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Phylogenetic analysis of fishes of the subfamily Schizothoracinae (Teleostei: Cyprinidae) from Indian Himalayas using *cytochrome b* gene

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ABSTRACT

Molecular phylogeny of two genera containing five species fish of the subfamily Schizothoracinae distributed in the north and north-east Himalayas was investigated based on the partial 307 bp *cytochrome b* gene sequences. The sequence analysis data showed that 48 sites out of 307 (16%) were variable without any insertion or deletion. Rate of transition (4.8%) was higher than transversion (0.65%). A total of 12 haplotypes (h) were identified. No haplotype was shared by the five species. The nucleotide diversity (π) ranged from 0.00561 to 0.06073 with least between *Schizothorax richardsonii* and *Schizothorax progastus*. The phylogenetic tree, constructed by neighbour-joining, minimum evolution and maximum parsimony methods revealed similar results suggesting that *S. richardsonii* and *S. progastus* were more closely related to each other than the other species in the subfamily, which was also confirmed by the genetic distance data. The results indicate that *cytochrome b* gene is useful in analysing genetic variation as well as in unravelling phylogenetic relationship in the subfamily Schizothoracinae.

Keywords: *Cytochrome b,* Genetic distance, Genetic diversity, Mitochondrial DNA, Phylogenetic relationship, Schizothoracinae

Introduction

Snow trouts belong to the subfamily Schizothoracinae (Family: Cyprinidae) which consists of 15 genera and over hundred species distributed all over the world (Mirza, 1991). The Indian snow trouts fall under seven genera, majority of which constitute an important part of coldwater fishery in the Himalayan region (Tilak, 1987). These are economically important fishes which inhabit fast flowing snow fed streams and lakes. Due to overexploitation, many of the species were listed as 'endangered' by the National Environmental Protection Agency and Endangered Species Scientific Commission (Yue and Chen, 1998). Classification of snow trouts at species level is generally based on classical, morphological and osteological methods. However, accurate identification of Schizothoracine fishes using morphological characters (*e.g.*, dorsal and caudal fin rays count, length and weight, structure of scales, structure of jaws and lips *etc*.) is difficult due to intraspecific morphological variability and therefore sometimes causes error in proper identification of closely related species. Although, all the species of snow trouts are classified under the subfamily Schizothoracinae, ambiguity remains under the genus level. The taxonomic positions of these species vary according to different sources leading to improper identification of the species.

There are several studies on classification of fishes under Schizothoracinae (Wu, 1984; Chen, 1998; Wu and Tan, 1991). Phylogenetic relationships among genera and species under Schizothoracinae have been investigated based on morphological characters, RAPD analysis (Chen and Chen, 2000; 2001) and mitochondrial *cytochrome b* gene sequence analysis (Dekui *et al*., 2004; Qi *et al.,* 2005). Mitochondrial DNA (mtDNA) has been one of the most widely used molecular markers for studying intraspecific and interspecies variation in animals because of its simple genomic structure, high nucleotide substitution rate, lack of recombination and maternal inheritance (Avise, 1986; Billington and Hebert, 1988). The availability of mtDNA data has provided new perspectives on taxonomically debatable taxa and confusing questions of phylogeny (Groves and Shields, 1996). Among many mitochondrial genes, the mitochondrial *cytochrome b* gene has been widely used to study genetic variation (McVeigh and Davidson, 1991), phylogenetic relationships (Groves and Shields, 1996; Gilles *et al*., 1998; Xiao *et al*., 2001; Perdices *et al*., 2004; Bajpai and Tewari, 2010; Kumar *et al*., 2011), biogeographical patterns (Gilles *et al*., 2001; Xiao *et al*., 2001; Durand *et al*., 2002) and taxonomy (Burridge, 1999; Xiao *et al*., 2001) in many fishes and higher nvertebrates. The rate of evolution of the *cytochrome b* gene is appropriate for investigating events that have occurred within the last 20 million years, such as the evolution of the Cyprinidae (Irwin *et al*., 1991). In the present study, we analysed the *cytochrome b* sequences of five species of

two genera of the subfamily Schizothoracinae, to infer the phylogenetic relationship among these species.

Materials and methods

A total of 20 individuals of 5 species belonging to two genera *viz*., *Schizothorax* (*S. richardsonii, S. progastus, S. esocinus* and *S. plagiostomus*) and *Schizopyge* (*S. niger*) were collected from north and north-east Himalayas during October 2009 to March 2010. Fin tissues were preserved in absolute ethanol in the field. The details of collection are given in Table 1.

