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DNA bar-coding of snow trout (*Schizothorax plagiostomus*) from Neelum and Jhelum rivers of Azad Jammu and Kashmir, Pakistan

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Abstract

The mitochondrial *Cytochrome oxidase 1 (CO1)* gene is used as a standardized, authenticated and reliable genetic marker for a global species-level bio-identification system. The present study was conducted to examine *COI* gene for *Schizothorax plagiostomus*, from Azad Jammu and Kashmir, to determine whether barcoding can help accurate species identification in fishes. The overall base composition of *Schizothorax* species were A, 25.7%; C, 28.0%; T, 28.1% and G 18.1%, A+T content 53.7% and G+C content 46.1%. The Ts/Tv bias (R) was 1.70 and 7 haplotypes were observed. The average value of haplotype diversity was Hd: 0.944±0.07 and the average value of nucleotide diversity (Pi) was 0.0032±0.001. Short fragment (721) was amplified using PCR and sequenced from nine fish samples collected from river Neelum and Jhelum, and identification of species were done in Genbank and BOLD. Two variable characters were parsimony uninformative, and 4 variable characters were parsimony informative. The overall mean intra-specific distance was 0.03±0.001 and mean inter-specific nucleotide sequence divergence was 0.404%. We emphasized that, DNA barcoding is an accurate, reliable and has the great potential for identification of freshwater fish species. The ability to assign species with high precision from DNA samples of different quality and origin has major utility in several fields, from fisheries and conservation programs to control of fish products authenticity and its sustainability.

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Introduction

Accurate and unambiguous identification of fish and fish products, from eggs to mature, is important in many areas. This accurate identification would improve the fish conservation and ecosystem research and contribute for long term fish management and its sustainability. For the accurate and unambiguous fish species identification, a large variety of DNA and protein based methods has been used (Tautz *et al*., 2002; Perez-Martin and Sotelo, 2003; Tautz *et al*., 2003). A short fragment of *CO1* gene (655 bp) has been used for identification of species from 30 years, but in different laboratories, different DNA sequences have been also used for species identification (Hebert *et al*., 2003). DNA barcoding is therefore, widely used for invertebrate (Costa *et al*., 2007; Mikkelsen *et al*., 2007) and vertebrate (Hebert *et al*., 2004; Hajibabaei *et al*., 2006) species identification. The boundaries between inter and intra specific morphological similarities and variation could be unclear based on morphological taxonomy.

The lack of universal characters through taxa, inapplicability of identification keys, and requisite of high levels of expertise among taxonomists are the additional drawbacks of morphology-based taxonomy. The approach of molecular taxonomy i.e. DNA barcoding resolves these issues and supported the taxonomists in different species identification (Asgharian *et al*., 2011). The mitochondrial DNA gene *Cytochrome C Oxidase I (COI)* is used as a suitable genetic marker for a global bio-identification system and would be sufficient to differentiate all, or at least the vast majority of animal species (Elmeer *et al*., 2012).

DNA barcoding was proposed as a resolution to the problem of species identification caused by a divergence between the species on our planet and taxonomists (Hebert *et al*., 2003). It is based on the sequencing of a short standardized fragment of DNA of target specimen and comparing this sequence to the Genbank from known species. This standard barcode sequence of mitochondrial DNA gene *Cytochrome C Oxidase 1 (CO1)* is 655 bp for animal species.

It has been discussed that the DNA bar-coding has many benefits for species identification and discovery (Hebert and Gregory, 2005) while, this concept remains to be fiercely disputed (Wheeler, 2005). Along with the identification of species, the barcode database construction could depict the novel DNA barcodes that may specify temporary new species (Hebert *et al*., 2004).

The family Cyprinidae containing the genus *Schizothorax*, are locally known as snow trout, containing 20 genera and more than 150 species throughout the world (Mirza, 1991). These fish species are dispersed in the cold waters from Jammu and Kashmir (Sunder and Bhagat, 1979) to Eastern Himalayas and Assam through Sikkim and Bhutan at an altitude of 1180-3000m (Jhingran, 1982). So far, in Himalayan and sub Himalayan region, almost 30 snow trout species have been reported.

Their essential biological features, such as slow growth to maturity, and short growth period are the specific restrictions for their resources and population increase (Goel *et al*., 2011). The genus *Schizothorax* containing the remarkably similar morphology. It is very difficult to distinguish the genus *Schizothorax* based on the external morphological characters.

