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Comparative Phylogenetic Study of four Coldwater Fishes (Family Cyprinidae) Based on Targeted 16S RNA Mitochondrial Gene

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Abstract : *S. richardsonii, T. putitora, B. Bendelisis and G. Gotyla are commercially important coldwater fish, widely used as a food fish, game fish and ornamentally fish in the Uttarakhand region of India. The present study examined the phylogenetic relationship within and between four coldwater fish species from Kumaun and Garhwal region of Uttarakhand. The mitochondrial 16S ribosomal DNA were used to elucidate phylogeny of the family Cyprinids. Analysis of 34 sequences of 16S rRNA gene (550 bp) revealed 12 haplotypes for both regions. The 16S rRNA dataset contained 93 and 94 variable site found for Garhwal and Kumaun region respectively. Rate of transition were higher than transversion for both region. The nucleotide diversity was lowest between the T. putitora and S. richardsonii and highest between the B. bendelisis and G. gotyla for both regions. The phylogenetic tree, constructed unweighted pair group average (UPMGA) methods revealed similar results suggesting that T. putitora and S. richardsonii have a close relationship to each other while maximum divergence was observed in B. bendelisis, which was also confirmed by the genetic distance data. The understanding of genetic variations of coldwater fishes will play a key role in conservation and management of this endangered fish species.*

Keywords : *S. richardsonii*, *T. putitora*, *B. bendelisis*, *G. gotyla*, 16S rRNA, Phylogenetic relationship.

introduction

In Uttarakhand Himalayas, coldwater fish species (Cyprinidae) is widespread endemic in nature of the natural water bodies system. About 218 fish species are found (both endemic and exotic) which belonging to 21 families (Cyprinidae) and 76 genera (Sehgal, 1987; Tripathi, 2005). Coldwater contributes more substantially to overall aquaculture production. But the major problems in coldwater fisheries are low productivity, facing challenge to change of environment, comparatively slow growth rate of the fish species, and low fecundity in fishes. There is depletion of these fishes in natural fish resources due to over utilization of fish stocks, pollution and other anthropogenic activities. Commercially these four fish species are very important *Tor putitora, Schizothorax richardsonii, Barilius bendelisis* and *Garra gotyla.* These are food, game and ornamental fishes. Species characterization using morphology and anatomical characters causes sometimes errors in proper identification of closely related species. Because of these issues, molecular markers have been used as a complementary tool for taxonomic identification. However the phylogenetic relationship of these species is still ambiguous. Because the classification of the family Cyprinidae have mainly been based on morphological characters, which have conflicted and not provided conclusive answers to the phylogenetic questions. Genetic relationship in the four predominant fish species viz., *Tor putitora, Schizothorax richardsonii, Barilius bendelisis* and *Garra gotyla* species an investigation is carried out using targeted mitochondrial DNA- PCR analysis.

mtDNA is useful for estimating genetic distances among the species (Brown *et al*.,

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1979) and inferring systematic positions of fishes at species level (Kocher *et al*., 1989, Britoo *et al*., 1997; Dergam *et al*., 2002; Shikano and Taniguchi, 2003). The mitochondrial 16S rDNA gene has proven a valuable evolutionary marker for fishes because it has produced robust phylogenies at various taxonomic levels (Brown *et al.* 1982; Karaiskou *et al.* 2003; Perez *et al.* 2005; Turan *et al*. 1998). In the past two decades, the mitochondrial 16S rRNA gene has been widely used to explore the phylogenetic relationships of fishes at varying taxonomic levels [e.g. at the order level (Ortı´ and Meyer, 1997); the familial level (Waters *et al*., 2000); the subfamily level (Ortı´ *et al*., 1996; Harris and Mayden, 2001); the generic level (Moyer *et al*., 2004); and the species level (Chakraborty and Iwatsuki, 2006). One approach to improve the phylogenetic performance of the mitochondrial 16S rRNA gene is to incorporate information regarding the molecular structure of the marker in analyses for more accurate phylogenetic inference. The aim of this study was to solve the problem of species characterization and also genetic variation at genus level of important coldwater fishes (*Schizothorax richardsonii, Tor putitora, Garra gotyla* and *Barilius bendelisis*) of two region of Uttarakhand (Kumaun and Garhwal).

