Intraspecific Genetic Distance and Phylogenetic Evolution of *Schizothorax Plagiostomus* Inferred from Mitochondrial D-Loop Sequences

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Abstract

The fish in the genus *Schizothorax* from the Cyprinidae family live in high-altitude Rivers and streams, are threatened by various anthropogenic stressors. This study aims to characterize *S. plagiostomus* across Pakistan and throughout the world available on NCBI using the mitochondrial D-loop region, and in particular, to assess the degree of intra-specific pairwise distance among these sequences, as well as to establish their phylogenetic relationships. The percent overall nucleotide composition was 32.6% (A), 33.6% (T), 19.8% (C), and 14.0% (G), which infers that *S. plagiostomus* control regions is AT-rich (66.2%) and poor in G contents. The mean pair-wise intra-specific nucleotide diversity (P_i) of all the *S. plagiostomus* was 0.022. While, the inter-specific nucleotide diversity of all the *Schizothorax* species was 0.049. D-loop sequences for intra-specific variations revealed 765 sites were invariable and 10 were variable, 8 parsimony informative sites and only 2 were singletons. The overall transition/transversion ratio is $R = 7.135$. Three domains in *S. plagiostomus* were observed, namely, the termination associated sequence (TAS) domain, the central conserved sequence block (CSB) domain, and the conserved sequence block (CSB) domain. No substitution saturation was detected as an I_{ss} value was significantly ($P < 0.001$) lower than the $I_{ss.c}$ in all cases indicating the suitability of the data for phylogenetic analysis. This study signifies the importance of the control region for the genetic analysis of *S. plagiostomus* and also provides a hypothesis of their phylogenetic relationships.

Introduction

The Cyprinids fish include in the genus of *Schizothorax* encompasses more than 200 species with a world-wide distribution [1, 2, 3]. *Schizothorax* species are economically important as fetching high prices in local market and inhabit cold water bodies including the Neelum and Jhelum Rivers in Azad Jammu and Kashmir [4, 5]. Over-fishing, demolition of their habitat due to road and dam construction and excessive quantity of heavy metals can be considered as the key factors behind the declines of the *Schizothorax* populations [6, 7]. In addition, the deterioration of catchment areas due to unseemly agrarian practices, deforestation, and contamination is lessening water quality, declining these cold-water adapted fishes in some water bodies [2–3, 8]. However, the genetic diversity, evolutionary history and affiliations of these fish species are poorly known [9] as are also their taxonomy and biogeography [10].

The information based on genetic diversity of fish species are applied in genetic improvement programmes and as well as to develop a suitable base population for sustainable use [11]. Preservation of genetic variation within and among populations is an important aspect in management and conservation of biodiversity. Conservation genetics become more important in recent decades [12, 13] and species associated to conservation programs should be genetically characterized and compared with other populations of the same species to set up an appropriate conservation strategies [14, 15]. It is notable that a decrease in genetic variation diminishes the capacity of a population to adjust to the natural changes and in this way diminishes its drawn-out endurance [16]. The success of breeding and conservation programs as well as effectiveness for management policies would benefit from better knowledge of intra- and interspecific genetic diversity and divergence.

Different approaches have been utilized to examine the genetic differentiation, phylogenetic affiliations, biogeographical patterns and taxonomic description of fish and higher vertebrate species [17, 18]. In vertebrates, most of the mitochondrial genome variation confined to a non-coding control (D-loop) region [19] therefore, generally considered as the highly variable in mitochondrial genome [20]. The mitochondrial D-loop region is used to study the genetic structure and phylogeography in many animal species [21, 22] This study constitutes the first attempt to investigate the intraspecific genetic distance and phylogenetic relationships among the *S. plagiostomus* distributed in allied region.

