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Molecular Phylogeny of *Schizothorax* Species Based on Concatenated *CO-I* and *Cyt b* Sequences

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Abstract: *Schizothorax plagiostomus* is found in the cold-water of Hindukush-Karakoram-Himalayan mountains/foothills and parts of Central Asia. The local cold-water fishery of Schizothoracinae supports the rural economy of this region. Despite of its wide range of presence and potential economic value, *Schizothorax* is a highly neglected ichthyo-fauna with disputed taxonomical status. In this study the sequence data of mitochondrial *Cytochrome c oxidase subunit I (CO-I)* and *Cytochrome b (Cyt b)* gene have been employed to explain the phylogenetic relationship(s) between the *S. plagiostomus* from different riverine system and the phylogenetic relationship between seven species of *Schizothorax* keeping *Schizothorax plagiostomus* as a reference organism. Our data revealed the genetic proximity shared between *S. niger, S. curvifrons , S. richardsonii, S. progastus , S. esocinus, S. labiatus and S. plagiostomus* & *S. esocinus*.

Keywords: *Schizothorax* • Phylogeny • Cytochrome c oxidase subunit I • Cytochrome b

Introduction

Schizothorax plagiostomus, commonly called snow trout is a food fish and a major animal protein source, which belongs to Cypriniformes order, Cyprinidae family and Schizothoracinae sub family (Jhingran, 1991; Mir et al., 2013). *S. plagiostomus* found in the cold-water of the rivers, streams, tributaries and lakes of the Hindukush-Karakoram-Himalayan mountains/foothills covering around 2400 km of parts of Central Asia (Jhingran 1991; Mir et al., 2013; Mirza 1991). There are three groups of sub family Schizothoracinae: primitive group, specialized group and highly specialized group (Mir et al 2013; He et al 2004; Qi et al 2012; Yonezawa et al 2014). There are a total of 63 species in the genus *Schizothorax*. Of these, 34 species are prominent in parts of Central Asia while the other 28 are found in the Indo-Himalayan region. The *Schizothorax* species found in the Himalayan region includes *S. curvifrons, S. nasus¸ S. richardsonii, O. sinuatus, S. planifrons, S. esocinus, Schizothoraichthys progastus, S. longipinnis, S. kumanonensis, O. molesworthii, S. hugelli, S. labiatus and S. micropogon* (Ma et al 2020; Zhang et al 2018; Menon 1999; Mishra 2003). The identification of Schizothoracinae species according to traditional taxonomy were established on the morphological reports based on the characters that were externally visible, morphometric analyses and length vs weight ratio measurements (Mir et al 2013). These approaches were insufficient to differentiate the exact identification of species. Recent advancements in cytogenetic and DNA-bar coding have been potentially contributory to study the genetic resources of the ichthyo-fauna globally (Ward et al 2005). Mitochondrial (Mt) DNA is chosen as it is haploid and maternally inherited, also Mt-DNA shows a faster rate of mutation due to a poor repair system during/after replication, lower effective single-nucleotide polymorphism (SNP) variations due to lack of

recombination. Mt-DNA shows an anti G bias that is seen in most teleost (Brown et al., 1979, Fiaz et al., 2016). By using mitochondrial *Cytochrome c oxidase subunit I (CO-I)* and *Cytochrome b (Cyt b)* gene we have earlier reported the phylogenetic relationship of *S. plagiostomus* with the other species of Schizothoracinae (Purohit et al 2023). Our study aims to obtain the sequence data by using mitochondrial *CO-I* and *Cyt b* gene and to employ the data to interpret the phylogenetic position of *S. plagiostomus* with the following objectives: i) to study the phylogenetic relationships of *S.plagiostomus* from different riverine system ii) to explore phylogenetic relationship of seven major species under genus *Schizothorax* found in Indian sub-continent.