Materials and Methods

Materials examined

Fishes were collected from two different region (Kumaun and Garhwal) of Uttarakhand for the period of two years i.e., from January 2009 to June 2010 (Fig. 1, Table 1). All fishes were collected with the help of local fishermen using different types of nets. A total sample size consisted of 60 individuals of four fishes *(Schizothorax richardsonii, Tor putitora, Garra gotyla and Barilius bendelisis)* were collected. Immediately fish photographs were taken prior to preservation in 10% formalin. Identifications done were based on keys for fishes of the Indian subcontinent (Jayaram, 1999; Talwar and Jhingran, 1991). The identification of the species was done mainly on the basis of the colour pattern, specific spots or marks on the surface of the body, shape of the body, structure of various fins etc. Caudal fin sample of each fish were cut and placed in 2 ml vials containing 75% ethanol and voucher fish specimens immediately fixed in 5% formalin, the fin samples were kept in at -20^0 C until DNA extraction.

DNA Extraction and PCR Amplifications

Isolated genomic DNA was precipitated with 2-2.5 volume of chilled ethanol. The DNA pellets was washed twice with 70 % ethanol, air dried and resuspended in 1X TE (10mM Tris-HCl, pH 8.0 and 1 mM ethylene diaminetetraacetic acid disodium salt) buffer and kept at -20^{0}C till further use. Contaminating RNA was removed by digested with RNAase A (60 min at 37^{o}C). DNA quality was checked by 0.8 % agarose gel electrophoresis and the DNA concentration was estimated with the help of UV-VIS spectro-photometer (Thermo Scientific, England). Amplification of each DNA samples was performed in a 50µl reaction mixture containing 50 ng template DNA, 10 x PCR-buffer (100 mM Tris, pH 9.0, 500 mM KCl, 15 mM $MgCl_2$, 0.1 % Gelatin), 2.5 mM of each dNTPs (Genei, India), 5 pmol of each primer (Ocimum Biosolutions, India) and 0.5U Taq DNA Polymerase (Genei, India). The thermal cycler (Eppendorf, Mastercycler Gradient) profile used to amplify 16S rRNA consisted of an initial denaturation of 95^{o}C for 5 min; followed by 30 cycles of 95^{o}C for 20 sec, 48^{o}C for 20 sec, 72^{o}C for 20 sec and a final extension at 72^{o}C for 7 min. PCR products were checked in 1.2% agarose gel in 1X TBE (Tris-HCl, boric acid, EDTA, pH 8.0) buffer and visualized with ethidium bromide (Sambrook *et al*., 1989) under UV-Gel-Documentation system (Alpha Imager 3400, Alpha Innotech Corporation, USA). Molecular weights were determined using 100 bp DNA marker (Fermentas, Canada) (Fig. 2 and 3).

Table1 Species, drainages, collection sites, and GenBank Accession Nos. of specimens for this study.

Species	No. of Specimen	Collection site of Kumaun with Coordination location	Collection site Garhwal with Coordination location	Genbank accession numbers
S richardsonii	60	Gauri river Champawat 29° 0'' N, 80°, 6'0''E	Alaknanda River Srinagar 30°, 13', 11"N 78°, 46', 47"E	JX 204403 to JX 204411
T. putitora	60	Kosi River, Ramnagar 29°, 24', 56"N 79°,08',01"E	Alaknanda River Srinagar 30°, 13', 11"N 78°, 46', 47"E	JX 204393 to JX 204402
G. gotyla	60	Gola River, Ranibagh 29°, 18', 02"N 79°, 32', 02''E	Alaknanda River Srinagar 30°, 13', 11"N 78°, 46', 47"E	JX 204419 to JX 204426
B. bendelisis	60	Gola River, Ranibagh 29°, 18', 02"N 79°, 32', 02''E	Alaknanda River Srinagar 30°, 13', 11"N 78°, 46', 47"E	JX 204413 to JX 204418

Fig. 1 Map showing the different sampling sites in the Uttarakhand region.

