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Population Structure of Indian Hill Trout (*Barilius bendelisis*) Inferred from Variation in Mitochondrial Dna Sequences

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Abstract: *Barilius bendelisis* is an important food fish and a demanding ornamental species in India. Despite its great economic importance, several wild populations have been suffering drastic reduction. The genetic variability within population is extremely useful for gathering information on individual's identity, breeding patterns, degree of relatedness and disturbance of genetic variation among them. In order to understand the genetic structure of three populations of *Barilius bendelisis,* sequences of mitochondrial gene, cytochrome b (307 bp) from three wild populations were sequenced and analysed. A total of 17 polymorphic sites and 14 parsimony informative sites were detected in cytochrome b gene (307bp) sequences in all three populations. The nucleotide diversity was 0.0237 between Saryu and Kosi river populations, 0.01831 between Saryu and Kalsa river populations and 0.01346 between Kosi and Kalsa river populations. The eight different haplotypes were detected among the three populations studied, single population specific haplotype was observed in Kosi river population. UPGMA dendrogram based on cytochrome b gene sequences of *B. bendelisis* shows that the Kosi river population make cluster with Kalsa river population rather than the Saryu river population. The genetic distance was 0.04 between Saryu and Kosi river population, 0.03 between Saryu and Kalsa river population and 0.02 between Kosi and Kalsa river population. The cytochrome b gene sequences revealed high level of genetic differentiation within and between populations of *B. bendelisis* and demonstrated the suitability of partial Cytochrome b gene (307 bp) sequence in determining the genetic diversity in *B. bendelisis* populations.

Key words: *B. bendelisis* · Indian Hill Trout · Population structure · Cytochrome b · mtDNA

INTRODUCTION

The genus *Barilius* (Hamilton-Buchanan) belongs to the family Cyprinidae, found all over Asia. So, far 20 species of *Barilius* have been reported in the Himalayan and sub-Himalayan regions. *Barilius bendelisis* (Hamilton, 1807), commonly known as Indian Hill Trout, plays significant role in the capture fishery in several parts of the Himalayan region of Uttarakhand and a demanding ornamental as well as potential food fish.

In spites of its greater importance few scattered reports on habitat characterization, feeding habits, food composition [1, 2] of *Barilius bendelisis* are available. Surprisingly, there was no information available on the genetic diversity of natural populations of this species. In recent year, different molecular techniques, using nuclear and mitochondrial DNA (mtDNA), have provided new information concerning the genetic variability of wild and cultivated populations of several fish species [3]. Mitochondrial DNA is maternally inherited without genetic recombination. 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 [4]. Mitochondrial DNA represents a significant marker system for use in population and phylogenetic studies. An extensive review of the advantages of mtDNA as a tool for population genetic analysis has been provided [5]. Among many mitochondrial genes, cytochrome b has been used successfully to identify genetic variation in many fish species [6]. Cytochrome b tend to show intra-specific variation mainly in 3rd position of codon which can be

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used to identify stocks. Variation in mtDNA Cytochrome b gene has been used for population studies in cyprinidae fishes [7]. There is no work done on genetic diversity of *Barilius bendelisis* previously. The aim of the present study was to present a preliminary assessment of the genetic variability of three wild populations of *B. bendelisis*, based on the nucleotide sequences of cytochrome b region of the mitochondrial genome. The results were useful not only to characterize *B. bendelisis* populations but also to give support to recovery efforts and to the biodiversity maintenance and breeding programme of this fish species.

MATERIALS AND METHODS

The sampling of *B. bendelisis* in selected rivers of Himalayan regions Saryu river near Champawat (n = 50), Kalsa river near Chanfi (n = 52) and Kosi river near Ramnagar (n = 53) were carried out using cast net. The fin tissues were cut and put immediately on 90% ethanol, than kept at -20°C until DNA extraction and the voucher fish specimens immediately fixed in 8% formalin.

