knowledge of reserves status of species, breeds and its numerous populations, to apply principle management on reserves(Zhu *et al.*, 2002). *Barbus* genus is from *cyprinidae* family and more than 800 species, are the dominant species of *cyprinidae* that have been developed throughout Europe, Africa and Asia (Nelson, 2006 and Wang *et al.*, 2004). The *Barbus* genus is from *Barbinae* sub-family (Tamura *et al.*, 2011). The *Cyprinidae* are the largest group of fish populations European - Asian breeds living in sweet water and so far, many researchers have examined the genetic structure of this family (Durand *et al.*, 2002). Phylogenetic relationships and taxonomic identification of *Barbus* fishes has long been controversial (Tsigenopoulos *et al.*, 1999). Genetic studies have been very effective based on protein electrophoresis to determine differences and estimates the relationships between groups of fish species. In addition to protein and nuclear DNA-based markers, different sequences of mtDNA (mitochondrial DNA) genes has been reported very useful to determine the level of diversity among species and within species. because the rapid rate of mtDNA evolution (approximately 5-10 times more than nuclear genes) and its inheritance along with native sequence, has changed mtDNA as an extremely useful genetic system for the study of species (Habib *et al.*, 2011). The Purposes of this study, are to determine sequencing from genes based in mtDNA in sub-populations of *B. sharpeyi* specie from *Barbus* fishes, determining the genetic structure of this specie in major habitats and drawing phylogenetic tree (population structure) of examined specie, and also showing how to communicate of sub-populations in different habitats. Given the importance of *Barbus* fishes such as *B. sharpeyi* specie in fisheries economics of Khuzestan province, we have tried to suggest the way for management to protect this specie by investigating the genetic structure of this specie and found strategy to minimize decrescent situation of genetic diversity. This specie is of native species in south Tigris and Karoon river area in southwest of Iran that have economic value (Abdoli, 2000).

Materials and methods

For this research in fishery farms, Jrahi river in Shadegan and Karkhe river in Hoveize ,A number of 16, 10 and 5 were sampled, respectively. A piece of 1-2 cm^2 take from the soft tissue of dorsal fin of each fish and stored in 95% ethanol. The amplification of the mtDNA gene of *COI* with 610 bp performed with a pair of primers. The sequences of which were as following :

COI-625 F: 5- TCAACCAACCACAAAGACATTGGCAC -3 and

COI-625 R: 5 - GACTTCTGGGTGGCCAAAGAATCA - 3.

The PCR (Polymerase Chain Reaction) thermal program consisted of 35 cycles of replicate with primary denaturing temperature 95 ° C for 5 min, then denaturation temperature of 95 ° C for 4 seconds, junction temperature of 62 ° C for 45 seconds, duplicattion temperature of 72 ° for 40 seconds, and final extension temperature of 72 ° C for 5 min. Reaction volume of 25 mililiter and its components, including 50 nonogram DNA, Taq (Thermus aquaticus) Polymerase enzymes 5 units, 0.2 milimolar dNTPs, 1 milimolar $MgCl_2$, 0.5 micromolar From each one primer, 2.5 microliters to 25 microliters of buffer 1x and distilled water(ddH_2O) until 25 microliters, respectively. In order to verify amplification of the exact region of the gene,the PCR products run on 1.5 % agarose gel of electrophoresis using ethidium bromide staining.

To determine of sequence for 25 samples, number 8, 14 and 3 was purified (over 80%) to fishery farms, Jrahi river in Shadegan and Karkhe river in Hoveizeh

sub-populations were used respectively, with ABI 3130 device and sequence method. For phylogenic relationships study, the Nei's genetic distance matrix(Nei, 1987) and dandrogram were made with UPGMA(Unweighted Pair Group Method with Arithmetic Mean) method, using MEGA5(Molecular Evolutionary Genetics Analysis Version 5.0)(Tamura *et al.*, 2006) software. The nucleutide diversity of the species determined with Arlequin 3.5 software(Excoffier and Licher,2010). The genetic structure of *B. sharpeyi* was performed by using Analysis of Molecular Variance (AMOVA)(Excoffier *et al.*, 1992).