The taxonomy of genus *Schizothorax* is controversial (Mirza, 1991; Talwar and Jhingran, 1991; Wu and Wu, 1992; Chen and Cao, 2000), and the phylogenetic relationships and biogeography of this group are unclear. It is very difficult to distinguish whether these are different species, different phenotypes of single species or an intermediate situation among these extremes (Raina and Petr, 1999).

A DNA bar-coding approach could be helpful for the fish identification. Due to this reasons, the *COI* barcode sequence was used for the snow trout identification. In the present study, the *COI* gene was used to determine whether DNA bar-coding can achieve unambiguous species recognition in fishes.

The current study would be useful for conservation of these species in the state of Azad Jammu and Kashmir.

Materials and methods

Sample Collection

The fish samples were randomly collected by using different fishing gears (cast nets and gill nets) from River Jhelum and Neelum, Muzaffarabad (Fig 1). The labeled samples were packed in polyethylene bags and brought back to the Laboratory of Molecular Genetics, University of Azad Jammu and Kashmir for further research analysis. After analysis, voucher specimens were preserved in 70% ethanol and stored in the Zoological museum hall of the University of Azad Jammu and Kashmir, Pakistan. Taking into account potential problems with the identification of species (Talwar and Jhingran, 1991; Mirza, 1991), all the *Schizothorax* samples were identified following Mirza (1991), Jhingran (1982) classification.