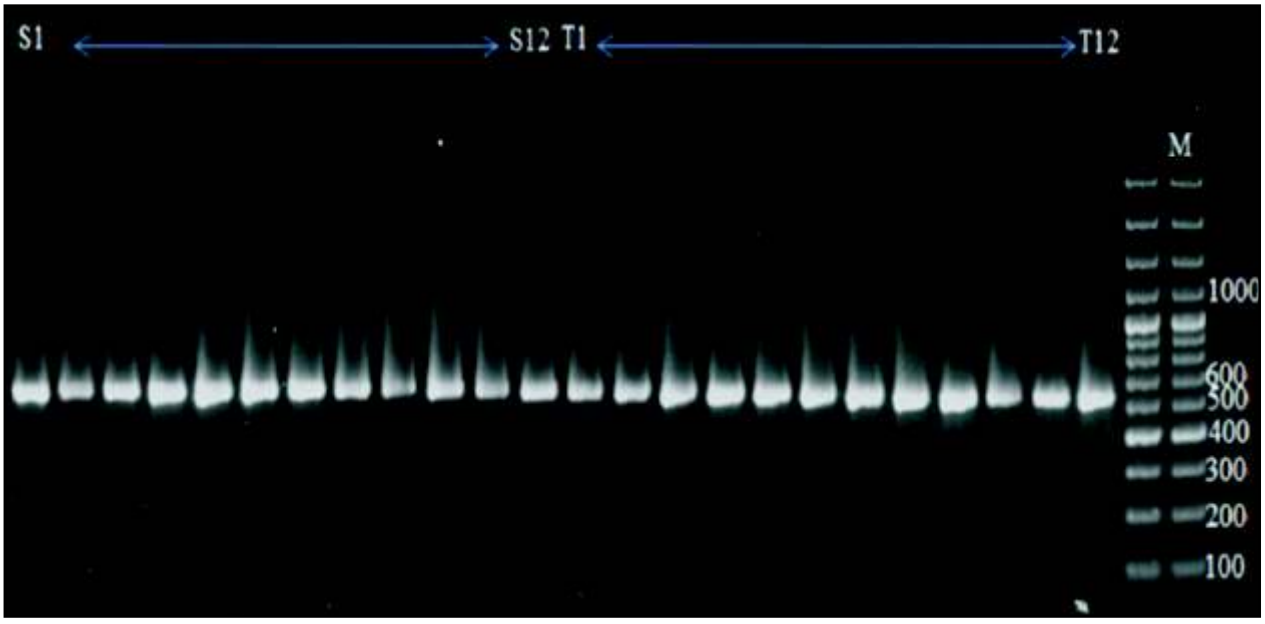

Fig. 2 16S rRNA gene amplification in *S. richardsonii* (S1-S12), *T. putitora* (T1-T12) for Kumaun and Garhwal region. M is 100 bp ladder.