Analysis of nucleotide composition, overall transition: transversion rate (including *cytochrome b* sequences of *S, richardsonii*, *S. progastus*, *S. esocinus*, *S. plagiostomus* and *S, niger*) and pairwise genetic distance with Kimura 2 parameter method (Kimura, 1980) of sequences were estimated by MEGA 4 (Tamura *et al*., 2007). Numbers of invariable, variable, singleton variable and parsimoniously informative sites of *cytochrome b* sequences were calculated using DnaSP *vers*. 4.50.2 (Rozas *et al*., 2003). The haplotype number, haplotype diversity (h) and nucleotide diversity (π) were also performed using DnaSP software.

Table 1. Species, drainages, collection sites, number of haplotypes and GenBank Accession nos. of specimens used in the study

Species	No. of specimens	Drainages, collection site	No. of haplotypes	Genbank Accession no.
Schizothorax richardsonii	4	Bhagirathi, Uttarkashi	3	JN600500- JN600503
S. progastus	5	Bhagirathi, Uttarkashi	3	JN600504- JN600508
S. esocinus	4	Indus, Leh	2	JN600512- JN600515
S. plagiostomus	4	Upper Siang River, Arunachal Pradesh	1	JN600516-JN600519
Schizopyge niger	3	Jhelum, Jammu & Kashmir	3	JN600509- JN600511

Total genomic DNA was isolated from 50 mg fin tissue samples preserved in absolute ethanol using phenol chloroform method (Sambrook *et al*., 1989). Partial sequence of the *cytochrome b* gene was amplified by PCR (Eppendorf, Mastercycler gradient) using universal Primers CytBF: 5'-AAAAAGCTTCCATCCAACATCTCAGC ATGATGAAA-3' and CytBR: 5'-AAACTGCAGC CCCTCAGAATGATATTTGTCCTCA-3' (Kocher *et al.,* 1989). Amplification was done in 50 µl volume containing 5 µl of 10x PCR buffer (100 mM Tris, pH 9.0, 500 mM KCl, 15 mM $MgCl_2$, 0.1% Gelatin) (B-Genei, India), and 1 unit of Taq DNA polymerase (B-Genei, India), 200 µM of each dNTPs (dATPs, dCTPs, dGTP, dTTPs) (B-Genei, India), 25 pmol of each primer and 50 ng of genomic DNA. The thermal profile used to amplify *cytochrome b* consisted of an initial denaturation of 95 ^{o}C for 5 min; followed by 34 cycles of 94 ^{o}C for 30 sec, 54 ^{o}C for 30 sec, 72 ^{o}C for 1 min and a final extension at 72 ^{o}C for 7 min . PCR products were stored at 4 ^{o}C. For each sample, 3 µl of PCR product was electrophoresed on 1.2 % agarose gel followed by ethidium bromide staining, and visualized under UV illumination in the Gel-Doc system (Alpha Imager 3400, Alpha Innotech Corporation, USA). Molecular weights were determined using 100 bp DNA markers (Fermentas, Canada) and the PCR products were sequenced (Chromas Biotech, Bangalore). A total of 307 base pairs of the *cytochrome b* gene fragment were successfully sequenced for 20 individuals representing five species of subfamily Schizothoracinae. The sequence data were aligned using BioEdit version 5.0.9 (Hall, 1999). All sequences representing *cytochrome b* gene were submitted to the GenBank (Accs. No: JN600500-JN600519).

Phylogenetic relationships among five species of two genera were constructed using nucleotide sequences of *cytochrome b* gene estimated by neighbour-joining, maximum-parsimony and minimum evolution. Phylogenetic analysis was conducted using MEGA 4. Bootstrap support was calculated from 1000 replications.

Results and discussion

The alignment of *cytochrome b* gene sequences showed the presence of a common conserved region in all the five species indicating that these species belong to the same subfamily. This was also confirmed on the basis of homology with previously published sequences from other fish species through NCBI Genbank. The nucleotide sequence alignment is shown in Fig. 1. The alignment data showed that 48 sites (16%) out of 307 bp were variable without any insertion or deletion. Among these 48 variable sites, 44 sites (92%) were Parsimony information polymorphic while 4 sites (8%) were singleton variable sites. Rate of transition (4.8%) was higher than transversion (0.65%). A high transition bias is well known in vertebrate mtDNA (Meyer, 1993). The majority of variable and phylogenetically informative sites of *cytochrome b* were found on first codon position and the rate of transition/ transversion (R=Si/Sv) was also higher in first codon position (R= 11.0). It indicated several million years of evolution involved in the genetic evolution of different cyprinid species (Springer and Douzery, 1996; Wang *et al*., 2002; Sivaraman *et al.,* 2009).