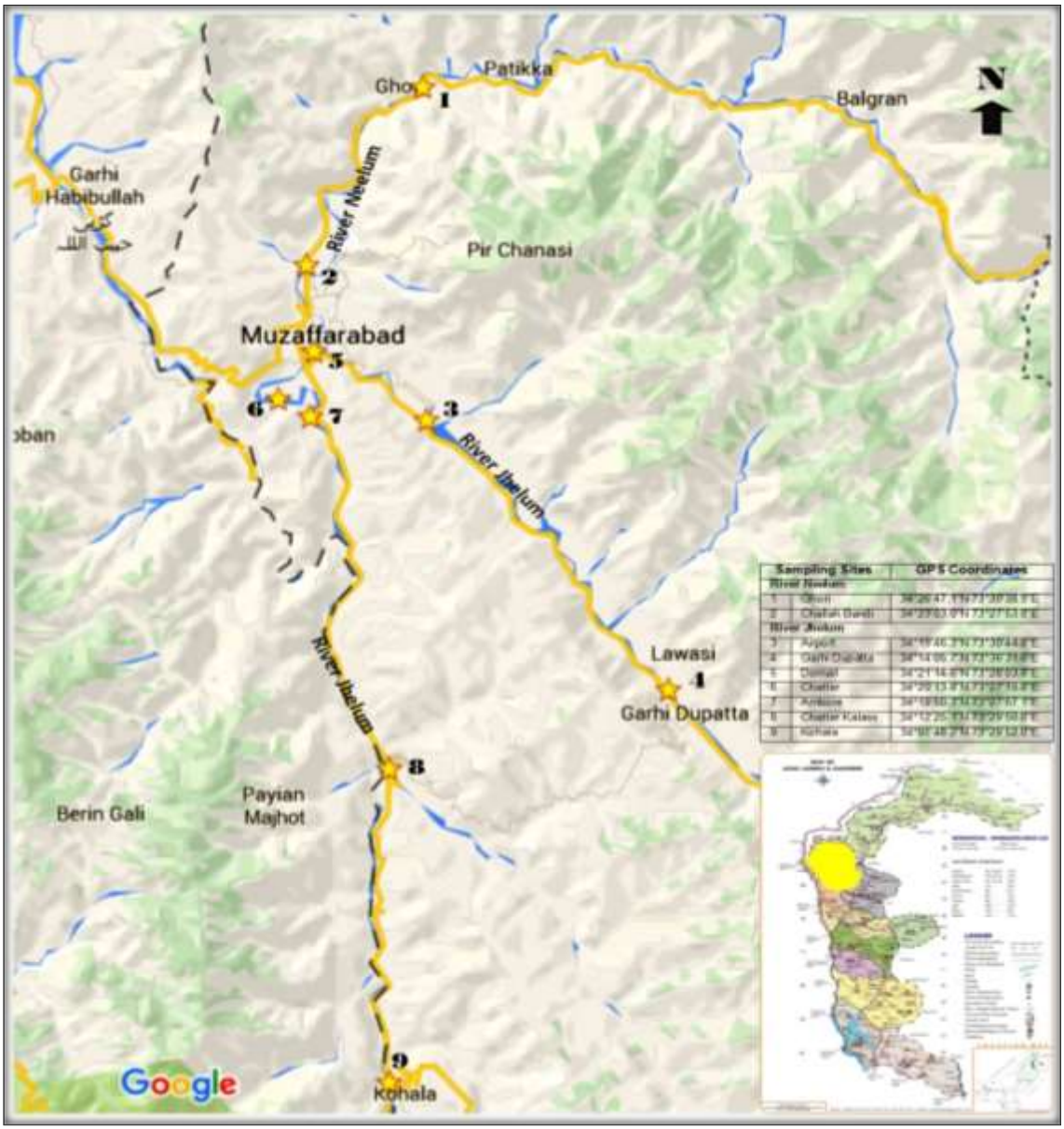


Fig. 1. Sampling sites for Schizothorax fishes from river Neelum and Jhelum, Azad Kashmir. The numbers refer to the catchments listed in Table 1.

DNA Extraction

Total DNA was isolated by standard phenol-chloroform extraction by Sambrook *et al.* (1989). About 0.1 g of tissue was sterilized with ethanol and washed three times with distilled water and stored at 4°C. The extracted DNA was allowed to run on 1% agarose gel and visualized under UV transilluminator and results were recorded by using a gel documentation system. The quantity was measured by spectrophotometer at 260/280 nm wavelength.

Primer Designing for Sequencing

Sequencing primers of *CO1* gene of *Schizothorax* samples were designed by using program Primer-3. A short fragment (721) of *COI* gene was amplified from mt DNA using one set of primer: CO1F (5´-AAC CAC AAA GAC ATT GGT AC-3´), CO1R (5´-GGT GTC CAA AGA ATC AGA AT-3).

DNA Amplification and Sequencing

Mitochondrial DNA was amplified by using PCR (Polymerase Chain Reaction). PCR was carried out in 25µl (standard amount) reaction volumes in each tube (14µl PCR water, 3µl template DNA, 2.5µl Taq buffer, 0.5µl dNTPs, primers 1µl of each (forward and reverse primers), 2.5µl magnesium chloride, and 0.5 µl of enzyme (Taq polymerase). For thorough mixing the reaction mixture kept for 30 s on 8,000 rpm for centrifugation. Thermal cycling comprised 95°C for 3 min, followed by 39 cycles of 95°C for 30 s, annealing at 53°C for 30 s, and an extension temperature of 72°C for 1 min. This was followed by a final extension of 72°C for 10 min. Prior to cycle sequencing, PCR products were purified with Exo Sap-IT (Affymetrix purification kit). Nucleotide (nt) sequencing were carried out in an ABI Prism 3100 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) using gene specific forward and reverse primers for *Schizothorax* genes. One microliter cleaned PCR product was used for each 10 µL reaction.

Sequence and Phylogenetic Analysis of CO1 Gene

For the *CO1* genes, the bidirectional nucleotide (nt) sequencing were carried out in an ABI Prism 3100 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) by using gene specific forward and reverse primers.

The editing of sequences were carried out by using the Bio Edit program (http://www.mbio. ncsu. edu/Bio Edit/) for nucleotides and amino acids variations.

The unknown species were identified from DNA samples by using the BOLD search engine. Furthermore, identification of species were also done by using the Basic Local Alignment Search Tool (BLAST) of Genbank/NCBI. These *Schizothorax* sequences were aligned by using the ClustalW algorithm of Meg Align program, LASERGENE software package (DNAS tar, Inc., Madison, WI). Multiple sequence alignment were performed to determine the sequence divergence among *Schizothorax plagiostomus* of AJK. A phylogenetic tree was generated using neighbor-joining method and Kimura 2-parameter supported by 1,000 bootstrap replicates (Jiang *et al.*, 2014; Khan *et al.*, 2015) in the MegAlign program of the LASERGENE software (Saitou and Nei, 1987).