Genomic DNA was isolated from all fin tissue samples by phenol-chloroform extraction method [8]. Isolated genomic DNA was precipitated with 2-2.5 volume of chilled ethanol. The DNA pellet was washed twice with 70% ethanol, air dried and re-suspended in 1X TBE (10mM Tris-Hcl, pH 8.0 and 1mM EDTA) buffer and kept at 4°C till further use. The quality of DNA was checked by 0.8% agarose gel electrophoresis and the concentration of DNA was estimated with the help of UV-VIS spectrophotometer (Thermo Scientific, England) by taking absorbance at 260nm and 280nm, as one OD_{260} equals 50 µg/ml of double stranded DNA. The intact DNA samples with absorbance ratio 1.6 to 1.8 were used for further experimental work.

The partial Cyt b gene were amplified in seven samples per population of *B. bendelisis* in 25 µL reaction volume, containing 2.5µL of 10X PCR-buffer (100mM Tris, pH 9.0, 500mM Kcl, 15 mM $Mgcl_2$, 0.1% Gelatin) (Bangalore, Genei, India), 200 µM of each dNTPs (Bangalore, Genei, India), 10 pmol of each primer "L14841" and "H15149" [9], (Table 1), 1 Unit of Taq DNA polymerase (Bangalore, Genei, India), 100 ng of genomic DNA and rest miliQ water with PCR amplification cycles as follows: preliminary denaturation step at 94°C for 4 min, 35 PCR cycles of 94°C for 45 sec (Strand denaturation), 54°C for 30 sec (annealing), 72°C for 1 min (primer extension) and final extension at 72°C for 7 min. One negative control (absence of DNA template) was included for each set of amplification. The PCR amplified products were checked in 1.2% agarose gel in 1X TBE (Tris-Hcl, boric acid, EDTA, pH 8.0) buffer at constant voltage of 3V/cm and the amplified fragment visualized with ethidium bromide staining [10], (Figure 1) under UV illumination in the Gel-Doc system (Alpha Imager 3400, Alpha Innotech Corporation, USA).

Molecular weight of target fragment was determined using 1Kb and100bp DNA ladder (Fermentas, Canada). The amplification products were purified before sequencing with Qiaquick columns (Qiagen, USA) followed by manufacturer's instructions. After purification, sequencing of PCR products of five individuals of *B. bendelisis* from each population were performed in both directions by Cycling sequencing with the primers we used for PCR amplification. Sequencing was performed in an ABI Prism 3100 automated sequencer (Applied Biosystems, USA) using Bigdye Terminator. Nucleic acid sequences were subjected to BLASTn [11] at the National Centre for Biotechnology Information (NCBI), Website (http://www.ncbi.nlm.nih.gov/blast). All the Sequences of the mtDNA gene (Cyt b) of *B. bendelisis* were submitted in Gene-Bank, with the accession number (HQ852726 to HQ852740).

The sequences were aligned using ClustalW software [12] website (htpp://www.ebi.ac.uk/clustalw). The genetic diversity (π) and haplotype diversity (h) were calculated using software DNASP version 5.10 [13]. The analysis of molecular variance (AMOVA) as implemented in Arlequin version 3.01 [14], used to assess the population structure of *B. bendelisis*. The phylogenetic relationship among three populations of *B. bendelisis* was constructed by unweighted pair group method with arithmetic mean (UPGMA), using software MEGA version 4.0 and bootstraps support was calculated using 1000 replication. The mean genetic distances between the populations were calculated using the software MEGA version 4.0 [15].

Table 1: Primers used for PCR and sequencing of mitochondrial cyt b gene in this study

Primer name	Sequence
L14841	AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA
H15149	AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA

