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Molecular Characterization of Subfamily Schizothoracinae (Teleostei: Cyprinidae) using Complete Sequence of Mitochondrial 16S rRNA Gene

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ABSTRACT

Molecular phylogeny of Schizothoracinae containing four species distributed in the north and north-east Himalayas was investigated based on the complete mitochondrial 16S rRNA gene sequences. The average nucleotide length of 16S rRNA in 45 samples ranged from 1527 to 1552 bps. Five haplotypes (h) were observed, with haplotype diversity (Hd) 0.5323±0.080. Among 5 haplotypes, 2, 2 and 1 haplotype were detected in *S. plagiostomus, S. niger* and *S. esocinus*, respectively. Haplotype diversity was lowest and same in *S. esocinus, S. niger* and *S. progastus* (0.0001) and highest in *S. plagiostomus* (0.5436). Average number of nucleotide differences of 4 species (Kt) was 0.737 and total nucleotide diversity (Pi) was equal to 0.0004 ± 0.0001. Out of 1552 sites, 1547 sites were found to be conserved and five were polymorphic, in which four were parsimony informative while, one was singleton. The rate of transition/transversion (R) was 3.131, which deviate from neutral evolution where, R= 0.5. The negative values of Tajima's D and Fu and Li's F* indicated that the genetic variations in 16S rRNA between species were not neutral which reflect the excess of external mutations. Results indicate that 16S rRNA gene is unable to fully resolve the inter-relationship of Schizothoracinae species.

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Authors' Contribution
TA and GA designed the experiments, analyzed the data and wrote the manuscript. TA conducted the experiments. NS helped in sample collection. NS and AR helped in preparation of manuscript.

Key words
Genetic diversity, Mitochondrial 16SrRNA gene, Phylogenetic analysis, Schizothoracinae, *Schizothorax niger*.

INTRODUCTION

The family Cyprinidae, which contains the genus Schizothorax, are locally known as snow trout and encompasses 20 genera and more than 150 species throughout the world (Mirza, 1991; Chen and Cao, 2000). These economically important fish inhabit fast flowing snow-fed streams, including the Neelum and Jhelum rivers in Azad Jammu and Kashmir (Akhtar *et al*., 2016). These fish species are dispersed in the cold waters from Jammu and Kashmir (Sunder and Bhagat, 1979) to the eastern Himalayas and Assam through Sikkim and Bhutan at an altitude of 1180–3000 m (Jhingran, 1991). Thus far, almost 30 snow trout spp. have been reported in the Himalayan and sub-Himalayan region. Their essential biological features such as slow growth to maturity and short growth periods are the specific restrictions on their resources and population increase (Goel *et al*., 2011). Schizothoracinae are going to extinct due to over-fishing and destruction of their spawning grounds (Liang *et al*., 2008; Ali *et al*., 2010).

DNA based markers are widely applied to offer a better resolution of systematic relationships among spp., especially mitochondrial DNA (mtDNA) markers. Many changes have taken place in the species composition, distribution, and abundance of the fish in this region. The deterioration of catchment areas due to inappropriate agricultural practices, deforestation, and pollution are reducing water quality, harming the cold-water fish stocks in some water bodies (Ahmad *et al*., 2014). Members of the genus *Schizothorax* exhibit remarkably similar morphology. It is very difficult to distinguish members of *Schizothorax* based on external morphological characteristics to determine whether they are different species, the diverse phenotypes of a single species, or a transitional condition among these extremes (Raina and Petr, 1999; Mir *et al*., 2013).

A simple genomic structure, high nucleotide-substitution rate, absence of recombination, and maternal inheritance make mitochondrial DNA (mtDNA) one of the most extensively employed molecular markers for the analysis of intraspecific and interspecies variations among animals. Available data on mtDNA has offered new insights on taxonomically questionable taxa and perplexing queries of phylogeny. Various mitochondrial genes have been used to study the genetic differences, phylogenetic associations, biogeographical patterns, and taxonomy (Xiao *et al*., 2001; Durand *et al*., 2002; Bajpai and Tewari, 2010; Meng *et al*., 2018) of numerous fish and higher vertebrates. Animal

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mtDNA is thought to have rigorous maternal transmission and is highly mutable within a species; thus, mtDNA provides a substantial resource for phylogenetic analyses and investigating genetic divergence (Wolf *et al*., 1999).