Materials and Methods

Fresh fish (*S. plagiostomus*) samples were collected from the river(s) Alaknanda and/or Nandakini either at Nandprayag (30.3320°N, 79.3205°E) and/or at Srinagar (30.2247°N, 78.7986°E) of Garhwal-Uttarakhand, India by deploying local fishermen. For molecular analysis, the species were initially selected on the basis of reported external morphology and after that around 100 mg tissue from muscle and fin of *S. plagiostomus* were preserved in 95% (v/v) ethanol. DNA was isolated from 25 mg of tissue by phenol/chloroform protocol with partial modifications in the initial step of homogenization. After DNA isolation, the TE buffer is used to dissolve the DNA pellet and the concentration was adjusted to 100ng/µl. For *CO-I* and *Cyt b* amplification, the reaction mixture consist of 10X Taq polymerase buffer (5 µl), 50mM $MgCl_2$ (2 µl), 0.05mM dNTP (0.25 µl), 0.01mM primer (0.5 µl), Taq polymerase (1.5 IU) and 200ng genomic DNA template (2 µl). The primer pair used for the *CO-I* was: *FishF1 5'TCAACCAACCACAAAGACATTGGCAC3'* and *FishR1 5'TAGACTTCTGGGTGGCCAAAGAATCA3'* (Ward et al., 2005). The primer pair for *Cyt b* was L14724 5′-TGACTTGAARAACCAYCGTTG-3′ and H15915 5′-CTCCGACT CCGGATTACAAGAC-3′ (Singh et al., 2012). The PCR cycle of *CO-I* consist of initial denaturation at 94ºC for 3 min. After the initial denaturation, there are 35 cycles at 94 ºC for 1 min, 54ºC for 40 sec and 72ºC for 1 min followed by 10 min at 72ºC for final extension. The thermal protocol for PCR for *Cyt b* includes an initial denaturation step of 3 min at 94 °C, 35 cycles of 1 min at 94 °C, 40 s at 49 °C, and 80 s at 72 °C, and a final extension step of 10 min at 72 °C. 1.5% and 1 % agarose gel was made for *CO-I* and *Cyt b* respectively to visualize the PCR products by using gel documentation system. Di-deoxynucleotide chain termination method was used to sequence the PCR products at the central facility of the National Bureau of Fish Genetic Resources, Lucknow, India (Sanger et al., 1977). DNA sequencing was done using an automated *ABI-3500 Genetic Analyzer*. The BigDye Terminator V.3.1 Cycle Sequencing 153 Kit (Applied Biosystems, Inc) was used for labeling the products.The following components made up the 10 µl cycle sequencing PCR reaction: BigDye reaction mix (2.5X), 4 µl, and sequencing buffer (5X), 1µl of 50 ng per 2µl of purified PCR product, 0.5µl of 10 µM primer and 2.5µl nuclease-free water. Conditions for the PCR cycle sequencing were 25 cycles of 96°C for 20 sec, 50°C for 15 sec, and 60°C for 4 min. For analysis and constructing phylogenetic tree, the forward sequence and inverted (reversed and complimented) reverse sequences were aligned to make a consensus sequence for each sample. Ambiguous bases were checked manually against the raw sequencing electropherogram files and corrected accordingly. Clustal-W, an integrated tool in Molecular Evolutionary Genetics Analysis (MEGA) software version 11

was used for the alignment of sequences (Tamura et al., 2021). The obtained consensus sequences were blasted in National Centre for Biotechnology Information (NCBI) GenBank for the nearest similar sequence matches. The phylogenetic tree was also constructed by using the MEGA 11 software with 1000 bootstrap replications (Felsenstein, 1985). The Neighbour Joining (NJ) method was also based on the Tamura 3-Parameter method (Saitou and Nei 1987).

Results and Discussion

The current study is focused to understand the genetic distance/proximity among species of *Schizothoracinae* by using *S. plagiostomus* as a reference organism. Globally, the Mt *CO- I* and *Cyt b* are highly recognized and dependable genetic marker for the molecular identification of highly diversified ichthyo-fauna (Ward et al 2005; Singh et al 2012), including *Schizothoracinae* (Ali et al 2014; Singh et al 2018) species. We sequenced *CO-I* and *Cyt b* gene of *S. plagiostomus* specimens captured from Alaknanda and Nandakini rivers /streams of Garhwal Himalaya, Uttarakhand. The sequences were submitted in NCBI GenBank (accession numbers OK484543-OK484560 for *CO 1* and OL588985- OL589002 for *Cyt b*).