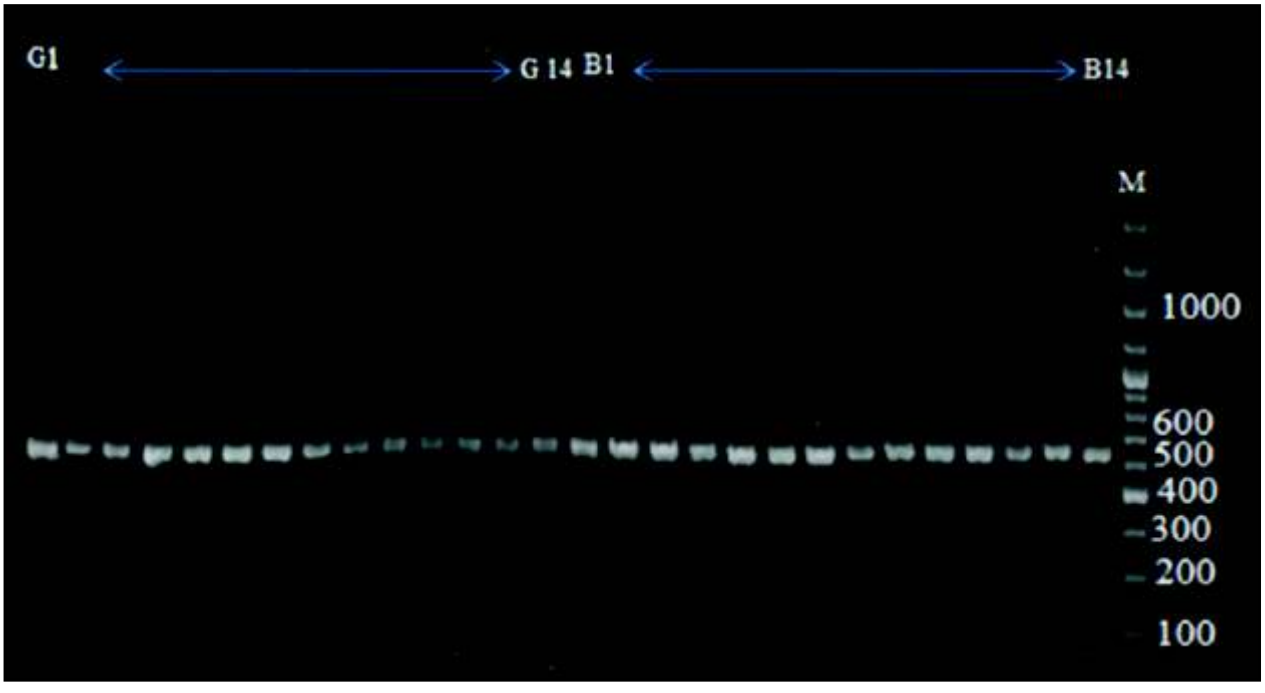

Fig. 3 16S rRNA gene amplification in *G. gotyla* (G1-G12), *B. bendelisis* (B1-B12) for Kumaun and Garhwal region. M is 100 bp ladder.

Table 2 Pairwise genetic distance for 16S rRNA gene sequences of four species of coldwater fishes from Kumaun and Garhwal region.

	G. gotyla (g)	*B. bendelisis* (g)	*T. putitora* (g)	*S. richardsonii* (g)
G. gotyla (g)	-----	-----	-----	-----
B. bendelisis (g)	1.778	-----	-----	-----
T. putitora (g)	0.348	1.936	-----	-----
S. richardsonii (g)	0.325	1.424	0.141	-----

Table 3 Pairwise genetic distance for 16S rRNA gene sequences of four species of coldwater fishes from Kumaun region.

	G. gotyla (k)	*B. bendelisis* (k)	*T. putitora* (k)	*S. richardsonii* (k)
G. gotyla (k)	-----	-----	-----	-----
B. bendelisis (k)	3.049	-----	-----	-----
T. putitora (k)	0.413	0.978	-----	-----
S. richardsonii (k)	0.562	0.774	0.126	-----

Table 4 Pairwise genetic distance for 16S rRNA gene sequences of four species of coldwater fishes from Kumaun and Garhwal region.

	G. g(g)	*G. g*(k)	*B. b*(g)	*B. b*(k)	*T. p* (g)	*T. p* (k)	*S. r* (g)	*S. r* (k)
G. g(g)	-----	-----	-----	-----	-----	-----	-----	-----
G. g(k)	3.426	-----	-----	-----	-----	-----	-----	-----
B. b(g)	6.644	20.622	-----	-----	-----	-----	-----	-----
B. b(k)	6.644	20.622	0.000	-----	-----	-----	-----	-----
T. p (g)	1.698	2.637	6.663	6.663	-----	-----	-----	-----
T. p (k)	1.698	2.637	6.663	6.663	0.000	-----	-----	-----
S. r (g)	1.695	3.617	5.255	5.255	0.177	0.177	-----	-----
S. r (k)	1.694	3.617	5.251	5.251	0.177	0.177	0.001	-----