Result and discussion

Sequence and Phylogenetic Analysis of CO1 Barcode Region

Unluckily, the morphometric characteristics of genus *Schizothorax* have not evidenced to be completely accurate and authentic in resolving inter and intra-specific relationships (Raina and Petr, 1999; Kullander *et al.*, 1999; Talwar and Jhingran, 1991; Balkhi, 2005). The DNA barcode should allow reliable, fast, cost-effective and automatable species identification by users with no or little taxonomic experience (Hebert *et al.*, 2003; Hebert and Gregory, 2005). Nine *CO1* gene sequences of *S. plagiostomus* were used for species identification through Genbank/NCBI and BOLD. *CO1* gene of *Schizothorax* species was sequenced and aligned by using the Clustal W program. This alignment permitted the grouping of species into specific clusters. The use of *Cytochrome C Oxidase I*, a mitochondrial protein coding genes (the standard DNA barcode for animal species) are capable of differentiating the cold water *Schizothorax* species with high accuracy. The absence of stop codons and well defined peaks indicated that co-amplification of nuclear pseudo-genes did not occur (Zhang and Hewitt, 1996).

Blast Search

Five sequences of the same species were taken from public domain on the basis of percentage similarity with *Schizothorax plagiostomus*. The sequences were downloaded from Genbank (NCBI). These sequences are *S. plagiostomus* (KT184924.1, NC_023531.1, KP712118.1, KP712120.1 and KF928796.1).

Multiple alignments of *CO1* mtDNA gene resulted in a consensus length of 721 base pairs. Of the 800 sites (base pairs and gaps), 721 were constant, 2 variable characters were parsimony uninformative and 4 variable characters were parsimony informative.

The estimated Ts/Tv bias (R) was = 1.70. The number of nucleotides of *CO1* gene sequenced was globally G-deficient (18.1%), whereas (A, 25.7%; C, 28.0%; T, 28.1%). Such type of nucleotide composition pattern has been widely stated in many other fish species with the smaller variations (Khan *et al*., 2015). The A+T content 53.7% and G+C content 461% showing an obvious anti-G bias as appear commonly in teleost fishes (Tzeng *et al*., 1992; Zhu *et al*., 2012). All these sequences were submitted in Genbank with accession no, sex and collection locality are shown (Table 1).

Table 1. List of samples used in this study, including species name, code, sample locality and Genbank Accession numbers.

No	Species name	Code	Sex	Genbank accession no.	Collection locality
1	*S. plagiostomus*	SP-A	Male	KU317682	River Neelum, Ghori
2	*S. plagiostomus*	SP-AH	Female	KU317683	River Jhelum, Air Port
3	*S. plagiostomus*	SP-AP	Female	KU317685	River Jhelum, Kohala
4	*S. plagiostomus*	SP-AR	Female	KU317686	River Jhelum, Kohala
5	*S. plagiostomus*	SP-G	Female	KU317696	River Neelum, Chella Bandi
6	*S. plagiostomus*	SP-BD	Male	KU317691	River Jhelum, Chatter Kalass
7	*S. plagiostomus*	SP-BF	Male	KU317692	River Jhelum, Chatter Kalass
8	*S. plagiostomus*	SP-K	Female	KU317697	River Neelum, Chella Bandi
9	*S. plagiostomus*	SP-C	Female	KU317695	River Neelum, Ghori

The average evolutionary divergence of all the sequence pairs is 0.03±0.001 while, the sequence divergence between present *S. plagiostomus* and international S. *plagiostomus* is 0.404%. The possibility of intra-specific sequence divergence, possibly due to hybridization and ancestral polymorphisms (Hajibabaei *et al*., 2006). The lack of difference in the mitochondrial sequence data of some *Schizothorax* species may be explained in terms of introgressive hybridization, incomplete lineage sorting, rapid radiation in lineages and multiple hits (homoplasy) (Tsigenopoulos and Berredi, 2000; He and Chen, 2006; Qi *et al*., 2007). Silas (1960) reported that inter-specific hybridization in nature takes place to a greater extent among the Schizothoracinae, and the major factors responsible for this in the Kashmir valley are the great numbers of each species present, overlaps in breeding time, and spatial distributions.

Phylogenetic Analysis of COI Barcode Sequences

The main purpose of present study was to examine whether the *Cytochrome Oxidase Subunit I* barcode provided an adequate resolution to identify *S. plagiostomus*. The Neighbour-Joining, maximum likelihood and maximum parsimony methods of analysis generated almost identical relationships. The Neighbour-Joining (Fig 2) analysis are presented and exhibited that the *COI* barcode is a reliable tool for species identification (Hebert *et al*., 2003).