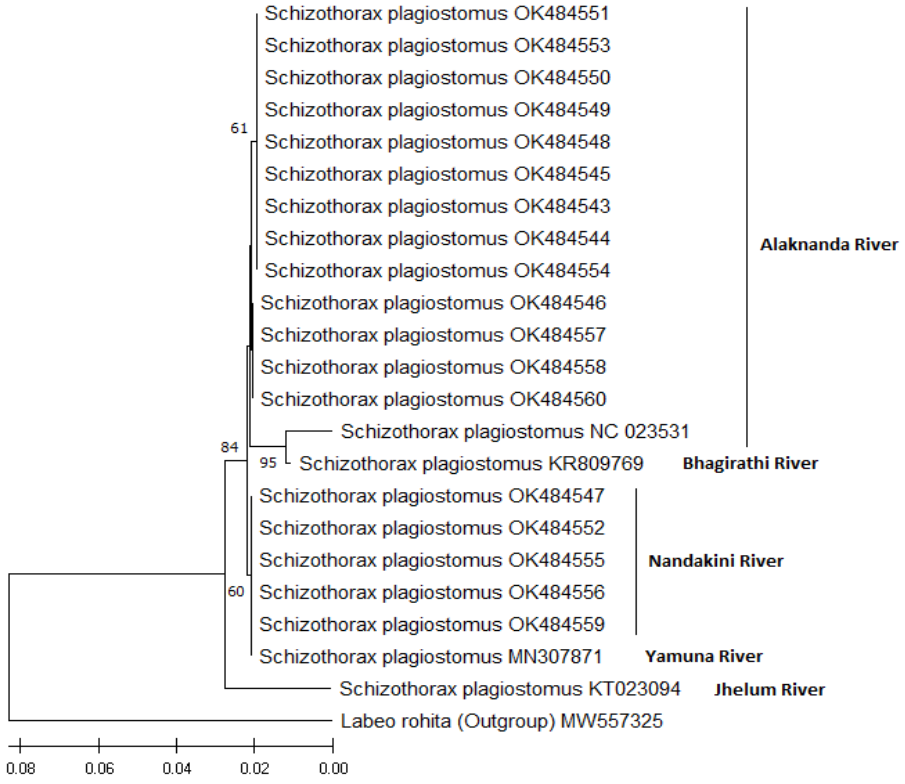

Figure 1A. Phylogenetic tree based on mitochondrial *CO-I* partial gene sequences of *Schizothorax plagiostomus* from different riverine system of India, obtained by the Neighbour Joining (NJ) method. Numbers above branches are percent bootstraps values based on 1,000 replicates.

Table 1 : Summarizes different *S.plagiotomus* from the different geographical areas shown in Fig 1.

SN	Fish Species	Gene Accession Number	River/Location	Latitude and longitude	Reference
1.	*S. plagiostomus*	OK484543-OK484560, OL588985-OL589002	Alaknanda, River, Srinagar, and Nandakani river, Nandprayag	30.2247^{0}N, 78.7986^{0}E and 30.33°N, 79.33°E	Present study
2.	*S. plagiostomus*	NC023531	Bhimtal, Nainital	29.3461° N, 79.5519° E	Goel C, Sahoo PK, Barat A. Complete mitochondrial genome organization of Schizothorax plagiostomus (Heckel, 1838). Mitochondrial DNA Part A. 2016 Jan 2;27(1):113-4.
3.	*S. plagiostomus*	KR809769	Bhagirathi, River, Uttarkashi	30.8500°N, 79.1486°E	Thapliyal, M., Pokhriyal, H., Sati, B. K., Nagpure, N. S., Singh, M., & Thapliyal, A. (2015). Molecular characterization of coldwater fishes of district Uttarkashi, Uttarakhand using DNA Barcoding. *Environment Conservation Journal*, *16*(3), 109-116.
4.	*S. plagiostomus*	KT023094	Jhelum River, Kashmir	75°2' 41.79''E, 33°38' 42.588''N	Bashir, A., Bisht, B. S., Mir, J. I., Patiyal, R. S., & Kumar, R. (2016). Morphometric variation and molecular characterization of snow trout species from Kashmir valley, India. *Mitochondrial DNA Part A*, *27*(6), 4492-4497.
5.	*S. plagiostomus*	MN307871	Yamuna River, Uttarkashi	30.7088^{0}N, 78.3537^{0}E	SINGH, U., NAUTIYAL, P., & DEWAN, S. (2018). Sub-speciation tendencies, genetic diversity and divergence of the Schizothorax progastus (McClelland 1839) in tributaries of the Ganga river in Indian Himalayas. *COLDWATER FISHERIES SOCIETY OF INDIA*, *1*(1), 54-59.
6.	*Labeo rohita*	MW557325	Karanpuli river, Chittagong, Bangladesh	22.3569° N, 91.7832° E	Direct submission. Siddiki,A.Z., Akter,S., Asek,A.A., Bhuiyan,M.A.B., Rahman,S. and Kibria,M.M
7.	*Ptychobarbus dipogon*	NC024537	Yarlung Tsangpo River, Tibet, China	29.3460^{0}N, 90.1115^{0}E	Direct Submission. Wei,J.