According to Stoeckle (2003), mtDNA genes are becoming an attractive target since these genes are shared across various taxa (species) and do not contain introns that complicate amplification. Mitochondrial DNA has been proved to be a powerful tool in fish species identification (Lemer *et al*., 2007; Rubinoff *et al*., 2006; Teletchea, 2009) and fish stock maintenance (Greig *et al*., 2005; Mohd-Shamsudin *et al*., 2011).

Mitochondrial 16S rRNA gene has been extensively employed to investigate the phylogenetic associations of fishes at different taxonomic ranks for about two decades. This gene cover 1/10 of entire mitochondrial genome (Yang *et al*., 2014). It offers enormous potential for interpreting the differences among the cyprinid ancestries and establishing the associations within Cyprinidae (Li *et al.,* 2008). The application of this gene as a genetic marker has become important due to highly conserve between closely related species. Therefore, the complete sequence of mtDNA 16S rRNA genes has been used to study the genetic relationship and phylogeny of the selected fresh water Schizothoracinae and to determine whether this gene is able to resolve the intra and inter-specific relationship or not?

MATERIALS AND METHODS

Sample collection

The fish samples were randomly collected from the Jhelum and Neelum rivers near Muzaffarabad (from Ghori to Kohala) with the use of different fishing gear (cast nets and gill nets). The collected fish samples were anesthetized by immersion in 1% benzocaine in water and euthanized by benzocaine excess. Considering the potential problems in identifying species (Talwar and Jhingran, 1991; Mirza, 1991), the four Schizothorax spp. were identified through barcoding and by following the classifications by Mirza (1991) and Jhingran (1991).

PCR amplification of mitochondrial DNA

Total DNA was isolated by a standard phenol-chloroform extraction method described by Sambrook *et al*. (1989).

Primers of complete 16S rRNA gene of *Schizothorax* spp. were designed by using program Primer-3. The 16S rRNA gene was amplified from mtDNA: 16SF1 (5′- AGA AGA CAT CCA TGC AAA CT -3′), 16SR1 (5′- TGC GTT TGC CGA GTT CCT T -3′), 16SF2 (5′- AGG AAG GAA CTC GGC AAA CG -3′), 16SR2 (5′- AGC GTT ACA GAT AGA AAC TGA CCT -3′), 16SF3 (5′- CAG TTT CTA TCT GTA ACG CT -3′), 16SR3 (5′- GCC TTT CGC AAT TTA CCA TG -3′).

Mitochondrial 16S rRNA was amplified in 25 µl (standard amount) reaction volumes containing 14 µl DMSO-water, 3 µl template DNA, 2.5 µl Taq buffer, 0.5 µl dNTPs, 1µl of each primer (forward and reverse), 2.5 µl magnesium chloride and 0.5 µl Taq polymerase. For thorough mixing, the reaction mixture was vortexed and centrifuged for 30 s at 8,000 rpm. Thermal cycling was comprised of 95°C for 3 min; 39 cycles of 95°C for 30 s (denature), 53°C for 30 s (anneal); and 72°C for 1 min (extension) and followed by a final extension at 72°C for 10 min. The PCR products were purified with Exo-Sap-IT (Affymetrix purification kit) prior to cycle sequencing.

Sequence and phylogenetic analysis

Bidirectional nucleotide (nt) sequencing was performed on the 16S rRNA gene in an ABI Prism 3100 Genetic Analyzer (PE Applied Biosystems; Foster City, CA, USA) using gene-specific forward and reverse primers. Sequence editing was performed using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/) to determine nucleotide and amino acid variations. The *Schizothorax* sequences were aligned by using the ClustalW algorithm of the MegAlign program in the LaserGene software package (DNAStar, Inc., Madison, WI). Multiple sequence alignments were performed to determine the sequence divergence among the *Schizothorax* spp. in Azad Jammu and Kashmir. A phylogenetic tree was generated using the neighbour-joining and Kimura 2-parameter algorithm supported by 1000 bootstrap replicates (Jiang *et al.*, 2014; Khan *et al.*, 2016) in the MegAlign program of the LaserGene software (Saitou and Nei, 1987).

Test of neutral evolution

Three different neutrality test were examined for the combined DNA sequence alignment: Tajima's D (1989), Fu and Li's D* (1993), Fu and Li's F* (1993). The DnaSP 5.0 program was used to calculate level of polymorphism and divergence for pairwise comparison of sequence both within and between populations and to test for the expected neutral evolution of the sequence. The null hypothesis of neutral evolution of the marker was tested by using Tajima's D, Fu and Li's D*, Fu and Li's F*.