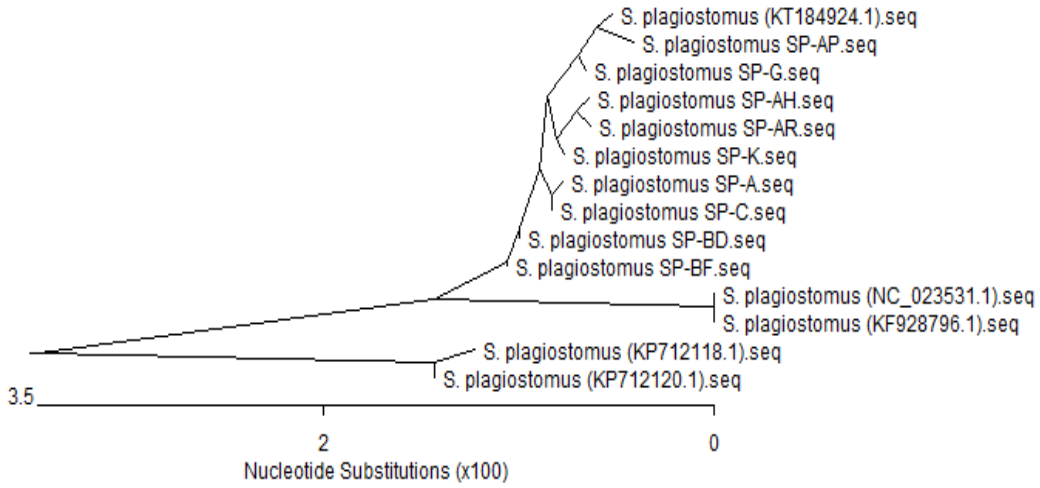
CO1 gene of *S. plagiostomus* was sequenced and aligned with other global sequences available in BOLD and Genbank database using the Clustal W program. This alignment allowed grouping of species into definite clusters. These nine *Schizothorax* samples were named as *S. plagiostomus* because they showed maximum sequence homology with the BOLD (99.32 to 99.86%) and NCBI (99.0%) reference sequence of *S. plagiostomus* (KT184924.1) of Northern Pakistan (Table 2).

But these sequences showed minimum homology (95.1 to 95.7%) and maximum divergence (4.5-5.1%) with the sequences of *S. plagiostomus* (KP712118.1 and KP712120.1) of USA.

These two sequences form a separate cluster with 100% bootstrap values as shown (Fig 3a,b).

Table 2. Identification results based on BOLD and BLAST for *Cytochrome Oxidase I (COI)* sequencing for nine fish samples from river Neelum and Jhelum Azad Kashmir.

No	Species Code	BOLD Species identification	Similarity (%)	Genbank/BLAST Species identification	Similarity (%)	Accession number
1	SP-A	*Schizothorax plagiostomus*	99.74	*Schizothorax plagiostomus*	99.00	KT184924.1
2	SP-AH	*Schizothorax plagiostomus*	99.58	*Schizothorax plagiostomus*	99.00	KT184924.1
3	SP-AP	*Schizothorax plagiostomus*	99.32	*Schizothorax plagiostomus*	99.00	KT184924.1
4	SP-AR	*Schizothorax plagiostomus*	99.44	*Schizothorax plagiostomus*	99.00	KT184924.1
5	SP-BD	*Schizothorax plagiostomus*	99.86	*Schizothorax plagiostomus*	99.00	KT184924.1
6	SP-BF	*Schizothorax plagiostomus*	99.86	*Schizothorax plagiostomus*	99.00	KT184924.1
7	SP-C	*Schizothorax plagiostomus*	99.72	*Schizothorax plagiostomus*	99.00	KT184924.1
8	SP-G	*Schizothorax plagiostomus*	99.60	*Schizothorax plagiostomus*	99.00	KT184924.1
9	SP-K	*Schizothorax plagiostomus*	99.58	*Schizothorax plagiostomus*	99.00	KT184924.1



(a)

Fig. 3a. Unrooted phylogenetic tree (a) of *Cytochrome Oxidase 1* based on nucleotide sequences of present study and international sequences (with accession number) of *S.plagiostomus*. The length of each pair of branches represents the distance between sequence pairs. The phylogenetic tree was constructed by the Clustal W method using the MegAlign program (DNASTAR).