Molecular diversity of S. plagiostomus with respect to the differential geographical distribution pattern

Our data shown in **Figure 1A** demonstrated the molecular comparisons of *S. plagiostomus* species taken from the different cold-water riverine systems of the Indian Himalayas based on the phylogenetic tree made by the NJ method by using *CO-I* keeping *Labeo rohita* as an outgroup. Specimens collected either by us and others from Alaknanda river, Bhagirathi river, Nandakini river, Yamuna river and Jhelum river

were analysed (GenBank accession numbers and other details are given in **Table 1**) and each formed well-supported clades. Specimens from the Alaknanda River were all clustered together and showed closer relatedness with specimens of the Bhagirathi River. Whereas, specimens from the Nandakini river showed closer genetic proximity with specimens of the Yamuna river. Specimen from the Jhelum river forms a different clade elucidating the geographical isolation. **Figure 2A** represents the molecular comparisons of *S .plagiostomus* species from different riverine systems of Indian Himalayas based on the phylogenetic tree made by the NJ method using *Cyt b* marker (GenBank accession numbers and other details are given in **Table 1)**. *Ptychobarbus dipogon* is used as an outgroup for this tree. There are two Clades formed. Clade A that includes the 18 samples and Clade B that includes the *S. plagiostomus* collected from the different riverine system. The two *S. plagiostomus* (NC 023531 & KF928796) in Clade B form monophyletic group. There are two different clusters formed in the Clade A. The first group is formed by the samples collected from river Alaknanda and the second group formed from the Nandakini river. It can be inferred that the samples taken from the same geographical area are clustered together.

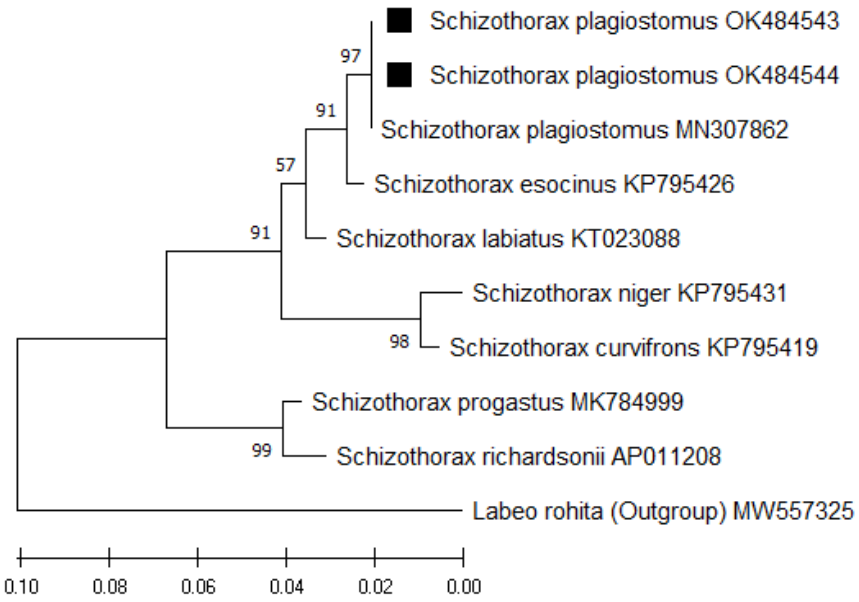

Figure1B. Phylogenetic relationship(s) of seven major *Schizothorax* sp. Found in Indian subcontinent obtained by the Neighbour Joining (NJ) method based on *CO-I* partial sequences. Numbers above branches are percent bootstraps values based on 1,000 replicates.

Genetic comparison(s) among seven major Schizothorax species found in Indian Sub-continent

The data shown in **Figure 1B** was used to elucidate the phylogenetic relationships among the seven major *Schizothorax* species, keeping *Labeo rohita* as an outgroup (GenBank accession nos. and other details given in **Table 2**). Our data shows two clades under genus *Schizothorax*. One clade (Clade A) contained *S.*

plagiostomus, *S. esocinus*, *S. labiatus*, *S. Niger* and *S. curvifrons*. The analysis further confirmed our specimen as *S. plagiostomus* (bootstrap value 97%) forming monophyletic group. *S. plagiostomus* showed closer relationship with *S. esocinus* (bootstrap value 91%), whereas *S. niger* and *S. curvifrons* formed a monophyletic group (bootstrap value 98%). The second clade (Clade B) constituted with *S. progastus* and *S. richardsonii* forming a monophyletic group (bootstrap value 99%).