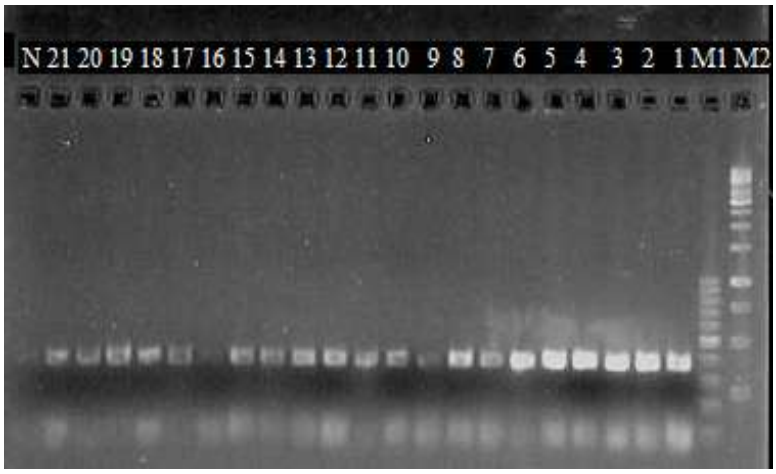

Fig. 1: Amplified Cytochrome b gene in three populations of *B. b* (1-7 Saryu, 8-14 Kosi and 9-21 Kalsa) with (M_2) 1Kb ladder, (M_1) 100bp ladder and (N) Negative control

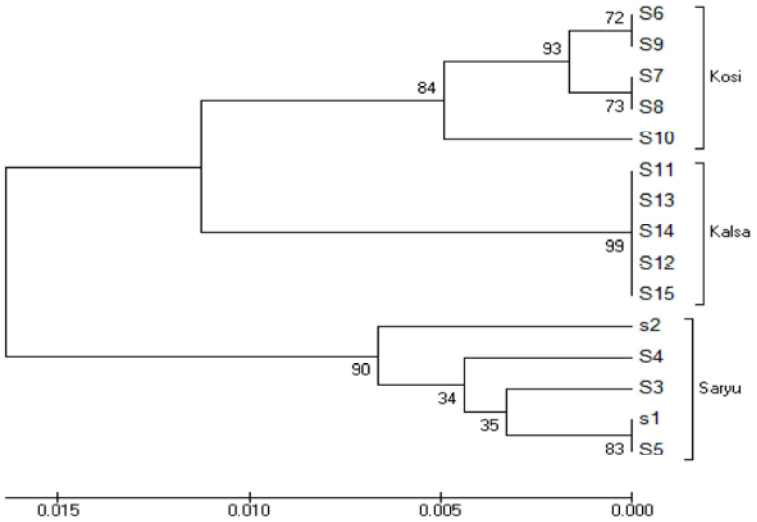

Fig. 2: UPGMA Dendrogram, based on the nucleotide divergence, showing the relationship between the three populations of *Barilius bendelisis (B. b)*.

RESULTS

Cytochrome b gene amplified in this study was 307 bp long. The average nucleotide frequencies among 15 samples of three populations of *B. bendelisis* was 0.196 (A), 0.316 (T/U), 0.276 (C) and 0.212 (G). Cyt b gene revealed 17 variable sites and 14 parsimonic informative sites in 307 bp long region. The nucleotide diversity was 0.0237 between Saryu and Kosi river populations, 0.01831 between Saryu and Kalsa river populations and 0.01346 between Kosi and Kalsa river populations. A total of eight distinct cyt b mtDNA haplotypes were identified in three populations of *B. bendelisis* (Table 2). The hierarchical analysis of molecular variance (AMOVA) of population structure reveals a highly significant subdivision between populations in the total sample (F_{ST} = 0.83333; P<0.005) (Table 3), percentage of variation among population is 16.67% and within population 83.33%. Population pair wise F_{ST} value ranged from 0.79245 to 0.88235 (Table 4).

Table 2: Number of haplotypes detected in three different populations of *B.b*

Haplotype	Saryu	Kalsa	Kosi
Hap 1	2	0	0
Hap 2	1	0	0
Hap3	1	0	0
Hap 4	1	0	0
Hap 5	0	2	0
Hap 6	0	2	0
Hap 7	0	1	0
Hap 8	0	0	5

Table 3: AMOVA analysis based on cytochrome b sequences of three populations of *B. b*

Source of variation	d. f	Sum of squares	Variance of components	Percentage of variation	Fixation index	P-value
Among populations	2	38.133	3.66667 Va	16.67		
Within populations	12	8.800	0.73333 Vb	83.33	0.83333	0.00000+-0.00000

Table 4: Population pair-wise F_{ST}

	Saryu	Kalsa	Kosi
Saryu	0.00000		
Kalsa	0.79245	0.00000	
Kosi	0.84444	0.88235	0.00000

UPGMA Dendrogram based on cyt b gene sequences shows that, three different populations of *B. bendelisis* make three different clusters (Figure 2). Mean genetic distance between populations range from 0.02 to 0.04, with the highest genetic distance between the Saryu and Kosi river population.