Nucleotide and haplotype diversity

The number of variable sites in the alignment was determined with MEGA version 6.06 software. The number of polymorphic sites (S), total number of mutations (η), haplotype diversity (Hd), and nucleotide diversity (Pi) were estimated using the DNASP 5.0 program, which

eliminated all positions containing gaps, except where otherwise stated. The minimum spanning network (MSN) was used for haplotype formation through Popart 1.7 software.

Table I.- Details of mitochondrial 16S rRNA gene sequences, sample code, sequence length (bps), collection sites and Genbank accessions number of four *Schizothorax* species.

Species	Sample code	Sequence length (bp)	Collection Site	A+T	G+C	NCBI Acc. No.
S. plagiostomus	SP-01	1551	River Neelum, Ghori	55.96	44.04	KX160502
	SP-02	1533	_	55.77	44.23	KX160503
	SP-03	1551	_	55.96	44.04	KX160504
	SP-04	1530	_	55.88	44.12	KX160505
	SP-05	1533	_	55.84	44.16	KX160506
	SP-06	1533	_	55.84	44.16	KX160507
	SP-07	1533	_	55.77	44.23	KX160508
	SP-09	1533	River Neelum, Chella Bandi	55.84	44.16	KX160509
	SP-10	1534	_	55.90	44.10	KX160510
	SP-11	1533	_	55.84	44.16	KX160511
	SP-12	1533	_	55.84	44.16	KX160512
	SP-13	1533	_	55.77	44.23	KX160513
	SP-15	1534	_	55.90	44.10	KX160514
	SP-19	1534	_	55.90	44.10	KX160517
	SP-20	1534	_	55.77	44.23	KX160518
	SP-21	1534	_	55.84	44.16	KX160519
	SP-22	1534	_	55.90	44.10	KX160520
	SP-23	1534	_	55.84	44.16	KX160521
	SP-24	1534	_	55.84	44.16	KX160522
	SP-35	1527	River Jhelum, Kohala	55.86	44.14	KX160523
	SP-37	1549	_	55.88	44.12	KX160524
	SP-39	1542	_	55.90	44.10	KX160525
	SP-40	1542	_	55.90	44.10	KX160526
	SP-44	1542	_	55.90	44.10	KX160527
	SP-46	1542	River Jhelum, Chatter	55.90	44.10	KX160528
	SP-48	1542	_	55.90	44.10	KX160529
	SP-48	1542	_	55.90	44.10	KX160529
	SP-50	1542	_	55.90	44.10	KX160530
	SP-52	1542	River Jhelum, Chatter Kalass	55.90	44.10	KX160531
	SP-56	1542	_	55.90	44.10	KX160532
	SP-58	1542	_	55.90	44.10	KX160533
	SP-59	1542	_	55.90	44.10	KX160534
	SP-60	1542	_	55.90	44.10	KX160535
S. esocinus	SE-08	1534	River Neelum, Chella Bandi	55.84	44.16	KX160494
	SE-38	1534	River Jhelum, Ambor	55.84	44.16	KX160495
	SE-53	1534	_	55.84	44.16	KX160496
	SE-54	1534	_	55.84	44.16	KX160497
	SE-57	1534	_	55.84	44.16	KX160498
S. niger	SN-33	1552	River Jhelum, Domel	56.09	43.91	KX160499
	SN-45	1552	_	56.09	43.91	KX160500
	SN-49	1552	_	56.09	43.91	KX160501
S. progastus	SPR-47	1542	River Jhelum, Garhi Dupatta	55.90	44.10	KX160536
	SPR-51	1542	_	55.90	44.10	KX160537
	SPR-55	1542	_	55.90	44.10	KX160538

RESULTS

For the phylogenetic analysis of the Schizothoracinae, the complete 16S rRNA gene of four *Schizothorax* spp. were sequenced. The total length of complete 16S rRNA gene varied from 1527 to 1552 bp in the four *Schizothorax* spp. The sequences were submitted to Genbank under different accession numbers (KX160494 to KX160538). Out of 1552 total sites, 1547 (99.67 %) were conserved (monomorphic) and only 5 were polymorphic. Out of 5 polymorphic sites, 4 were parsimony informative while 1 was singleton. The sequences were found generally AT rich ranging from 55.77 to 56.09% with average value being 55.88 %, while the GC rich ranging from 43.91 to 44.23 % with average value being 44.11% (Table I). The 16S rRNA gene displays base compositions of Adenine (A) 36.21%, thymine (T) 19.59%, cytosine (C) 23.69%, and guanine (G) 20.50% across the four *Schizothorax* spp. With respect to the 16S rRNA gene, the highest intra-species similarity (99.9 to 100%) was observed in all selected species. The model of evolution of 16s rRNA region was HKY.

