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Genetic Differentiation and Phylogenetic Relationships Among *Barbus xanthopterus* (Cyprinidae) Populations in Southwest of Iran Using Mitochondrial DNA Markers

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Abstract: This study was conducted to evaluate the genetic structure of *B. xanthopterus* populations in Karoon, Krkhe and Jrahi rivers in southwest of Iran using Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA). Amplified mtDNA (cytochrome b and part of $tRNA^{Glu}$) genes were digested with nine restriction enzymes in order to check the presence of recognition sites. RFLP analysis revealed a total of nine composite haplotypes in 100 individuals. The estimated sequence divergence between all pairs of haplotypes ranged from 0.8 to 4.4%. The haplotype and nucleotide diversity within populations ranged from 0.3785 to 0.6456 and 0.0052 to 0.0125, respectively. The average nucleotide diversity (π_{xy}) and the net nucleotide divergence (δ) among 3 populations ranged from 0.0108 to 0.0127 and 0.00 to 0.39%, respectively. The neighbour-joining dendrogram which was constructed based on the net nucleotide sequence divergence showed that *B. xanthopterus* populations separated in two groups. The *B. xanthopterus* populations in Karoon and Krkhe rivers are categorized in one group and Jrahi river in a second group. The obtained phylogenetic relationships was approved by Fitch-Margoliash method using Slatkin linearized FSTs. The obtained results at the present study showed a low genetic diversity between *B. xanthopterus* of Karoon, Krkhe and Jrahi rivers in southwest of Iran. The results also suggested that for maintaining of genetic diversity of *B. xanthopterus* at appropriate levels, the exchange and transfer of *Barbus* stocking between Jrahi and other river basin should be avoided.

Key words: mtDNA, RFLP, PCR, genetic diversity, *Barbus xanthopterus*

INTRODUCTION

With about 1800 km of coastline along the Persian Gulf and the Sea of Oman and about 990 km on the southern shore of the Caspian Sea, plus some inland fresh water (rivers, lakes, large water reservoirs behind dams, aquaculture ponds, etc.), has provided a great variety of aquatic fauna: mollusks, crustaceans, chelonians, mammals (dolphins, whales, seals) and particularly, fishes in Iran. Thus the country has rich aquatic resources and considerable potential for fishing and aquaculture.

Cyprinids (Cyprinidae) are the major component of Eurasian temperate fresh water fish fauna (Coad, 1998). The role of this family within freshwater ecosystems is therefore central and to date, many research groups have been investigated the genetic structure of this family (Durand *et al*., 2002). The genus *Barbus* constitutes the dominant component of cyprinids, with more than 800 species spread over Europe, Africa and Asia (Wang *et al*., 2004). Protein electrophoresis has been used to discriminate species of *Barbus* but this technique has been unable to distinguish completely all these species. The mtDNA gene that are inherited as a single non-recombining genealogical unit through maternal lines and are therefore useful for analysis of intraspecific phylogeography (Wilson *et al*., 1985). The evolutionary rate as well as the genetic differentiation of mtDNA among populations is thought to be approximately 5-10 times higher than that exhibited by nuclear genes (Birky *et al*., 1989). Recently, using mtDNA for phylogenetic studies revealed unexpectedly high divergence between populations of a *Barbus* species from an European river (Danube), suggesting the presence of several geographically separated genetic lineages within

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this species (Tsigenopoulos *et al*., 2002). A considerable intraspecific variation and small differences among species have also been reported (Kotlik and Berrebi, 2002).

In Iran several parameters such as excess fishery, water-flow regulation, pollution and habitat destruction may cause danger to *Barbus* at the population level. Thus, an conservation and management program should be developed with considering the genetic structure of *Barbus* populations both at macro- and micro-geographical scales. Current knowledge on genetic structure and phylogenetic lineages of *B. xanthopterus* is still very poor in Iran. Despite the advantage of mtDNA analysis in population genetic studies, no such studies concerning *B. xanthopterus* populations have been presented so far in Iran. The aim of the present study was to provide some basic data using estimating of genetic variation and differentiation of *B. xanthopterus* populations where may be used in genetic conservation and management programs for this stock where is an important commercial and recreational fishery species in three major rivers in southwest of Iran (Karoon, Karkhe and Jrahi) as well as Persian Gulf's coastline.