Materials And Methods

For the current study the fish samples (n = 60) were randomly collected from the Jhelum and Neelum rivers (from Ghorī to Kohala) with cast and gill nets during 2013–2014. Only the sixteen samples were amplified and their information is shown in Table (1). The collected fish were anesthetized by immersion in 1% benzocaine in water, and euthanized with over dose of benzocaine. Following the analysis, these samples were stored in 90% ethanol and deposited at UAJK Museum. Approximately 0.1g of tissue was sterilized with ethanol, and then washed three times with distilled water. The total DNA was extracted through a standard phenol-chloroform extraction method of Sambrook et al [23]. The region was amplified with the newly designed primers: D Loop-F (5'-CAT ATA TGT ATT ATC ACC ATT-3'), D Loop-R (5'-GTT TGA CAA GGA TAA CAG GA-3') by using the Primer-3 program (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Sequencing was done for the D-Loop region in Genetic Analyser (ABI Prism 3100, USA). The BioEdit program (<http://www.mbio.ncsu.edu/BioEdit>) was used for sequence editing to determine nucleotide variants. These sequences were deposited to Genbank (KX364221 to KX364236). To study the population of Jammu and Kashmir India, the D-Loop sequences were retrieved from NCBI GenBank resources (Fig. 1 and Table 1). Sequence analyses were carried out using MEGA X [24] software. DNASP 5.0 program [25] was used to calculate no. of haplotypes, haplotype diversity (Hd), and nucleotide diversity (Pi). The Median Joining Network (MJN) was used for haplotype construction through Network 10.2 software. DAMBE (v7.2.14) was applied to calculate the entropy-based substitution saturation and its critical value [26]. The *Barbus barbus* (NC008654) was used as outgroup to confirm the monophyly of the *S. plagiostomus*. The evolutionary divergence was calculated by ML method based on the general time reversible with the gamma distribution shape parameter (GTR + G) model. In addition, the demographic history was inferred by analysing mismatch distributions through pairwise differences between all individuals of each population by using Fu's Fs [27] and by departure from mutation-drift equilibrium with Tajima D test.

Table 1

List of samples subjected to genetic analyses used in this study, showing the specimens, locality with site code, GPS Coordinates and their accessions.

	Code	Locality with site code	GPS Coordinates	Accession no.	Length
Population 1	SP-02	River Neelum, Ghorī (I)	34°26'47.1"N 73°30'38.9"E	KX364221	772
	SP-03	River Neelum, Ghorī (I)	34°26'47.1"N 73°30'38.9"E	KX364222	772
	SP-30	River Neelum, Ghorī (I)	34°26'47.1"N 73°30'38.9"E	KX364223	772
	SP-31	River Jhelum, Domel (III)	34°21'14.6"N 73°28'03.8"E	KX364224	772
	SP-34	River Jhelum, Domel (III)	34°21'14.6"N 73°28'03.8"E	KX364225	772
	SP-35	River Jhelum, Domel (III)	34°21'14.6"N 73°28'03.8"E	KX364226	772
Population 2	SP-39	River Jhelum, Ambor (IV)	34°19'50.3"N 73°27'57.1"E	KX364227	772
	SP-40	River Jhelum, Ambor (IV)	34°19'50.3"N 73°27'57.1"E	KX364228	772
	SP-42	River Jhelum, Ambor (IV)	34°19'50.3"N 73°27'57.1"E	KX364229	775
	SP-44	River Jhelum, Ambor (IV)	34°19'50.3"N 73°27'57.1"E	KX364230	777
	SP-46	River Jhelum, Chatter (V)	34°20'13.8"N 73°27'10.8"E	KX364231	763
	SP-48	River Jhelum, Chatter (V)	34°20'13.8"N 73°27'10.8"E	KX364232	768
	SP-50	River Jhelum, Chatter (V)	34°20'13.8"N 73°27'10.8"E	KX364233	772
	SP-52	River Jhelum, Chatter Kalas (VI)	34°12'25.1"N 73°29'50.8"E	KX364234	772
	SP-56	River Jhelum, Chatter Kalas (VI)	34°12'25.1"N 73°29'50.8"E	KX364235	772
	SP-60	River Jhelum, Kohala (VII)	34°05'48.2"N 73°29'52.0"E	KX364236	772
^a D-Loop sequences retrieved from NCBI					
^b Outgroup specie					