DISCUSSION

Understanding of population genetics structure of *B. bendelisis* species provides critical information for developing conservation, management and fish production strategies. Result obtained from 307 bp mtDNA sequences in present study, revealed high genetic differentiation in *B. bendelisis* populations collected from three different rivers. Cytochrome b gene amplified in *B. bendelisis* populations has been reported to be useful in detecting variation in *B. bendelisis*. The universal primer (Kocher *et al.* 1989) used in this study for amplifying 307 bp region of mtDNA, found to be polymorphic in *B. bendelisis* populations. This region found to be polymorphic and has been used successfully for intraspecific genetic diversity analysis in various other fish species, like *Salmo trutta* [16]; *Cyprinodon variegates* [17]; *Sardina pilchardus* [18] and *Lates calcarifer* [19]. Nucleotide sequences of Cytochrome b gene in *B. bendelisis* were A+T rich (51.2%), which are similar to many fishes [20]. The nucleotide diversity was 0.0237 between Saryu and Kosi river populations, 0.01831 between Saryu and Kalsa river populations and 0.01346 between Kosi and Kalsa river populations. A total of 8 different haplotypes were found among three populations of *B. bendelisis*, from which Hap1, Hap2, Hap3 and Hap 4 were found in Saryu river population, Hap5, Hap6 and Hap7 were found in Kalsa river population and Hap 8 was found in Kosi river population. A common haplotype was not observed in any of the population of the species. The haplotype diversity for Saryu river population was 0.90000, for Kalsa river population was 0.80000 and for Kosi river population was 0.00000. The haplotype and nucleotide diversity for Kosi river population was 0.0000, as Kosi river population give a single haplotype, indicating low gene flow among these three wild populations of the *B. bendelisis*. The highest nucleotide and haplotype diversity in Saryu river population might be due to the isolation by distance. AMOVA revealed high within population variation (83.33%) and low among populations variation (16.67%). It is reported that a migratory fish species has 85% and 15% of its diversity within and between its local populations, respectively and 67.6% and 32.4% for a non migratory fish [21]. The level of genetic divergence between populations of *B. bendelisis* observed in this study was slightly lower than that reported for a migratory fishes, which indicated the migratory nature of *B. bendelisis*. F_{ST} value also supported the presence of significant genetic differences between populations of Saryu, Kosi and Kalsa rivers. Such high intra-specific diversity could be expected as the rivers belong to different river basins. Therefore, it is likely that populations under study could have evolved in isolation after fragmentation from common ancestor. It is accepted t hat high mtDNA variation, exiting in Saryu river population than Kalsa and Kosi river populations. Mean genetic distance between population 1(Saryu) and population 2 (Kosi) is 0.04, between population 1 (Saryu) and population 3 (Kalsa) is 0.03 and between population 2 (Kosi) and 3 (Kalsa) is 0.02. The Kosi river population makes a cluster with Kalsa river population which shows that *B. bendelisis* population of Kalsa and Kosi River are genetically closer to each other than Saryu river population. Result obtained demonstrated that partial cyt b fragment (307 bp) is observed to be a potential marker for studying variation within as well as among populations in *B. bendelisis*. Such information has provided useful information in case of many other fishes. The success of conservation programs and effective management policies depend on the level of

genetic divergence within and between species and developing strategies to maintain the natural genetic diversity [22]. The analysis of mtDNA (cyt b) represents an important tool for the characterization of distinct populations of *B. bendelisis* for conservation, breeding and management programmes.