MATERIALS AND METHODS

Sample collection and DNA isolation: A total number of, 100 individuals of fresh water fish were sampled from three major rivers (Karoon, Karkhe and Jrahi) in southwest of Iran (Fig. 1) using traps. All individuals were transferred in dry ice to the laboratory and stored at -20°C until used. Total genomic DNA was isolated from each individual according to the protocol of Jackson *et al*. (1991), after minor modifications. About 50 mg of muscle tissue was ground in 400 μL homogenized buffer (20 mM Tris-HCl pH: 7.5, 200 mM NaCl, 20 mM EDTA). Then 100 μL of 10% SDS and 5 μL proteinase K (20 mg mL^{-1}) were added and the mixture was incubated at 50°C for 30 min. DNA was purified with standard phenol: Chloroform extractions, precipitated with ice cold absolute ethanol and resuspended in 50 μL TE (Tris-EDTA pH: 8) buffer. The DNA suspension was stored at 20°C until used for assay.

PCR-RFLP analysis: A mtDNA fragment (aprox. 1216 bp) of cytochrome b and part of $tRNA^{Glu}$ genes was amplified from DNA samples of 33 to 34 fishes of each Barbus populations. The primer pairs for the amplification of these genes. The PCR was carried out in a final volume of 50 μL containing 100 μM dNTPs mix, 50 pmol of each primer, 5 μL of 10 × PCR assay, 1.5 mM $MgCl_2$, 0.5 units

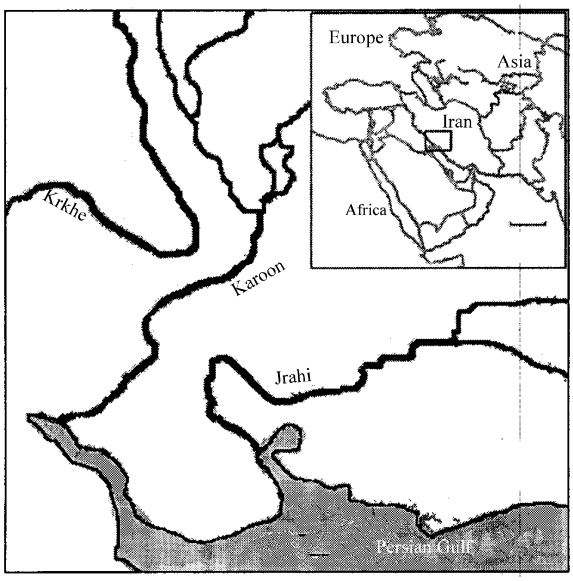

Fig. 1: Sampling sites of *B. xanthopterus* populations in three major Rivers (Karoon, Krkhe and Jrahi) in southwest of Iran

of Taq DNA polymerase and 80-100 ng of purified fish genomic DNA. PCR amplification conditions were as follows: one preliminary denaturation step at 94°C for 4 min followed by 35 PCR cycles. Strand denaturation was at 94°C for 15 sec, annealing at 64°C for 30 sec and primer extension at 72°C for 1 min. A final extension at 72°C for 5 min was performed.

The amplified PCR products were digested with nine restriction endonucleases: *Alu*I, *Hae*III, *Hinf*I, *Hpa*II, *Mbo*I, *Eco*RI, *Rsa*I, *Hind*III and *Taq*I in order to check the presence of recognition sites. The digested PCR products were then separated electrophoretically on 3% agarose gels, in 1× TBE buffer, stained with ethidium bromide and visualized under UV light. The sizes of DNA fragments were compared to the PCR marker (Fermentas) run on the same gel. Distinctive restriction fragment patterns were identified by letter codes and subsequently combined to produce composite mtDNA haplotypes for individual fish.

Data analysis: The genetic relationship among haplotypes *(dij)* were analysed based on Fitch-Margoliash method using the PHYLIP 3.6 statistical package (Felsenstein, 1993). Nucleon diversity (h) was calculated for each locality according to Nei and Tajima (1981). Nucleotide sequence diversity and nucleotide sequence divergence were calculated with the Restriction Enzyme Analysis Package (REAP, version 4.0; McElroy *et al*., 1991). Phylogenetic trees were constructed based on Nei's distance (Nei, 1987) by the unweighted

pair group method with arithmetic mean (UPGMA), based on net nucleotide divergence (δ) between populations and the confidence of the branches was evaluated (1000 replicates) by the application of the bootstrap method using the PHYLIP 3.5 statistical package (Felsenstein, 1993). Population structure in *B. xanthopterus* was also evaluated by using nested analysis of molecular variance (ANOVA), Excoffier *et al*., 1992). AMOVA input consisted of instance matrix containing genetic distance values for all possible pairs of the nine observed mtDNA haplotypes. Total genetic variation was partitioned into two components, within and among populations. The pairwise mismatch distribution between all individuals was estimated based on sudden expansion models using the Arlequin computer package version 3.01 (Excoffier *et al*., 2005).