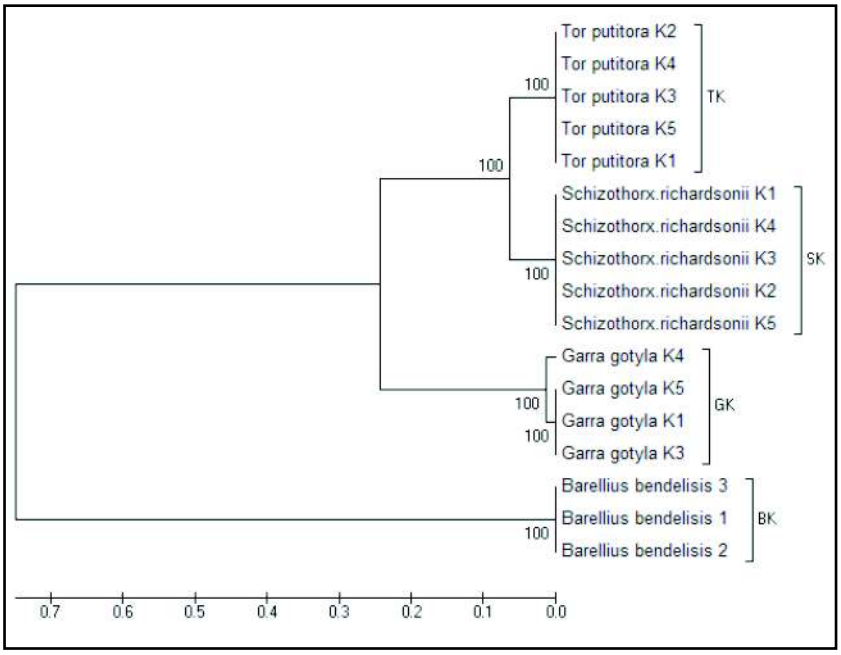

Fig. 4 Phylogenetic tree (UPGMA tree) based on 550 bp mitochondrial *16S rRNA* DNA sequences for four coldwater fishes (*S. richardsonii, T. putitora, B. bendelisis and G. gotyla*) from Kumaun region.

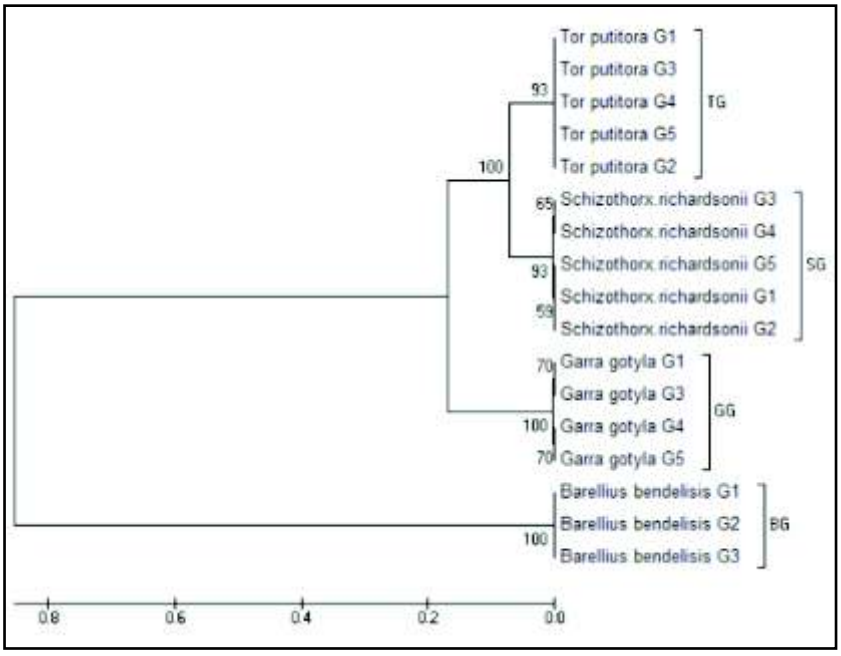

Fig. 5 Phylogenetic tree (UPGMA tree) based on 550 bp mitochondrial *16S rRNA* DNA sequences for four coldwater fishes (*S. richardsonii, T. putitora, B. bendelisis and G. gotyla*) from Garhwal region.