Among 20 individuals of 5 species belonging to two genera *viz*., *Schizothorax* and *Schizopyge,* 12 haplotypes

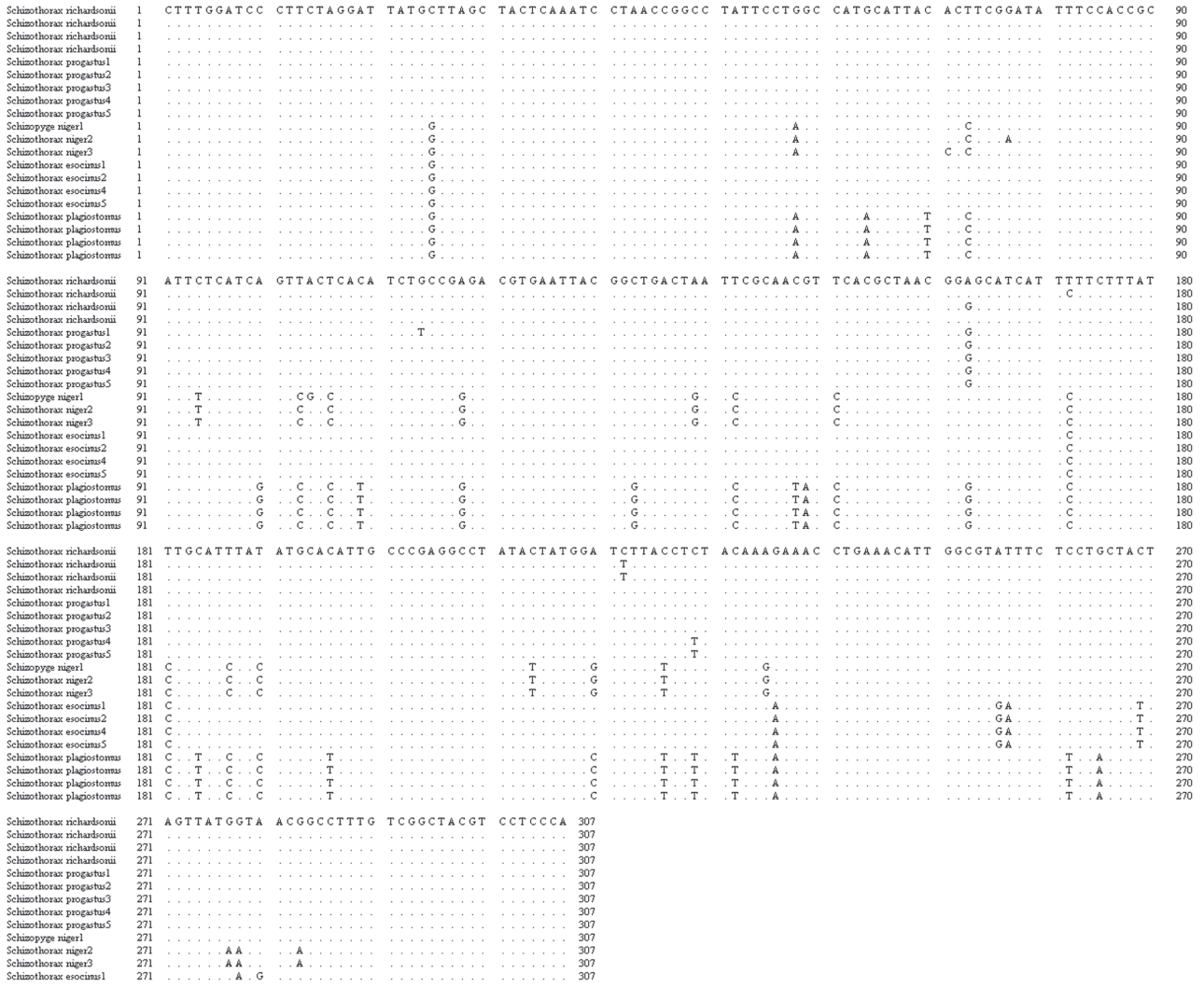

Fig. 1. Aligned sequences for the 307-nucleotide segment of *cytochrome b* gene in five species of subfamily Schizothoracinae (variable positions are indicated with nucleotide).