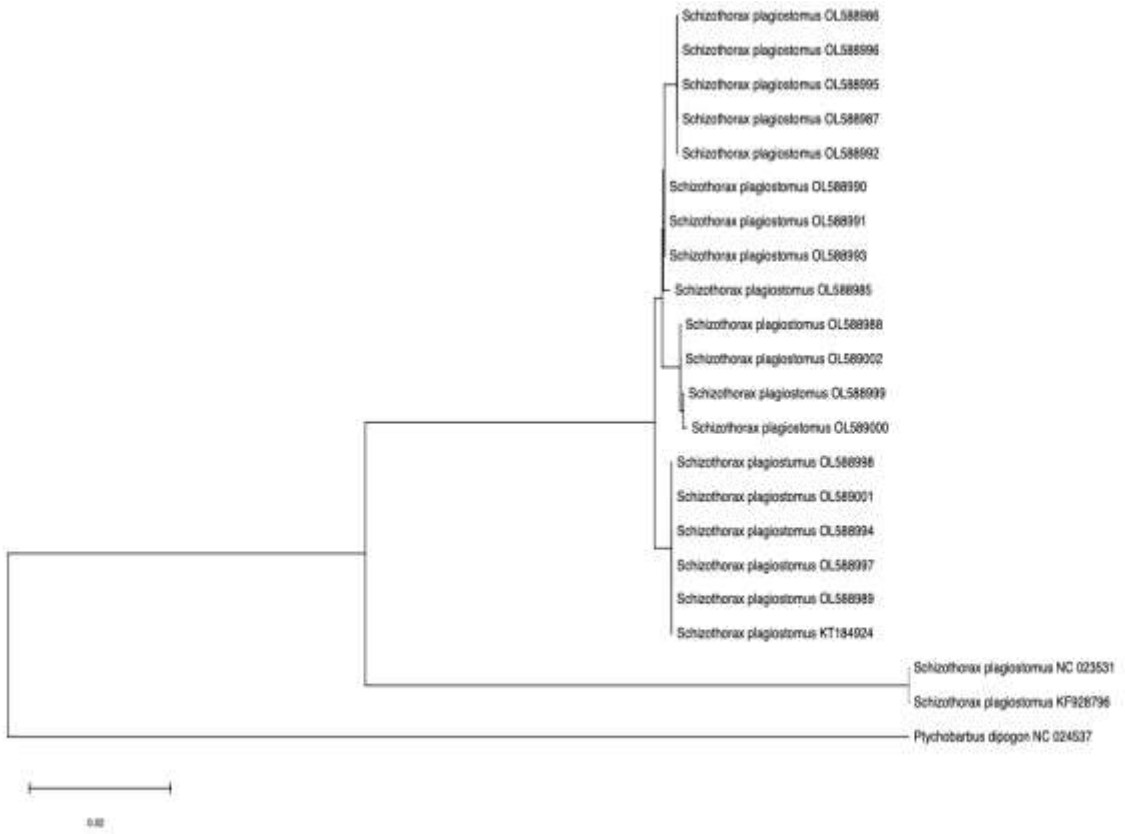

Figure 2A: Phylogenetic tree based on mitochondrial *Cyt b* partial gene sequences of *Schizothorax plagiostomus* from different riverine system of India, obtained by the Neighbour Joining (NJ) method. Bootstraps values based on 1,000 replicates.

Figure 2B represents the seven major *Schizothorax* species found in the Himalayan region namely *S. labiatus*, *S. esocinus*, *S. progastus*, *S. richardsonii*, *S. curvifrons*, *S. plagiostomus* and *Schizopyge niger* using *Barbus trimaculatus* as an outgroup. The Neighour joining method (NJ) tree made by using the sequences of *Cyt b* gene by Tamura-3-parameter model, to elucidate the relationship among the seven major *Schizothorax* species. In the tree two clades were formed. Clade A that include *S.labiatus*, *S. esocinus*, *S. progastus*, *S. richardsonii*, *S. curvifrons*, and *S. plagiostomus*. Clade B includes *S. niger* that forms a monophyletic group. *S.plagiostomus* is most closely related to *S. curvifrons* and most distantly related to *S. niger*. Ahmad *et.al* have investigated the Mt-DNA variability by

examining the mitochondrial markers (*16S rRNA, Cyt-b* and *D-loop*) to found the evolutionary relationships between five *Schizothoracinae* species obtained from the Kashmir valley (Ahmad et al., 2014).