	Code	Locality with site code	GPS Coordinates	Accession no.	Length
Population 3		River Jhelum Kakapora Pulwama	33°95" N, 74°92"E	a JX101883	786
		River Jhelum, Buchbagh Sambora, Pulwama	33°88"N, 74°88"E	a JX101885	786
		River Jhelum Haji peer Uri, Baramullah	34.20" N, 73.98"E	a JX885847	783
		River Jhelum, Hajan Bandipora	34.29" N, 74.61" E	a JX885848	787
		River Jhelum, Buchbagh, Sambora, Pulwama	33.88" N, 74.88" E	a JX978536	786
		River Alaknanda near Srinagar, Uttarakhand	30°15"N 78°55 "E	a KF928796	786
<i>B. barbatus</i>				b NC008654	936
^a D-Loop sequences retrieved from NCBI					
^b Outgroup specie					

Results

Control Region Sequence Structure

The percent overall nucleotide composition was 32.6% (A), 33.6% (T), 19.8% (C), and 14.0% (G), which infers that *S. plagiostomus* CR is AT rich (66.2%) and poor in Gcontents. The D-loop region was AT-rich, and the 5' end was more conserved than the 3' end. The mean pair-wise genetic distance of all the studied samples calculated using the Kimura 2-parameter model (K2P) was 0.003 while, the average number of nucleotide differences (k) was 2.16. D-loop sequences for intraspecific variations revealed 765 sites were invariable in our sample and 10 were variables, 8 parsimony informative sites and only 2 were singletons. The transition/transversion rate ratios are $k_1 = 6.087$ (purines) and $k_2 = 22.446$ (pyrimidines). The overall transition/transversion ratio is $R = 7.135$, where $R = [A \cdot G \cdot k_1 + T \cdot C \cdot k_2] / [(A + G) \cdot (T + C)]$.

We have observed three domains in *S. plagiostomus*, namely, the termination associated sequence (TAS) domain, the central conserved sequence block (CSB) domain and the conserved sequence block (CSB) domain. In these sequences, we detected three TAS motif – TACAT – in 5' region and its reverse complement (RC) 'ATGTA' was also found near the 5' end. After TAS, three central CSB blocks (CSB-F, CSB-E and CSB-D) were also observed. The conserved sequence of CSB-F was ATGTAGTAAGAAACCACCAA,

which distinguished the central CSB block domain from the TAS domain. CSB-E was positioned after CSB-F, whose conserved sequence was AGGGACAACAACCTGTGGGGG. CSB-D was located downstream to CSB-F with its conserved sequence TACTGGCATCTGGTTCCT (Fig. 2). The CSB-F and CSB-D were highly conserved as compared to CSB-E with the few intraspecific variation- transitions (G to A-2nd residue, C to T – 10th and 13th residues).

Generally, these key sequences were highly conserved and easily documented. In the CSB domain, three conserved sequence blocks (CSBs): CSB-1, CSB-2, and CSB-3 of the *S. plagiostomus* were found at the 3'-end of D-Loop. The length of these CSBs was 22, 18, and 14 nucleotides, respectively. Base composition was extremely specific to each CSB as follows: CSB-D, T rich; CSB-I, AT rich; CSB-II, C rich; and CSB-III, AC rich. Moreover, pyrimidine block (TTTTTTCCTTTTTTC) consist of 15 bps was also detected between the TAS-4 and CSB-1 regions (Fig. 2).

Intra-specific D-loop Sequence Variations

The comparative analyses of the current study with all the available sequences of *S. plagiostomus* (above 700 bps) on NCBI, the length of Displacement looprange from 763 to 800 bps. Insertions and deletions (indels) were also found in both the left and right hypervariable domain of these sequences. After the alignment, the 762 sites were found conserved and 105 were observed as variable. Out of variable sites, 69 were parsimony-informativewhile rest were singletons. The overall nucleotide composition consist 32.3–33.3% (A), 32.7–35% (T), 12.9–15.5% (G) and 18.9–20.1% (C), highlighting AT rich vertebrate mtDNA composition. The intraspecific K2P distances ranged from 0.00 to 0.10% (0.025 ± 0.003). Due to sequence similarity, the intraspecific distance of most of the sequences was 0.00. The mean pair-wise intra-specific nucleotide diversity (Pi) of all the *S. plagiostomus* was 0.022. While, the inter-specific nucleotide diversity of all the *Schizothorax* species was 0.049. The maximum divergence (0.01–0.10) was observed among reference sequences retrieved from different countries.