Results and discussion

The Spectroscopy results show the quality and quantity of extracted DNA(deoxyribonucleic acid) was desirable. Electrophoresis of PCR products on Agarose gel 1.5 %, showed the fragment for cytochrome oxidase I with proximately 610 bp length amplified well. To compare of obtained sequences, firstly, all sequences in BioEdit 5.0.9 software (Hall, 1999) were converted to Fasta format, then were aligned with MEGA5 software. To draw dendrogram, the genetic distance matrix were used with Distance between groups method (Tamura *et al.*, 2006). Phylogenetic tree obtained from cytochrome oxidase I gene sequence analysis were drawn with UPGMA method under MEGA5 software (Tamura *et al.*, 2006).

Table 1. Matrix of genetic distance average from B.sharpeyi specie sub-populations (with MEGA 5 program and by using Kimura 2-Parameter model).

	1	2	3	4	5	6
1. B.sharpeyi-Fishery Farm 1						
2. B.sharpeyi- Fishery Farm 2	0.033					
3. B.sharpeyi-jrahi 1	0.155	0.157				
4. B.sharpeyi-jrahi 2	0.025	0.031	0.149			
5. B.sharpeyi-karkhe1	0.025	0.035	0.153	0.025		
6. B.sharpeyi-karkhe2	0.013	0.027	0.147	0.013	0.011	

Table 2. Nucleotide and heterozygosity diversity within sub-populations of B.sharpeyi in Southwest of Iran and in sampling area (with the Arlequin 3.5 software).

Sub- populations	Heterozygosity	Nucleotide Diversity
Fishery farms	0.03288± 0.09213	0.028615±0.015329
Jrahi- shadegan	0.07957±0.18747	0.078505±0.043549
Karkhe- hovaizeh	0.00752±0.07393	0.007220±0.00613

Based on dendrogram of Figure 1 and Table 1, most nearby and proximity of Karkhe river in Hoveizeh with farm sub-populations is in compared with Jrahi river in Shadegan. The relationships between the Karkhe river and Jrahi in Shadegan have been indicated in a study that have examined phylogenetic relationships of *B. xanthopterus* specie by using mtDNA (Fayazi *et al.*,2006).

As is shown in figure 2, relative sub-populations of *B. sharpeyi* is identified in Karkhe river in Hoveizeh and Fishery farms and distance of Karkhe river sub-populations with Jrahi is more indicated. These result was also obtained in figure 2.

By using MEGA 5 software and aligning nucleotide sequences of cytochrome oxidase I gene in Barbus family samples in Iran, the process displacement, deletion or addition of each base was determined based on staining intensity (Fig. 3).

Higher color intensity is reason of its greater protection of nucleotides in desired area. From 602 investigated nucleotides, 481 number (80%) of them were fully protected during studied areas (Fig. 3). sample graph of *COI* gene barcode of B.sharpeyi

specie with sequence using chromas lite 2.1 software is shown (Fig. 4).it's a way for identifying this specie using *COI* sequence.

Nucleotide diversity with Arlequin 3.5 (Excoffier and Licher,2010) within Fishery farms , Jrahi and Karkhe rivers that were 0.0286, 0.0785 and 0.0072, respectively (Table 2).This result showed a low genetic diversity within sub-populations in *B.sharpeyi*. The highest amount of heterozygosity and nucleotide diversity were revealed in sub-population in Jrahi showed that there is genetic distance between Jrahi and other places(Table1, Table 2). This result is showed in Figure 1, and have been indicated by *Fayazi et al.* 2006.

Table 3. Analysis of molecular variance to examine the genetic structure of investigated sub-populations of B. sharpeyi.

Source of Changes	Degrees of freedom	The sum of squares	Variance components	Percent
Between sub-populations	2	49.831	1.91661 Va	14.54
Within populations	22	247.929	11.26948 Vb	85.46
Total	24	297.760	13.18609	100

Fst index is equal to 0.14535

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

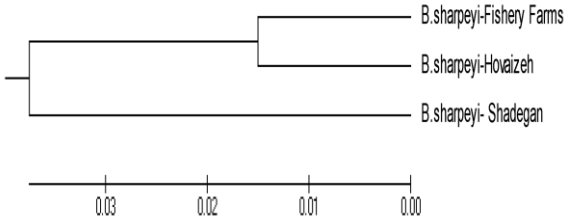

Fig. 1. Phylogenetic tree obtained from comparison of the nucleotide sequences of cytochrome oxidase I gene in B.sharpeyi with UPGMA method by MEGA5 software.