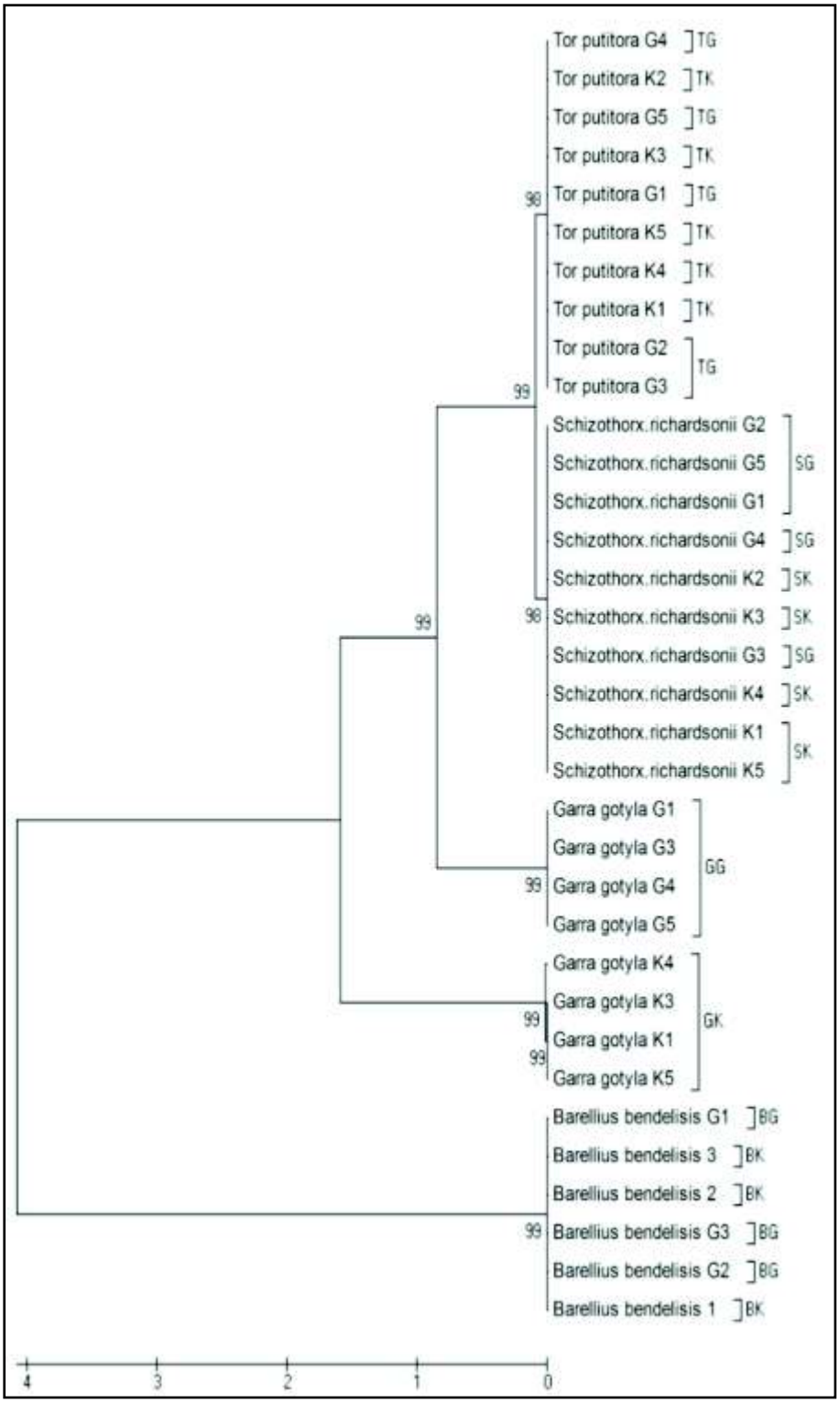

Fig. 6 Phylogenetic tree (UPGMA tree) based on 550 bp mitochondrial *16S rRNA* DNA sequences for four coldwater fishes (*S. richardsonii, T. putitora, B. bendelisis and G. gotyla*) from Kumaun and Garhwal region.

The amplicons were purified before sequencing with Qiaquick columns (Qiagen, USA) as per manufacturer's instructions. Sequencing was performed in ABI Prism 3100 automated sequencer (Applied Biosystems, USA) using Bigdye terminator with same primers used for amplification of the target gene.

Sequence Data analysis

The CHROMAS (Version 1.45) program was used to display the fluorescence based DNA sequencing analysis. The multiple sequence alignments were done using the CLUSTAL X program version 1.81 (Thompson *et al*., 1997). Numbers of invariable, variable, singleton variable, parsimoniously informative sites and number of haplotypes were calculated using software DNAsp version 4.5 (Rozas *et al*., 2003). Sequence divergence within and between the species were calculated using DNA STAR. The MEGA version 4.0 software (Tamura *et al*., 2007) was used to construct the phylogenetic relationship among four species of coldwater from two region of Uttarakhand based on UPMGA, maximum-parsimony (MP) *method*. Bootstraps support was calculated using 1000 replication.

Results and Discussion

The mtDNA 16S rRNA gene of 550 bp length was successfully amplified and sequenced for 34 individuals in this study for both region. The sequences obtained were aligned and compared with other GeneBank 16S rRNA sequences. All the sequences representing 16S rRNA gene were submitted to the GenBank with accession numbers given in Table 1.