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REFERENCES

1. Farswan Y.S., J.P. Bhatt and S.N. Bahuguna, 1989. Effects of some plant toxins on feeding and growth rate of *Barilius bendelisis* (Ham). Acta Ecthylogica., 19(1): 59-69.
2. Shehgal, K.L., 1999. Coldwater fish and fisheries in the indian himalayas: rivers and streams. In Petr, T. (Ed.), Fish and fisheries at higher altitudes: Asia, FAO Fisheries Technical Paper. No.385. Rome, FAO., 1999. 304.
3. Was, A. and R. Wenne, 2002. Genetic differentiation in hatchery and wild sea trout (*Salmo trutta*) in the Southern Baltic at microsatellite loci. Aquaculture., 204: 493-506.
4. Birky C.W., T. Maruyana and P. Fuerst, 1983. An approach to population and evolution genetic theory for genes in mitochondria and chloroplasts and some results. Genetics., 103: 513-527.
5. Avise, J.C., 1991. Ten unorthodox perspectives on evolution prompted by comparative population genetics findings on mitochondrial DNA. Annu. Rev. Genet., 25: 45-69.
6. McVeigh H. P., S. E. Bartlett and W. S. Davidson, 1991. Polymerase chain reaction/direct sequence analysis of the cytochrome b gene in *Salmo salar*. Aquaculture., 95: 225–233.
7. Fayazi J., M. Moradi, G. Rahimi, R. Ashtyani and H. Galledari, 2006. Genetic differentiation and phylogenetic relationships among *Barbus xanthopterus* (Cyprinidae) populations in south west of Iran using mitochondrial DNA markers. Pak J. Biol. Sci., 9(12): 2249-2254.
8. Sambrook J., E.F. Fritsch and T. Maniatis, 1989. Molecular Cloning: a Laboratory Manual. Coldspring Harbor Laboratory Press, Coldspring Harbor, NY.
9. Kocher, T.D., W.K. Thomas, A. Meyer, S.V. Edwards, S. Paabo, F.X. Villablanca and A.C. Wilson, 1989. Dynamics of mitochondrial DNA evolution in mammals: Amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA., 86: 6196-6200.
10. Sambrook, J. and D.W. Russell, 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
11. Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman, 1990. Basic local alignment search tool. J. Mol. Biol., 215: 403-410.
12. Thompson, J.D., D.G. Higgins and T.J. Gibson, 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res., 22: 4673-4680.
13. Rozas, J. and R. Rozas, 1999. DnaSP version 3 an integrated program for molecular population genetics and molecular evolutionary analysis. Bioinformatics., 15: 174-175.
14. Excoffier, L.G. and L.S. Schneider, 2005. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evol. Bioinformatics Online., 1: 47-50.
15. Tamura, K., J. Dudley, M. Nei and S. Kumar, 2007. MEGA 4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol., 24: 1596-1599.
16. Apostolidis, A.P., C. Triantaphyllidis, A. Kouvatsi and P.S. Economidis, 1997. Mitochondrial DNA sequence variation and phylogeography among *Salmo trutta* L (Greek brown trout) populations. Mol. Ecol., 6(6): 531-542.
17. Finne, K.L., 2001. Phylogeographic structure of the Atlantic pupfish, *Cyprinodon variegatus* (Cyprinodontidae), along the eastern coast of North America. Unpublished M.S. thesis, Virginia Polytechnic Institute and State University., Blacksburg, Virginia.
18. Tinti, F., C. Di Nunno, I. Guarniero, M. Talenti, S. Tommasini, E. Fabbri and C. Piccinetti, 2002. Mitochondrial DNA sequence variation suggests the lack of genetic heterogeneity in the adriatic and ionian stocks of *Sardina pilchardus*. Mar Biotechnol., 4: 163-172.

19. Marshall, C.R.E., 2005. Evolutionary genetics of barramundi (*Lates calcarifer*) in the Australian region. Unpublished Ph. D. thesis, School of Biological Sciences and Biotechnology, Murdoch University., Perth, Western Australia.
20. Jones, G.C. and J.C. Avise, 1998. A comparative summary of genetic distance in the vertebrates from the mitochondrial cytochrome b gene. Mol. Biol. Evol., 15: 1481-1490.
21. Vrijenhoek, R.C., 1998. Conservation genetics of fresh-water fish. J. Fish Biol., 53: 394-412.
22. Lakra, W.S., M. Goswami and A. Gopalakrishnan, 2009. Molecular identification and phylogenetic relationships of seven Indian Sciaenids (Pisces: Perciformes, Sciaenidae) based on 16S rRNA and cytochrome c oxidase subunit I mitochondrial genes. Mol. Biol. Rep., 36: 831-839.