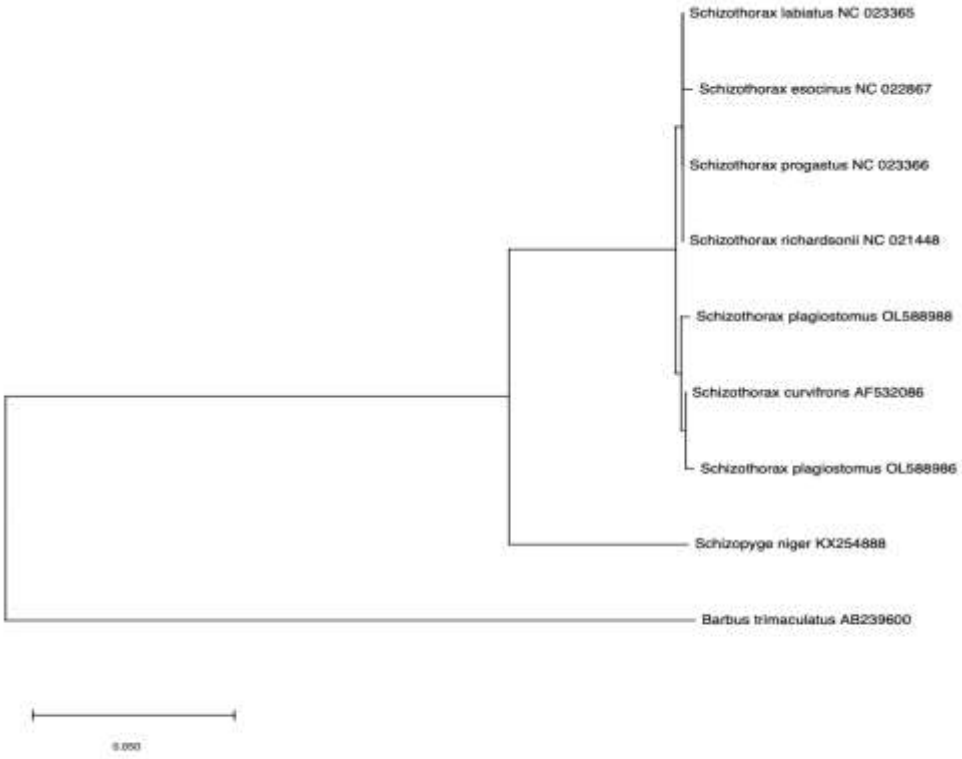

Figure 2B: Phylogenetic relationship(s) of seven major *Schizothorax* sp. Found in Indian subcontinent obtained by the Neighbour Joining (NJ) method based on *Cyt b* partial sequences. Bootstraps values based on 1,000 replicates.

Table 2: Summarizes the specimen details for comparing Seven major Indian *Schizothoracine* species shown in Fig 2.

SN	Fish Species	Accession Number	River/Location	Latitude and longitude	Reference
1.	*S. plagiostomus*	OK484543	Alaknanda River, Srinagar(Garhwal)	30.2247°N, 78.7986°E	Present study
2.	*S. plagiostomus*	OK484544	Alaknanda River, Srinagar, Garhwal	30.2247°N, 78.7986°E	Present study
3.	*S. plagiostomus*	MN307862	Yamuna River, Uttarkashi	30.7088^0N, 78.3537^0E	Direct submission Singh,U., Dewan,S.and Nautiyal,P. Genetic variability in sympatric Schizothoracinae stocks from the isolated Yamuna, Ganga and Kali Riverbasins
4.	*S. esocinus*	KP795426	helum River,	34°5'19.722''N	Bashir A, Bisht BS, Mir JI, Patiyal