Analysis of Molecular Variance (AMOVA) indicated that most of genetic variance (46.85%) is in *B. sharpeyi* sub-populations and variation between sub-populations is low (Table 3). This issue indicates that there is association and gene flow between regions and rivers. These results shows that Nucleotide diversity within sub-populations were low, that one reason of that is to reproduce and release this specie by fishery organization. Also nucleotide diversity of Karkhe sub-populations is lower than other sub-populations. It's recommended that fisheries in proliferation and release this species, use from new productive, especially in Karkhe river. These study was the first attempt to identify the genetic structure of *B.sharpeyi's* sub- populations in southwest of Iran by using *COI* marker. Although used marker in this research provided very strong and appropriate performance and results with high accuracy in identifying the genetic structure of species, but it's costly. It's suggested that *D-Loop* gene is also used for a closer investigation of genetic structure within a species with the same technique.

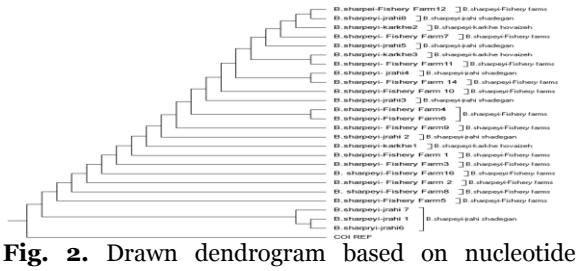

Fig. 2. Drawn dendrogram based on nucleotide sequences of cytochrome oxidase I gene in sub-populations of B. sharpeyi with UPGMA method based in maximum composite likelihood model in MEGA5 Software.

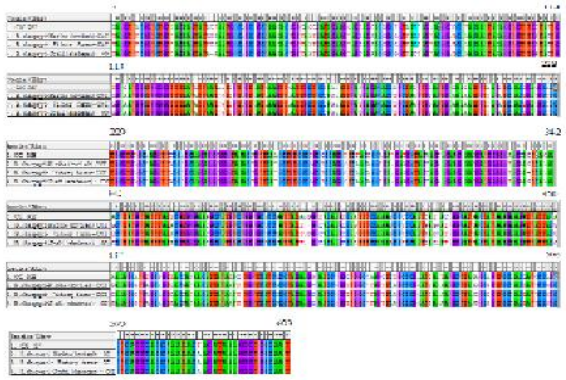

Fig. 3. Gene nucleotide sequence of Cytochrome oxidase I of B. sharpeyi populations and present in Khuzestan province (using CLUSTAL X software).

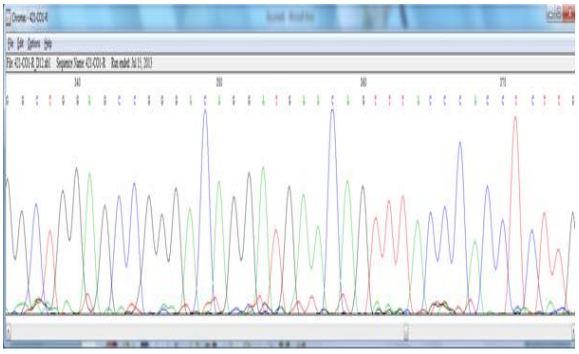

Fig. 4. Sample graph of COI gene barcode of B.sharpeyi specie with sequence using chromas lite.

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Abbreviations

units of measure and key terms:

DNA : deoxyribonucleic acid

mtDNA: mitochondrial deoxyribonucleic acid

COI:Cytochrome Oxidase I

PCR: Polymerase Chain Reaction

PCR- RFLP: Polymerase Chain Reaction- Restricted Fragment Length Polymorphysm

Taq :Thermus aquaticus

dNTP: deoxy Nucleotide Tri Phosphate

MEGA 5: Molecular Evolutionary Genetics Analysis Version 5.0.

Arlequin 3.5: A new series of programs to perform population genetics analyses under Linux and Windows

BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT

AMOVA: Analysis of Molecular Variance

UPGMA: Unweighted Pair Group Method with Arithmetic Mean

mL : milliliter

µM : micromolar

mM :milimolar

µL: : microliter.