RESULTS

The size of the PCR-amplified was found to be about 1216 bp for cytochrome b and part of $tRNA^{Glu}$ of mtDNA segment. The restriction enzymes *Mbo*I and *Alu*I, *Hae*III, *Hin*fI, *Taq*I and *Rsa*I had three and two recognition site in the amplified region, respectively. The restriction enzymes *Hind*III and *Eco*RI did not detect any site, but the *Hpa*II endonuclease has yielded only monomorphic pattern in all examined individuals. A total of nine composite haplotypes were identified among the *B. xanthopterus* populations (Table 1). The highest number of haplotypes and consequently the highest diversity values were obtained within Krkhe, Karoon and Jrahi population, respectively. The sequence divergence between all pairs of haplotypes was ranged between 0.8 to 4.4%. The genetic relationships between the nine different mtDNA haplotypes and the UPGMA dendrogram constructed from Nei's distance based on the net nucleotide divergence are shown in Fig. 2 and 3. Genetic distances, bootstrap procedures and neighbor-joining trees using the PHYLIP 3.5 statistical package showed the separation of the *Barbus* populations, followed by two main groups: one consisting of the Jrahi's population in one cluster and the second cluster contained Krkhe and Karoon's populations (Fig. 3). The gene and nucleotide diversity among three populations are shown in Table 2. The average nucleotide diversity (πxy) and the net nucleotide divergence (δ) among populations was ranged from 0.0108 to 0.0127 and 0.00 to 0.39%, respectively (Table 3). AMOVA analysis revealed that the majority of mtDNA variation (81%) occurred within populations (Table 4). The pairwise mismatch distribution was calculated as an estimation of differences in number of site changes between all individuals. The conservative nature of tests based on mismatch distribution did not confirm the inference of recent population growth (data are not shown).

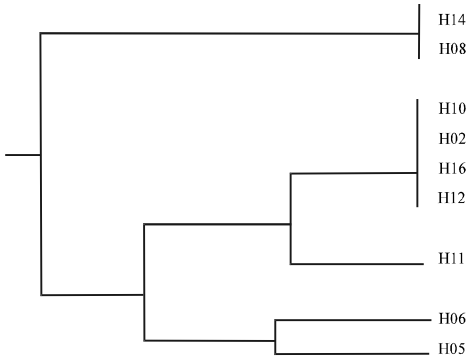

Fig. 2: Fitch-Margoliash dendrogram showing the relationships between the nine mtDNA haplotypes of *B. xanthopterus* populations detected in three major Rivers (Karoon, Krkhe and Jrahi) in southwest of Iran

Table 1: Haplotype code, Composite haplotype, haplotype frequency and sample size of *B. xanthopterus* populations in three major Rivers (Karoon, Krkhe and Jrahi) in southwest of Iran

Code	Composite haplotype	Karoon	Krkhe	Jrahi	Total
H02	BBBABBA	0.42 (11)	0.26 (9)	0.78 (31)	0.51 (51)
H05	BBBAABA	0.42 (11)	0.47 (16)	0.05 (2)	0.29 (29)
H06	BBCAABA	0.08 (2)	0.09 (3)	-	0.05 (5)
H08	ABBABBA	-	-	0.15 (6)	0.06 (6)
H10	BCBABBA	0.04 (1)	0.06 (2)	-	0.03 (3)
H11	BCCABBA	0.04 (1)	0.06 (2)	-	0.03 (3)
H12	BBCABBA	-	0.03 (1)	-	0.01 (1)
H14	ABBABBA	-	0.03 (1)	-	0.01 (1)
H16	BCBABBA	-	-	0.03 (1)	0.01 (1)
Total No. of individuals		26	34	40	100
Total No. of composite haplotype		5	7	4	9

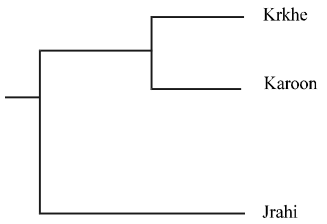

Fig. 3: UPGMA Dendrogram, based on the net nucleotide divergence, showing the relationships among *B. xanthopterus* populations in three major Rivers (Karoon, Krkhe and Jrahi) in southwest of Iran

Table 2: Haplotype and nucleotide diversity in within populations of *B. xanthopterus* in three major Rivers (Karoon, Krkhe and Jrahi) in southwest of Iran

Sampling site	Haplotype diversity	Nucleotide diversity
Karoon	0.6456±0.0386	0.0102
Krkhe	0.7024±0.0421	0.0125
Jrahi	0.3785±0.0621	0.0051
Average	0.5755±0.0099	0.0093±0.0000047

Table 3: Average nucleotide diversity (π_{xy}) (upper diagonal) and net nucleotide divergence (δ) (lower diagonal) in between populations of *B. xanthopterus* in three major Rivers (Karoon, Krkhe and Jrahi) in southwest of Iran