Eight sequences from the same species (two each) were downloaded from Genbank (NCBI) to construct the collective phylogenetic tree based on 16S rRNA gene. These sequences were *S. plagiostomus* (KT184924.1, NC_023531.1), *S. esocinus* (KT210882.1, NC_022867), *S. niger* (KF600712.1, NC_022866), and *S. progastus* (KF739399.1, NC_023366.1).

Nucleotide and haplotype diversity

Total number of segregating sites (S) and haplotypes (h) based on complete 16S rRNA gene were 5. Among 5 haplotypes, 2, 2 and 1 haplotype were detected in *S. plagiostomus, S. niger* and *S. esocinus*, respectively and haplotype 1 was shared by *S. plagiostomus* and *S. esocinus*. Haplotype diversity (h) ranged from 0.0001 to 0.5436 with average value of haplotype diversity (Hd) 0.5323±0.080. Haplotype diversity was lowest and same in *S. esocinus, S. niger* and *S. progastus* (0.0001) and highest in *S. plagiostomus* (0.5436). Nucleotide diversity (Pi) was 0.0004 in *S. plagiostomus* and 0.0001 for other three species. Average number of nucleotide differences (Kt) of 4 species was 0.737 and total nucleotide diversity (Pi) was 0.0004 ± 0.0001.

Along the 1552 bp alignment, only nucleotide substitutions were detected and insertions or deletions were not found. The overall transition/transversion bias (R) = 3.131, where R = [A*G*k1+T*C*k2]/[(A+G)*(T+C)] and the transitional substitutions are outnumbered the transversional substitutions. Simultaneously, the proportion of sites estimated to be invariable is 0.001%, indicating that across the gene less sites are virtually invariable.

Phylogenetic analysis

The nucleotide sequences of 16S rRNA gene were aligned in order to determine the phylogenetic relationships among the four species. This alignment showed the presence of a common conserved region in all the four species indicating that these species belong to the same genus. This was also confirmed on the basis of homology with previously published sequences from same species through NCBI Genbank. The phylogenetic tree, generated using three methods (Neighbour-joining, Minimum evolution, Maximum parsimony) was similar. The Neighbour-joining (NJ) tree (Fig. 1) of 16S rRNA gene sequences of the four species clearly clusters all samples in to two distinct clades without any out-grouping. This gene indicate an average ability to show diversification among selected samples. The nodes at the base of clades and groups were supported by > 50% bootstrap values. The resulted NJ tree clearly indicates two different groups of *S. plagiostomus* and *S. niger* populations. The population of *S. plagiostomus* constitute one clade with *S. esocinus*; further they constitute another clade with *S. progastus* and formed a monophyletic group.

Other groups of *S. niger* clustered separately with maximum bootstrap values. This fish is restricted to upper part of river Jhelum due to damming of waters. It was found that the species belonging to the northern Himalayas grouped together while species from north-eastern Himalayas remained separate. The result based on the 16S also supports the high genetic similarity among *S. plagiostomus, S. esocinus* and *S. progastus*. The neutrality tests were conducted to determine the neutral evolution among the genus *Schizothorax* based on 16S rRNA. Values obtained for different test were, Tajima's D = -0.88024 (P>0.10); Fu and Li's D* = 0.16028 (P>0.10); and Fu and Li's F* = -0.18796 (P>0.01).

When the present study was compared with sequences of eight other international *Schizothorax* spp. from NCBI, only 6.90 percent sites were found monomorphic while, the rest are polymorphic. Maximum number of nucleotide substitutions in 16S rRNA gene of *Schizothorax* spp. along eight *Schizothorax* spp. from NCBI were estimated (Fig. 2). It was observed that the present species form a distinct group while, eight other retrieved *Schizothorax* spp. were assorted in separate cluster with higher bootstrap values ranging from 52 to 100 percent. The overall mean divergence between present study and international sequences was 0.312 ± 0.035. The maximum similarity and minimum divergence (0.037 ± 0.002) was found between *S. plagiostomus* and *S. progastus*, whereas the less similarity and maximum divergence (1.033 ± 0.161) was recorded between *S. niger* and *S. esocinus*. Lacking the rate of heterogeneity among sites, the distributions of

the number of substitutions per site must follow a Poisson distribution. This assumption is tested for the overall sequences of the cyprinid 16S rRNA gene sequences using comparisons between the observed and the expected numbers of substitutions.