Using DAMBE the substitution saturation was assessed for D-Loop region. In these sequences no saturation was observed as shown a linear correlation when the transitions and transversions plotted against genetic distance (Fig. 3). It was also confirmed from a significantly higher ($P < 0.001$) Iss.c value of both of symmetrical (0.742) and asymmetrical (0.497) as compared to Iss values (0.027). These results depicted the suitability of the data for phylogenetic study. Also it was observed that transitions were outnumbering transversions. Phylogenetic analysis was used to estimate relationships among the studied and reference sequences (retrieved from NCBI) of *S. plagiostomus* to assess historical information of mitochondrial D-Loop region. If we consider the time divergence in mya (million years ago), then we might conclude that *S. plagiostomus* were radiated from other *Schizothorax* species about 0.91 mya while this species itself radiate in 0.05 mya due to the geological event that causes the uplifting of Himalaya as shown in Fig. (4a-b).

Population Wise D-loop Sequence Variations

All the studied samples of *S. plagiosomus* were categorized into population 1 (KX364221-KX364226), population 2 (KX364227-KX364236) and compared with the population (population 3) of Jammu and Kashmir (JX101885.1, JX101883.1, JX885847.1, JX885848.1, JX978536.1, KF928796.1). Average length of D-loop sequences was 867 bps. While, the number of sites (excluding sites with gaps / missing data) were 685. Among these, the 70 were segregating sites in which parsimony informative sites were 28 while 42 were singleton. The total number of 17 haplotypes were observed in *Schizothorax* species and their haplotype diversity (Hd) was 0.970. The haplotype network constructed for D-loop sequences of three *Schizothorax* population was presented in Fig. 5. Hap_8, Hap_9 and Hap_10 were shared among the population 1 and population 2. This haplotypes sharing seems to be the result of hybridization and deficient taxonomy. As shown in Fig. (5) all the sequences of population 3rd form the separate loop and have the unique haplotype and not shared with any other population.

The nucleotide diversity (P_i) of 3 populations was 0.018 and the average number of pairwise nucleotide differences (k) was 12.35. The neutrality tests were conducted to determine the neutral evolution among the three populations of *S. plagiosomus*. The values obtained for different test were, Tajima's $D = -1.566$ ($P > 0.10$); Fu and Li's $D^* = -1.991$ ($P > 0.10$); and Fu and Li's $F^* = -2.177$ ($P > 0.01$). The negative values of Tajima's D shown that the genetic variations among these populations were not neutral under the random effects of genetic drift and mutation which reflect the excess of external mutation.

Discussions

D-Loop is highly mutable and a specific non-coding region (compared with nDNA) in the mtDNA genome due to its fast rate of evolution [28]. The D-loop region (~ 770 bps) was identified by comparing with mitochondrial reference gene sequences from NCBI as per Lalitha and Chandavar [29]. All the D-Loop sequences were subjected to nucleotide BLAST with *S. plagiosomus*, showed the maximum similarity (E -value is less than or equal to 0) authenticating to rule out the risks of numts (nuclear copies of mitochondrial origin). The numts are actually shuffling of mtDNA fragments into nuclear genome [30]. Sorenson and Quinn [30] also suggested to apply newly designed primers using reference sequences available instead of using universal primers. Accordingly, we designed and apply the new primers from the reference sequences of *S. plagiosomus*.

The sequences of D-Loop region of *S. plagiosomus* were conserved with the no deletion/insertion however 1.28% of variable sites were found. The overall nucleotide composition was 32.6% (A), 33.6% (T), and 19.8% (C), and 14.0% (G), emphasizing AT-rich contents of animal mitochondrial genome. The best-fit ML model for control region was found to be T92 based on lowest AIC (Akaike Information Criterion, corrected) criterion values [31]. Although the control region is highly mutable and rapidly evolving portion of mtDNA [32], structurally, it consists of three domains like, TAS domain, central CSB domain and CSB domain, as found in freshwater turtles of order- Geoemydidae [32] and Trionychidae [33]. The TAS domain with the sequence of -TACAT- and its RC sequence -ATGTA- near 5' end were also reported in Cryptodiran and Pleurodiran turtles [33]. These sequences were also observed in current study.