In Garhwal region alignment data showed that 100 sites out of 550 were variable. Among 93 variable sites were Parsimony information polymorphic sites. Rate of transition (65.3%) were higher than transversion (34.6%) for Garhwal region. Empirical base frequency among species Garhwal were A=31.6%, C= 23.9%, G= 22.7%, and T=21.8%. High AT content was observed in all sequences. The overall haplotype diversity (h=0.860) and Nucleotide diversity (π=0.06554) were high. 6 haplotypes were found in Garhwal region. Among them 1, 2, 2, 1 haplotype were detected in the *B. bendelisis* (G), *G. gotyla* (G), *S. richardsonii* (G), and *T. putitora* (G) respectively. Haplotype diversity value (h) was 0.0 for *B. bendelisis* (G), 0.666 for *G. gotyla* (G), 0.0 for *S. richardsonii* (G) and 0.0 for *T. putitora* (G). Nucleotide diversity (π) was 0.0, 0.00122, 0.00109, and 0.0 for the *B. bendelisis* (G), *G. gotyla* (G), *S. richardsonii* (G), and *T. putitora* (G) respectively. The nucleotide diversity was lowest between the *T. putitora* (G) and *S. richardsonii* (G) (π=0.02311) and highest between the *B. bendelisis* (G) and *G. gotyla* (G) (π=0.07951).

In Kumaun region 94 sites were variable out of 550. Among these 94 variable sites 85 sites were Parsimony information polymorphic while 9 were singleton variable sites. Rate of transition (64.4%) were higher than transversion (35.5%) for Kumaun region. 6 haplotypes were observed for Kumaun region. Among them 1, 3, 1, 1 haplotype were detected in the *B. bendelisis* (K), *G. gotyla* (K), *S. richardsonii* (K), and *T. putitora* (K) respectively. Empirical base frequency were A=31.4%, C= 23.7%, G= 22.8%, and T=22.1%. Overall haplotype diversity was 0.824 and Nucleotide diversity was 0.06096. Haplotype diversity value (h) was 0.0 for *B. bendelisis* (K), 0.833 for *G. gotyla* (K), 0.000 for *S. richardsonii* (K) and 0.0 for *T. putitora* (K). Nucleotide diversity (π) was 0.0, 0.01865, 0.0, and 0.0 for the *B. bendelisis* (K), *G. gotyla* (K), *S. richardsonii* (K), and *T. putitora* (K) respectively. The nucleotide diversity was lowest between the *T. putitora* (K) and *S. richardsonii* (K) (π=0.02129) and highest between the *B. bendelisis* (K) and *G. gotyla* (K) (π=0.07584).

The overall haplotype diversity (h=0.860), (h=0.824) and nucleotide diversity (π=0.06554), (π=0.06096) were found for Garhwal and Kumaun region respectively. The

nucleotide diversity was lowest between the *T. putitora* and *S. richardsonii* for both regions. Pairwise genetic distance data showed close relationship between *Schizothorax richardsonii* and *Tor putitora* while high divergence were observed for *B. bendelisis* for both region. The UPGMA tree of 16S rRNA gene sequences of the four species showed that *Schizothorax richardsonii* and *Tor putitora* formed a monophyletic group and then they constitute one clad with *Garra gotayla* while *Barilius bendelisis* formed a different cluster.

This study may be useful in determining evolutionary characteristics of these four species and compare the genetic diversity in different geographically isolated natural populations. The present study mtDNA (16S rRNA) gene of four cyprinidae fishes of Kumaun and Garhwal region of Uttarakhand revealed that Garhwal region having higher rate of genetic diversity than Kumaun. The marker information available in this study would be useful for future Genetic improvement program of these species as these species are valuable for both food and ornamental purpose. This would be important for breeding programmes because maintaining genetic diversity within breeding populations is necessary to maximize hybrid vigour, because this species is not a migratory species and possibly suffers from inbreeding depression.

The present study of mtDNA (16Sr RNA) gene of four cyprinidae fishes of Kumaun and Garhwal region of Uttarakhand revealed that Garhwal region species having higher rate of Genetic diversity than Kumaun. The phylogenetic tree of these four cyprinidae fishes showed two different clusters consisting of *T. putitora* and *S. richardsonii* in one cluster and the other consisting *B. bendelisis* and *G. gotyla*. The present study suggested that mtDNA is valuable molecular tools for species characterization and phylogenetic relationship between the species. The marker information available in this study would be useful for future Genetic improvement program of these species as these species are valuable for both food and ornamental purpose.

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