Observed and expected values

Nucleotide diversity, Pi: 0.0004, with the average number of pairwise differences, k: 0.737. Similarly, observed variance of k: 0.677, with C.V. of k: 1.122. Expected total variance of k, V (k): 0.311 and the expected C.V. of k: 0.75. Similarly, expected sampling variance of k, Vs (k): 0.014. Results showed that the number of invariant and highly variable sites were greater than expected.

Haplotype analysis through Minimum spanning network

The haplotype network derived from 16S rRNA complete sequences was presented in Figure 3. Two haplotypes represents individuals of *S. plagiostomus,* 2 in *S. niger* and 1 in *S. esocinus*. The most common haplotype 1 was found in 13 individuals of *S. plagiostomus*, but this was not detected in *S. niger* individuals. This haplotype was also found shared among individuals from *S. esocinus* and *S. progastus*. In this study, most instances of haplotype sharing seem to result from hybridization and deficient taxonomy.

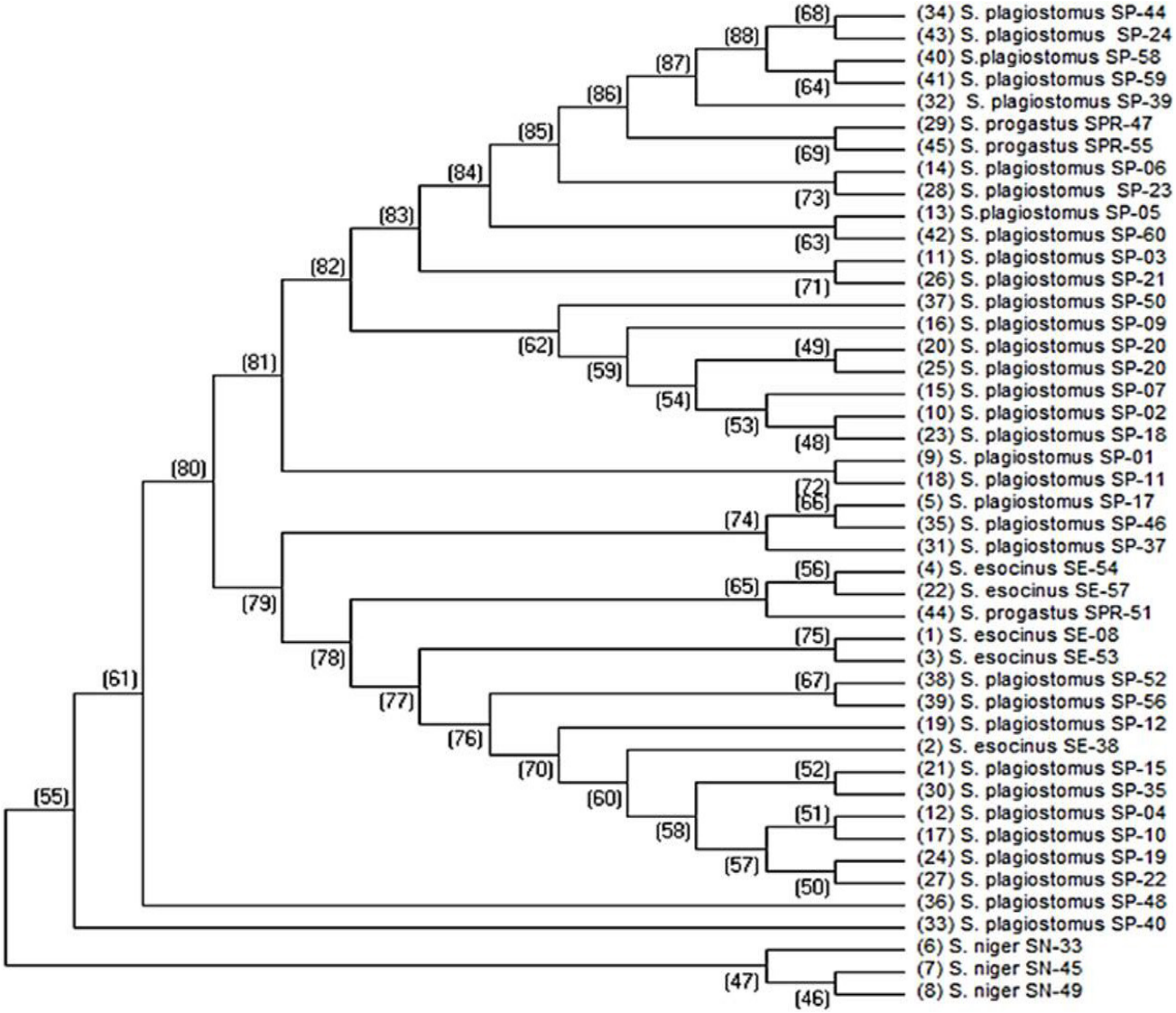

Fig. 1. The phylogenetic analysis of Schizothorax species by the neighbor-joining method using MEGA 6.06. Phylogenetic analysis of Schizothorax species based on complete 16S rRNA gene sequence.

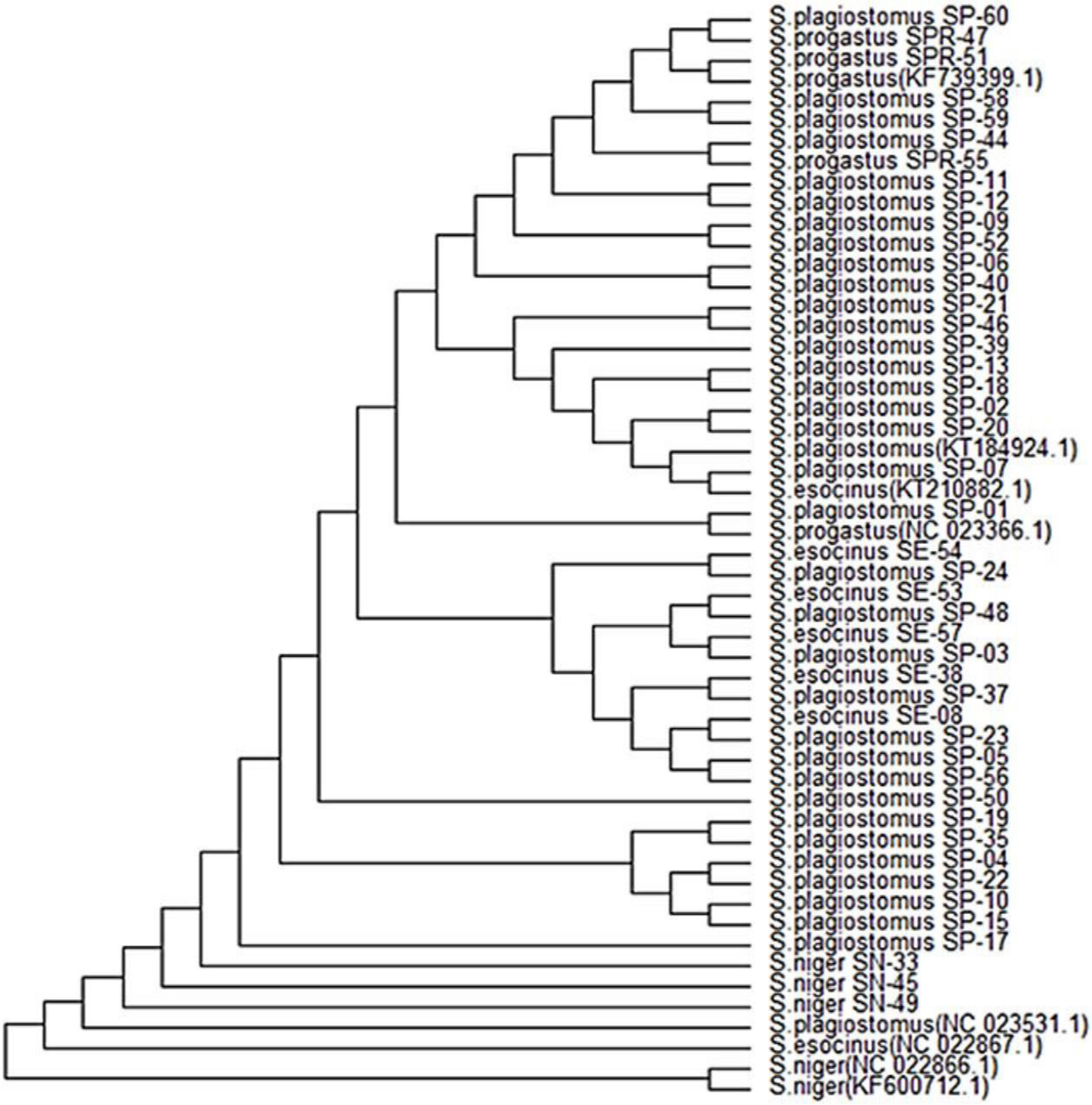

Fig. 2. The evolutionary history was inferred using the neighbor-joining method with eight other Indian Schizothorax species (with accession number).