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
1		100.0	94.6	94.8	95.4	98.1	97.9	98.2	98.0	98.4	98.4	98.0	98.5	98.3	1	S. plagiostomus (NC_023531.1).seq
2	0.0		94.6	94.8	95.4	98.1	97.9	98.2	98.0	98.4	98.4	98.0	98.5	98.3	2	S. plagiostomus (KF928796.1).seq
3	5.6	5.6		100.0	95.4	95.2	95.4	95.1	95.1	95.6	95.6	95.2	95.4	95.2	3	S. plagiostomus (KP712118.1).seq
4	5.4	5.4	0.0		95.6	95.4	95.6	95.3	95.3	95.7	95.7	95.4	95.6	95.4	4	S. plagiostomus (KP712120.1).seq
5	4.8	4.8	4.8	4.6		99.6	99.8	99.4	99.7	99.9	99.9	99.9	99.7	99.6	5	S. plagiostomus (KT184924.1).seq
6	1.9	1.9	5.0	4.8	0.4		99.6	99.9	99.3	99.7	99.7	99.7	99.6	99.7	6	S. plagiostomus SP-AH.seq
7	2.2	2.2	4.8	4.6	0.3	0.4		99.7	99.5	99.9	99.9	99.6	100.0	99.9	7	S. plagiostomus SP-A.seq
8	1.8	1.8	5.1	4.9	0.6	0.1	0.3		99.2	99.6	99.6	99.6	99.7	99.9	8	S. plagiostomus SP-AR.seq
9	2.0	2.0	5.1	4.9	0.3	0.7	0.5	0.8		99.6	99.6	99.6	99.4	99.3	9	S. plagiostomus SP-AP.seq
10	1.7	1.7	4.6	4.5	0.1	0.3	0.1	0.4	0.4		100.0	99.7	99.9	99.7	10	S. plagiostomus SP-BF.seq
11	1.7	1.7	4.6	4.5	0.1	0.3	0.1	0.4	0.4	0.0		99.7	99.9	99.7	11	S. plagiostomus SP-BD.seq
12	2.0	2.0	5.0	4.8	0.1	0.3	0.4	0.4	0.4	0.3	0.3		99.6	99.7	12	S. plagiostomus SP-G.seq
13	1.5	1.5	4.8	4.6	0.3	0.4	0.0	0.3	0.6	0.1	0.1	0.4		99.9	13	S. plagiostomus SP-C.seq
14	1.7	1.7	5.0	4.8	0.4	0.3	0.1	0.1	0.7	0.3	0.3	0.3	0.1		14	S. plagiostomus SP-K.seq
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		

(b)

Fig. 3b. Table of identities and divergence according to analysis.

Species identifications are generally made by comparing unknown and ambiguous sequences against the DNA barcodes of known species via alignment searching (e.g., BLAST; Altschul *et al.*, 1997), distance-based tree construction (Hebert *et al.*, 2003, 2004), and recently suggested methods such as the characteristic attribute organization system (Kelly *et al.*, 2007). Seven haplotypes were observed among these samples.

The average value of haplotype diversity was Hd: 0.944±0.07. The average value of nucleotide diversity (Pi) was 0.0032±0.001.

The relative frequencies of specific substitutions (A→G, C→T, etc.) for mitochondrial-encoded *CO1* gene in ways that affect the acquisition and retention of phylogenetic signal by each molecule. The transitional substitutions are outnumbered the transversional substitutions.

Conclusion

The efficacy of *COI* barcodes has strongly been validated by this study for identifying *Schizothorax* species from western Himalaya. The straight forward amplification and sequencing was proved by the *COI* barcode region, and this would enable the accurate identification of specimens and also aid consequent generation of barcode database. Moreover, the *COI* barcodes provide sufficient solution to isolate the cold water *Schizothorax* species. Both means i.e. intra-specific and inter-specific divergences in *COI* sequence differed by more than an order of magnitude.

The prompt and accurate identification of species would be aided by technique known as barcoding that would be very helpful in the application of molecular taxonomy evidence. On the basis of results for *Schizothorax*, it is predictable that in the identification of other snow trout species, DNA barcoding could be effective. Further investigations should confirm this viability and for identification cases, and form the consistency of the techniques for different application and introducing fishes in practical importance. For fish identification purpose, DNA barcoding had been established as an accurate, reliable and broadly applicable technique.

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Declaration of interest

The authors alone are responsible for the content and writing of the paper. The authors report no conflicts of interest.

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