Brzuzan and Ciesielski [34], also reported these TAS motifs in coregonid species and involved in termination of replication process.

Present data revealed that among these CSB, the CSB-3 (GTCAAACCCCTAAA) at position 738–751 show the mutation only in *S. plagiosomus* (KT833100) of China. The *S. plagiosomus* (KT833100) showed two transitions at position 739 (T to C) and position 750 (A to G) and 2 transversions (C to A) at position 740 and (A to T) at 749 position. While the rest of the CSBs, TAS and pyrimidine block were same in all the sequences. Zeng and Liu [35] and Guo et al [36] identified only the central conserved sequence block (CSB-F, CSB-E and CSB-D) in fishes. While, in current study the central CSB block domain (CSB-D, CSB-E, CSB-F) and conserved sequence block domain (CSB 1–3) were also identified in *S. plagiosomus*. CSB-F was used to separate the central CSB domain from the TAS domain. The relative position of these regions has been reported also in some other vertebrates [37, 38]. The consensus sequence of CSB-F, CSB-E and CSB-D in *S. plagiosomus* was highly conserved and consistent with those described in other fishes studied [39, 40]. The CSBs and TAS were also identified in *S. esocinus* of Pakistan [41]. A GTGGG-box (common to euteleosts), next to CSB-D was also identified, and also reported by Syed et al [42] in Indian Schizothoracinae. Moreover, a pyrimidine block (TTTTTTCCTTTTTC) consists of 15 bps was also detected between the TAS-4 and CSB-1 regions. This pyrimidine motif is similarly described in Indian Schizothoracinae [42].

In the primitive Schizothoracinae, the genetic divergence time was estimated through mitochondrial genome. Li et al [43] reported that specifically *Schizothorax* species radiated from Early Pleistocene to Late Miocene (1.0–10.2 Ma) notably the time period of uplifting of Plateau [44]. Possibly the ancestors of *Schizothorax* species were separated through this tectonic unrest, and cause the successive speciation. Present study reported that *S. plagiosomus* were radiated from other *Schizothorax* species about 0.91 mya while this species itself radiate in 0.05 mya due to the geological event that causes the uplifting of Himalaya.

In this study, a relatively high haplotype diversity (0.961) and low nucleotide diversity (0.013) were observed. The combination of high haplotype diversity and low nucleotide diversity also reported from previous studies [45, 46, 47, 48]. This is likely due to rapid demographic expansion from a small effective population size [49, 50]. Most of the haplotypes were shared between population 1 and population 2. The haplotype sharing and its connection with other lowest frequencies indicated that the population undergone a series of expansion event in recent time [51].

The deviation from neutrality estimates through the neutrality tests with Tajima's D and Fu's Fs statistics, that is based on the expectation of a constant population size at mutation-drift equilibrium. Here, a negative Tajima's D (-1.566) indicates an excess of low rate polymorphisms relative to expectation, indicating population size expansion or positive selection [52]. The Tajima's D was also used to estimate of selective neutrality, population bottlenecks and range expansion. The overall Tajima's D value was negative with an insignificant *p*-value, indicating deviation from evolutionary neutrality. Similarly, the Fu's Fs test, indicating the rare mutations in the populations compared to what is expected under a neutral

model of evolution. The significant negative F_u 's F_s statistical value provides strong support for previous population expansion, and exclude the possibility of background selection and evolutionary forces that fabricate a pattern to population expansion [27, 48].

Many fish species are threatened by different factors like, introduction of invasive-exotic species and human activities [53]. These constitute serious challenges which are threatening viability of many fish species, including those of endemic species. The phylogenetic, systematic and taxonomic affinities of cyprinids species of Pakistan, and those of indigenous taxa in particular, are still poorly resolved and highly fragmentary. Due to lack of reliable management plan in this region, natural populations of this species are exposed to overfishing by fishermen. It is almost impossible to bring them back when they are lost. This is the first study to report genetic data of *S. Plagiostomus* from AJK state, where there is a need for devise conservation and management plans for the exploited cold-water fish species. We report the genetic data and phylogenetic relationships among cyprinids, and especially of the *S. plagiostomus*. It is mandatory to prevent overfishing, particularly to prohibit fishing throughout reproductive season.