Amino acids substitutions

Due to conserved nature of 16S rRNA, the substitution rate of nucleotides and amino acids was less as compared to other genes such as D-Loop and Cytochrome b genes. Most of the substitutions were present at first and second codon. In the three species of *S. niger* (SN-33, SN-45 and SN-49), the amino acid H (Histidine) was replaced by amino acid Y (Tyrosine) at position 145. Similarly, in SP-17, the amino acid Y (Tyrosine) was substituted by amino acid C (Cysteine) at position 340. In the SP-02, SP-07, SP-13, SP-18 and SP-20 the amino acid D (Aspartate) was substituted by amino acid N (Asparagine) at position 374. Therefore, these differences could be valuable molecular characters to distinguish these putative species.

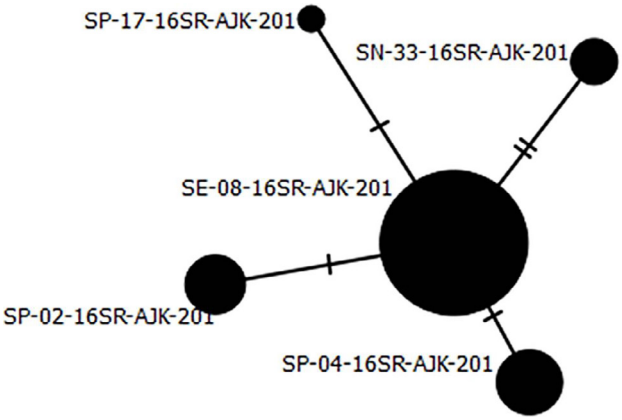

Fig. 3. The minimum spanning network (MSN) of each population by pair-wise distance between each haplotype. Mutations were shown through hatch marks.

DISCUSSION

Complete sets of degenerate oligonucleotides were used to clone DNA fragments encoding portion of complete 16SrRNA gene. The total length of complete 16S rRNA gene varied from 1527 to 1552 bps in the four *Schizothorax* species. Multiple sequence alignments resulted in a consensus length of 1527 sites, excluding sites with gaps and indels. Total 1552 sites, 1547 sites were found to be conserved (monomorphic) and only five were polymorphic. Out of five polymorphic sites, four were parsimony informative while one was singleton. The 16S rRNA gene displays base compositions across the four *Schizothorax* spp, similar to the findings of Zhao *et al.* (2004) for the 12S rRNA gene for cyprinids and by Ahmad *et al.* (2014) for Schizothoracinae (Teleostei: Cyprinidae).

The highest intra-specific similarity (99.9 to 100%) was observed for all *Schizothorax* spp. with respect to the 16S rRNA gene. In case of some individuals, variations in nucleotide sequences among *S. plagiostomus, S. progastus* and *S. esocinus* spp. were negligible, and it is very difficult to conclude that these species are different on the base of 16S rRNA gene. The absence of differences in mtDNA of Schizothoracinae could be elucidated by incomplete lineage sorting, multiple hits (homoplasy) and rapid lineage radiation (Tsigenopoulos and Berrebi, 2000; He and Chen, 2006; Qiao *et al*., 2013). Silas (1960) mentioned that among the *Schizothorax* spp. inter-specific hybridization took place in Kashmir due to the presence of large number of species present, overlays in breeding time, and spatial distributions. Presence of low intra-specific divergence indicated a threatening condition and it is very essential to pay appropriate care of investors for the sustainability of these fishes in wild habitat.

The total number of haplotypes (h) were five, with the high haplotype diversity (Hd) and low nucleotide diversity (Pi). Through the analysis of 16S gene, very low genetic diversity was observed which is caused by many factors such as, background of population, minimum rate of evolution, incomplete lineage sorting and inbreeding due to the small size of population. The loss of genetic diversity is directly correlate with the loss of population (Frankham, 2009). Several genetic factors such as inbreeding depression, decrease in long term survival, reduced adaptation and fitness has been produce by loss of genetic diversity (DeSalle and Amato, 2004; Frankham, 2009). It is more important to notice whether loss of genetic diversity is the cause of population reduction or vice versa. Loss of genetic diversity has been considered crucial genetic factors that tend to produce inbreeding depression, reduced adaptation and fitness and decrease in long term survival (Frankham, 2009; Desalle and Amato, 2004).