			Kashmir	75°51' 24.118''E	RS, Kumar R. Morphometric variation and molecular characterization of snow troutspecies from Kashmir valley, India. Mitochondrial DNA Part A. 2016 Nov1;27(6):4492-7.
5.	*S. labiatus*	KT023088	helum River, Kashmir	34°16'5.299''N 74°25'57.643''E	Bashir A, Bisht BS, Mir JI, Patiyal RS, Kumar R. Morphometric variation and molecular characterization of snow troutspecies from Kashmir valley, India. Mitochondrial DNA Part A. 2016 Nov1;27(6):4492-7.
6.	*S. niger*	KP795431	Dal lake, Kashmir	34°51'19.722''N 74°51'24.118''E	Bashir A, Bisht BS, Mir JI, Patiyal RS, Kumar R. Morphometric variation and molecular characterization of snow troutspecies from Kashmir valley, India. Mitochondrial DNA Part A. 2016 Nov1;27(6):4492-7.
7.	*S. curvifrons*	KP795419	helum river, Kashmir	33°46'6.752''N 75°12'16.785''E	Bashir A, Bisht BS, Mir JI, Patiyal RS, Kumar R. Morphometric variation and molecular characterization of snow troutspecies from Kashmir valley, India. Mitochondrial DNA Part A. 2016 Nov1;27(6):4492-7.
8.	*S. progastus*	MK784999	Gomti River, Tripura, India	23.9408 ° N, 91.9882° E	Direct submission Chaoba Devi,N., Parhi,J., Debbarma,B., Priyadarshi,H.,Radhakrishnan,K.V. andPandey,P.K
9.	*S. richardsonii*	AP011208	Hanami river, Chiba, japan	35.6074° N, 140.1065° E	Miya M. Whole mitochondrial genomesequences in Cypriniformes. Unpublished manuscript, Natural History Museum & Institute, Chiba, Japan. 2009.
10.	*S. labiatus*	NC023365	Bhimtal, Nainital	29.3461° N, 79.5519° E	Direct submission. Goel, C., Sahoo, P.K., Bhat,F.A.,Balkhi,M.H. and Barat, A.
11.	*S. esocinus*	NC022867	Bhimtal, Nainital	29.3461° N, 79.5519° E	Direct submission. Sahoo, P.K., Goel, C., Bhat,F.A.,Balkhi,M.H. and Barat, A.
12.	*S. progastus*	NC023366	Bhimtal, Nainital	29.3461° N, 79.5519° E	Direct submission. Sahoo, P.K.,Barat, A. and Goel, C.
13.	*S. richardsonii*	NC021448	Bhimtal, Nainital	29.3461° N, 79.5519° E	Direct submission Goel, C.,Sati,J., Patiyal, R.S., Ali, S., Barat, A. ans Sahoo,P.K.
14.	*S. niger*	KX254888	Neelum and Jhelum River	Neelum- $34^0$23'23" N, $75^0$07'19" E and Jhelum- 33°46'6.752''N 75°12'16.785''E	Akhtar,T. and Ali, G.
15.	*Barbus*	AB239600	Kanagawa, Japan	$35^0$30'59.9976"N	Direct submission. Saitoh,K.

	trimaculatus			and 139^{0}41'59.9892"E	
16.	*Labeo rohita*	MW557325	Karnphuli river, Chittagong, Bangladesh	22.3569° N, 91.7832° E	Direct submission. Siddiki,A.Z., Akter,S., Asek,A.A., Bhuiyan,M.A.B., Rahman,S. and Kibria,M.M

Their study suggests that unlike *Cyt-b* and *16S rRNA*, the *D-loop* shows greater length variations in $(TA)_n$ microsatellite repeats at 3'. Interestingly, $(AT)_n$ microsatellite of these species is not associated with longer tandem repeats in the 3' end of the mitochondrial control region, and therefore can be the most valuable marker providing a reliable cladogenic insight into the mode of evolution and distribution of the species with restricted fossil records (Ahmad et al 2014). Silas (1960) suggested the distinctions between *S. curvifrons* and *S. niger* are insufficient to justify their different species status and further classified *S. niger* as a subspecies of *S. curvifrons*. Nevertheless, our analysis revealed that *S. niger* is a distinct species. However, the mitochondrial sequence differences between *S. curvifrons* and *S. niger* are too small in some individuals to distinguish them as distinct species. Introgressive hybridization, poor lineage sorting, fast radiation in lineages, and numerous hits (homoplasy) may explain such lack of variation in mitochondrial sequencing data amongst *Schizothorax* species (Tsigenopoulos and Berrebi 2000; Qi et al 2007; He and Chen 2006).

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