Declarations

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Author contributions Tasleem Akhtar and Ghazanfar Ali designed the research. Tasleem Akhtar conducted the experiments. Tasleem Akhtar and Ghazanfar Ali analyzed the data. Tasleem Akhtar, Muneeb M. Musthafa and Noor Us Saher interpreted the results and edited the manuscript. All authors read and approved the manuscript.

Conflict of interest The authors report no conflicts of interest.

Compliance with ethical standards

Ethics approval The Board of Advanced Studies and Research at the University of Azad Jammu and Kashmir in Muzaffarabad, Pakistan provided the permit to conduct this study in the Jhelum and Neelum rivers. No specific permission was required for the collection sites.

Consent to participate All individual participants included in the study consent to this manuscript to participate.

Consent for publication All individual participants included in the study consent to this manuscript for publication

Disclosure Statement The authors alone are responsible for the content and writing of the paper.

Financial interests All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Data availability statement

The data that support the findings of this study are openly available in repository under Accession: KX364221 to KX364236 <https://www.ncbi.nlm.nih.gov/nuccore/KX364221> to KX364236.

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Figures

Figure 1

Sampling sites for the three populations of *Schizothorax plagiostomus* (Google map).

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      TAS-1      TAS-2  RC      TAS-3  RC
GGGGTACCACCCCTTATGGTTAGTACATAATATGCATAATATTACATTAATGTACTAGTACATATATGTATTATCACCATTTTATTATCTTAA

CCATAAAGCAGGTAATAATTAAGGTATGCATAAGCATAATATTAACCACTACAAATAATTTTATTTTAAATGGGTAATATATTAATTC

CATAAAAATTTGACCTCAAATTTTCTTGAAATAAACAACTAAATCCCACTAACCATATTAATGTAGTAAGAAACCACCACTAATTT

ATATAAAGGTATATCATGCATGATAGAATCAGGGACAATAACTGTGGGGTTGCACACTGTGAATTATTACTGGCATCTGGTTCCTATTT

CAGGAACATATATTGTAGTATCCACCCCTCGGAAAATTATACTGGCATTGATTAATGGTGTAGTACATATGTCTCGTTACCCACCCAGCC

GAGCGTTCTCTTATATGCATAACGTATTTTCTTTTTCATTTTCATCTGGCATCTCAGAGTGCAGGCTCAAATGTTATTTAAGGTAGAA

CACTTTTCTGTATGTGATAATAAATATTAATTATTGTAAGACATAACCTTAAGAACCACATATTTTAAAGTCAAGTGCAATACATATTCATC

TCTTGTTAGCTTATCTTATATAGTGCCCCCTTTTGGTTTTTGGCGGACAAACCCCTACCCCTACGCTCAGCGAATCCTGTTAT

CCTTGTCAAACCCCTAAACCAAGGAGGACTCAAGAACGC

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Figure 2

The structure and sequence of mitochondrial D-Loop region of *S. plagiostomus*. The termination associated sequence (TAS), central conserved sequence blocks (CSB-F, CSB-E, CSB-D), and conserved sequence blocks (CSB-1, CSB-2, CSB-3), motifs and its palindromic motif were highlighted.

Figure 3

Substitution saturation plot of the control region. The number of transitions (s) and transversions (v) is plotted against F84 genetic distance. A linear correlation is sustained for both transitions and transversions as expected in the absence of saturation.

Figure 4

Divergence time estimates for (a) *Schizothorax* species and (b) *S. plagiostomus* by the Maximum Likelihood method. Branch lengths are proportional to divergence times (MY).

Figure 5

The Median Joining Network (MJN) haplotype construction of *Schizothorax* species. Numbered circles represent haplotypes (Hap), with the circle size corresponding to haplotype frequency. Yellow color indicates Population 1 while, Green Population 2, and Purple Population 3.