Sample sites	Karoon	Krkhe	Jrahi
Karoon	*	0.011054	0.010827
Krkhe	0.000	*	0.012725
Jrahi	0.315	0.389	*
π_{xy}	0.011535±0.0000004		
δ	0.2243±0.0017		

Table 4: Hierarchical nested analysis of molecular variance on genetic distance between populations of *B. xanthopterus* in three major Rivers (Karoon, Krkhe and Jrahi) in southwest of Iran

Variance component	df.	Sum of squares	Variance	% of variation
Among populations	2	4.910	0.06613	18.93
Within populations	97	27.470	0.2832	81.07
Total	99	32.38	-	-

DISCUSSION

A total of nine composite haplotypes were detected among *B. Xnanthopterus* populations studied. The mtDNA analysis at the present study provided no uniform pattern for distribution of the haplotypes within *B. xanthopterus* populations. The frequency of haplotypes H02 (51%) and H05 (29%) was higher than other haplotypes where the remaining seven haplotypes occurred at low frequency (≤10%). Haplotype H02 was dominant at all localities while the haplotype H05 was dominant in karoon and krkhe populations. The haplotype 8 occurred in a low frequency only in Jrahi not in any other river basin. On the other hand, haplotype 2 and 5 shared among all river basin, appears not to be localized. The most likely explanation for this pattern might be the occurrence of gene flow from distributions of unique mtDNA haplotypes and their presumed precursors among these populations. According to Birky *et al.* (1989) this haplotypes could also be an ancestral one, which was retained in these populations.

Our results revealed the existence of two clearly distinguishable types of *B. Xnanthopterus* populations. The first one consists of the Krkhe and Karoon populations, while the second one contains the Jrahi population. The levels of divergence observed between the nine mtDNA haplotypes studied range from 0.8 to 4.4% which is higher than that reported by Apostolidis *et al.* (1996) in *Salmo trutta* populations (0.21 to 3.42%), one of the most structured fish species. The low levels of genetic variability revealed at the present study are consistent with the observation of Richardson and Gold (1995), in *Cyprinella lutrensis* population with the range of 0.14 to 9.24% and Imsiridou *et al.* (1998) in *Leuciscus cephalus* population with the range of 0.313 to 6.79%. The highest number of haplotypes (seven haplotypes) were found in Krkhe river, which might suggest that we are either dealing with a mixed group or a more ancestral population in this river basin.

The overall genetic diversity obtained in *B. xanthopterus* populations at the present study was higher than with the observation of Bernatchez and Dodson (1991) in *Coregonus clupeaformis* populations (0.000-0.351) in North America, King *et al.* (2000) in *Salmo salar* populations (0.00-0.682) in Atlantic, Machordom *et al.* (2000) in *Salmo trutta* populations (0.000-0.860) in Danish river system. AMOVA analysis revealed that most of the total mtDNA variation in *B. xanthopterus* at the present study was due to variation in within populations, indicating a very low level of differentiation among populations. The low level of genetic differentiation among populations is also highlighted by a low level of nucleotide diversity (π_{xy}) and nucleotide divergence (δ) among populations at the present study. It has been reported that a migratory species has 85 and 15% of its diversity within and between local populations, respectively. In contrast, a non-migratory species has 67.6 and 32.4% of its diversity within and between local populations, respectively (Vrijenhoek, 1998). The obtained results at the present study are indicating that, these populations are likely to be a migratory population due to a low level of genetic diversity between populations.

Recent theoretical results indicate that tests for population expansion that are based on mismatch distribution are more conservative than the tests based on either the distribution of mutation frequencies (the frequency spectrum) or the distribution of haplotypes

(genetically linked polymorphisms) (Ramos-Onsins and Rozas, 2002). So we used this method to test the theory of population expansion from few founder individual. The conservative nature of tests based on mismatch distribution did not confirm the inference of recent population growth. Despite, the neighbor-joining trees separated the *Barbus* populations in two groups with a more tendency for karoon/krkhe cluster, but the differentiation between the rivers was very low and this may be as a gene flow occurrence between this rivers. The possible hypothesis to explain these results, every so often, due to intensive rain and snow melting in Zagross mountain (origin of these rivers) causes a severe water flood and consequently connect the rivers in some places. These conditions may provide an appropriate situation for migration of fish and therefore occurrence of gene flow between rivers. This study represents the first attempt to identify the genetic structure of *B. xanthopterus* populations in southwest of Iran using mtDNA markers. Although, the technique employed in our study proved sufficiently powerful to detect regional population structure but, higher resolution mutation screening analysis or direct sequencing of the mitochondrial D-loop region might provide greater resolution of geographic structure than the RFLP technique employed here.

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