Based on 16S rRNA gene, the values obtained for the four fish populations indicate that there is a high differentiation between *S. niger* with other three species from genetic point of view. Along the 1552 bp alignment, only nucleotide substitutions were detected and insertions or deletions were not found. The transition–transversion rate bias has been observed in most vertebrate mitochondrial genes (Meyer, 1993; Sloss *et al*., 2004). The majority of variable and phylogenetically informative sites of 16S rRNA were found on first codon position and the rate of transition/transversion (R=Si/Sv) was also higher (R= 3.131). It indicated several million years of evolution involved in the genetic evolution of different cyprinid spp. (Sivaraman *et al*., 2009).

From a likelihood-based perspective, the Gamma distribution, used to measure the degree of rate variation among sites (Yang, 1996), also supported the hypothesis that most sites have high substitution rates. Simultaneously, the proportion of sites estimated to be invariable is 0.001%, indicating that across the gene less sites are virtually invariable. Thus, the likelihood based models with rate variation among sites and proportion of invariant sites serve as a potential tool to effectively account for situations of this nature (Yang, 1996). The model of evolution of 16s rRNA region was HKY. This model was utilized to determine the substitution types and the incorporation of gamma rate distribution.

A phylogenetic tree was built by aligning the 45 sequences of complete gene of 16S rRNA. Interestingly, contradicting evidence for this gene and its phylogenetic utility comes from its applications among Hymenoptera (Simon *et al*., 1994; Whitfield and Cameron, 1998) and in Schizothoracinae (Ahmad *et al*., 2014). This study

also showed that, the mitochondrial 16S rRNA is unable to provide better resolution of interspecific relationships in Schizothoracinae. The neutrality tests were conducted to determine the neutral evolution among the genus *Schizothorax* based on 16S rRNA. The negative values of Tajima's D and Fu and Li's F* indicated that the genetic variations between species were not neutral which reflect the excess of external mutation. In the current study, based on 16S rRNA gene, the neutrality test rejected the neutral changes. The negative value of Tajima's D test displays the purifying selection of population expansion (Tajima, 1989). It causes the bottleneck effect subsequent the selection pressure on fishes. The negative value of F* indicated that the tests were non-significant thus showing the excess of external mutation. Phyletic classification based on molecular method was clearly showing the genetic differentiation as compared to morphological characters. Identical work has been reported by Saitoh *et al*. (2006) in Cypriniformes.

Due to the conserved nature of 16s rRNA gene, it is commonly used in both eukaryotes and prokaryotes to identify the systematic and taxonomic status of the species, but it is widely used in fish molecular studies (Li *et al.,* 2008). This conserved gene was also used to examine the genetic variability of two Tor species and it was observed that this gene was very helpful among *Tor* brood stocks for analysis of genetic variation (Nguyen *et al*., 2006). In some marine taxa, such as sparids and percoid, the sequence analyses of 16S rRNA gene lacked the ability to resolve relationships (Orrell *et al*., 2004). However, Vinson *et al*. (2004) reported high value of nucleotide divergence among the sciaenid species from Brazil using 16S sequences, and Chakraborty *et al*. (2006a, b) also showed similar results in ribbon fishes and silver biddies, indicating the usefulness of this gene sequence for accurate identification of species.

This study provides an insight into the molecular phylogeny of the species encountered and enhances our comprehension of the historical and taxonomic connections obtained from morphological and ecological studies. This is the first study to report data from a reservoir of the cold-water bodies in Azad Jammu and Kashmir, which hold great potential for the conservation of cold-water fish. In summary, we have initiated a path towards a unifying hypothesis regarding the phylogeny of the Schizothoracinae by analyzing mitochondrial DNA sequences and previous morphological data. Through current study, we were unable to provide more useful insights into phylogenetic relationships and molecular identity with significant polymorphism prevailing in four important *Schizothorax* spp. of AJK. The authors recommend that the combined sequence information of other mitochondrial genes can capably be used as a diagnostic molecular markers in identification and resolution of taxonomic ambiguity of *Schizothorax* spp. in the rivers of Azad Jammu and Kashmir, Pakistan.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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