were observed. Among them 3, 3, 2, 1, 3 haplotype were detected in *S. richardsonii, S. progastus, S. esocinus, S. plagiostomus* and *S. niger* respectively and no haplotype was shared by the five species. Haplotype diversity (h) ranged from 0.000 to 1.000. The haplotype diversity was lowest in *S. plagiostomus* (0.000) and highest in *S. niger* (1.000). Haplotype diversity value (h) was 0.0833 for *S. richardsonii*, 0.8000 for *S. progastus* and 0.667 for *S. esocinus.* Nucleotide diversity (π) was 0.005429, 0.003257, 0.002172, 0.000000 and 0.013029 for *S. richardsonii, S. progastus, S. esocinus, S. plagiostomus* and *S. niger* respectively. The nucleotide diversity was lowest between *S. richardsonii* and *S. progastus* (π=0.00561) and highest between *S. richadsonii* and *S. plagiostomus* (π=0.06073).

The nucleotide sequences of *cytochrome b* gene were aligned in order to determine the phylogenetic relationships among the five species. The phylogenetic tree, generated using three methods (Neighbour-joining, Minimum evolution, Maximum parsimony) was similar. The Neighbour-joining (NJ) tree (Fig. 2) of *cytochrome b* gene sequences of the five species showed that *S. richardsonii* and *S. progastus* formed a monophyletic group (a result

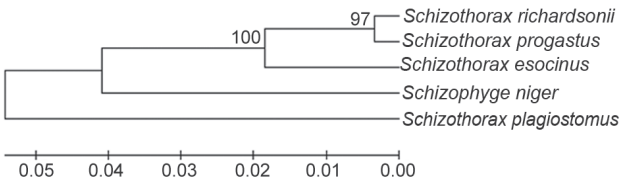

Fig. 2. Phylogenetic tree (Neighbour-joining tree) based on 307 bp mitochondrial *cytochrome b* DNA sequences for five species of Schizothoracinae

strongly supported by high bootstrap value of 97%) and then constitute one clade with *S. ecosinus*; further they constitute another clade with *S. niger* while *S. plagiostomous* formed a different cluster. It was found that the species belonging to the northern Himalayas grouped together while species from north-eastern Himalayas remained separate.

Pairwise genetic distance between the species is presented in Table 2. The mean genetic distance among five species of Schizothoracinae ranged from 0.006-0.116. The lowest pairwise genetic distance was observed between *S. richardsonii* and *S. progastus* while the maximum divergences were observed between *S. richardsonii* and *S. plagiostomous*. This revealed a closer phylogenetic relationship between *S. richardsonii* and *S. progastus* than other species under Schizothoracinae.

Table 2. Pairwise genetic distance (nucleotide Kimura 2 parameter) for *cytochrome b* gene sequences of five species of *Schizothoracinae* fishes

Species	*S. richardsonii*	*S. progastus*	*S. esocinus*	*S. plagiostomus*	*Schizopyge niger*
S. richardsonii	-	-	-	-	-
S. progastus	0.006	-	-	-	-
S. esocinus	0.034	0.037	-	-	-
S. plagiostomus	0.116	0.110	0.113	-	-
Schizopyge niger	0.076	0.079	0.081	0.087	-

The nucleotide diversity, genetic distance and phylogenetic relationship data showed distinct association with similar geographical distribution. The species that are distributed in the same drainage system (*S. richardsonii* and *S. progastus*) as well as the species distributed in the northern Himalayas (*S. richardsonii, S. progastus* and *S. esocinus*) exhibited closer phylogenetic relationship. Dekui *et al*. (2004) also observed that there was close relationship among the species that were distributed in the same drainage system. The present study did not completely resolve the phylogenetic relationships among the five species of subfamily Schizothoracinae, but study of *cytochrome b* sequences in these five species provides useful insights into the taxonomic status of these fishes and sets the stage for future investigations dealing with phylogenetic, taxonomic and conservation issues in this important group. Further studies are required on the phylogenetic relationships of these fishes based on more mtDNA genes, as the recent developmentsin molecular techniques based on these genes are very much useful for establishing taxonomical and phylogenetic relationships among different species.

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