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The complete mitochondrial genome of the stream loach (*Schistura sikmaiensis*) and its phylogenetic implications

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Abstract
Stream loaches belonging to the genus *Schistura* are important components of the aquarium trade as well as subsistence fishery in many Southeast Asian countries. Currently, these species are threatened by escalating habitat loss and therefore need instant conservation initiatives. Effective conservation measures, however, require meaningful taxonomic diagnoses of species groups within this genus. To provide an insight into the phylogeny, we sequenced the complete mitogenome of *S. sikmaiensis* for the first time and further studied its phylogenetic implication within Nemacheilidae. The full-length mtDNA of *S. sikmaiensis* was 16581 bp long and consisted of 22 transfer RNA (tRNA) genes, 13 protein-coding genes (PCGs), two ribosomal RNA (rRNA) genes, and a major non-coding control region. All of the genes were encoded on the H-strand, except for eight tRNAs and ND6 gene. The gene order and composition observed in the mitochondrial genome of *S. sikmaiensis* corresponds to the presumed teleost ground pattern. Phylogenetic analyses based on 13 PCGs yielded a well-supported topology that confirmed the non-monophyletic state of the genus *Schistura*. Besides, it also revealed that *S. sikmaiensis* is genetically closer to *S. reticulofasciata* compared to other *Schistura* species. The results presented herein will further promote investigations on molecular taxonomy, genetic diversity, evolution and conservation strategies for *Schistura* and other Nemacheilidae fishes.

Keywords: Stream loach, *Schistura*, mitogenome, phylogeny, Nemacheilidae

Introduction
Genus *Schistura* affiliated to the family Nemacheilidae has more than 228 valid species and still, new species are regularly being discovered under this genus [1]. In aquarium fish trade, *Schistura* species are commonly called as stream loaches, and they are renowned for their predominant size and mesmerising colourful banding patterns. They inhabit among stones in moderate to fast-flowing streams and rivers covering most parts of continental Asia and adjacent islands of Europe and Northeast Africa [2]. Most of the *Schistura* species are currently threatened by habitat loss and therefore enlisted in the Red List of International Union for Conservation of Nature (IUCN) [3]. Effective conservation measures, however, require meaningful taxonomic diagnoses of species. Nevertheless, the accurate identification of *Schistura* species is often a difficult task due to their morphological similarity, overlapping meristic features and lack of genetic data [4, 10]. Additionally, there is a consensus that the genus *Schistura* is polyphyletic [1]. Therefore, the usage of genetic markers for the identification of *Schistura* species would probably help to resolve the taxonomic ambiguity.
The complete mitochondrial genomes have demonstrated their ability in resolving persistent controversies over higher level relationships of teleost due to their small compact genome, conservative gene organisation, high substitution rate and easily accessible nature [11, 12].
In recent years, few studies involving mitochondrial genes such as cytochrome b and D-loop have attempted to establish phylogenetic relationships among a few *Schistura* species and other Nemacheilidae fishes [1, 4, 13, 14]. Nevertheless, many species in *Schistura* have never been sampled or have not been well represented in earlier studies. Consequently, the relationships between the species of *Schistura* remain unclear.
To date, the availability of mitochondrial genome sequence is limited to only eight *Schistura* species out of 228. Therefore, in the present study, for the first time, we have characterized and compared the complete mitochondrial genome of *S. sikmaiensis* with other Nemacheilidae fishes. Further, we reconstructed a comprehensive phylogenetic tree based on 13 PCGs and

identified the phylogenetic position of *S. sikmaiensis* within Nemacheilidae. The generated mitochondrial genome will be a valuable resource for further studies on molecular taxonomy, genetic diversity and population structure of *Schistura* species and also provide new insights for better conservation strategies.

Materials and Methods

Sample collection and DNA extraction

The *S. sikmaiensis* specimen was collected from Khonglah, Meghalaya, India. The morphological identification of this specimen was done by published taxonomic keys [15]. Fin samples were preserved in 95% ethanol and stored at –80 °C until used for DNA extraction. Total genomic DNA from 30-50 mg caudal fin clip was isolated using phenol-chloroform method as described by (Sambrook and Russel 2001). The concentration of isolated total DNA was determined by Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). Furthermore, the quality of extracted DNA was assessed by electrophoresis on a 0.8% agarose gel.

NGS sequencing, assembly and annotation

The genomic DNA library was constructed with Nextera DNA Library Prep kit following the manufacturer's protocols. The constructed library was initially quantified by a Qubit fluorimeter (Thermo Fisher Scientific, USA) followed by quantitative PCR. Finally, the library was sequenced on Illumina's Miseq platform (Genotypic Technology Pvt. Ltd. Bangalore, India) to obtain 2 x250bp reads, which yielded a total of 402,942,305 high quality reads. These high-quality reads were de novo assembled with CLC Genomics Workbench v.7.5.2 (CLC Bio, Aarhus, Denmark) using default parameters. Subsequently, the assembled sequences were mapped back to the complete mitochondrial genome of a congeneric species *Schistura reticulofasciata* (NC_008679). Sequences mapping to *S. reticulofasciata* mtDNA were considered as *S. sikmaiensis* mtDNA.

Annotation of the assembled mitogenome and further circular map illustration were done using the MitoAnnotator web server [15]. Additionally, gene boundaries were compared and confirmed with the annotated mitogenome sequences of the other *Schistura* species. The tRNAScan-SE server version 2.0 was used to identify the 22 tRNAs as well as predict their cloverleaf secondary structures, with default parameters and sequence source set to vertebrate mitochondrial with a cut-off score of 0.1 wherever necessary [16]. BLAST searches were made to identify the rRNA genes by their similarity to published gene sequences (http://www.ncbi.nlm.nih.gov/BLAST/). Base composition, AT-skew and GC-skew were determined for the mitogenome in order to understand the degree of base bias between all PCGs. AT skew and GC skew were calculated according to the formulas: AT-skew = (A%– T%)/(A% + T%) and GC-skew = (G% – C%) / (G%+ C%). Besides, base compositions and mtDNA genetic code were estimated using MEGA X [17]. The overlapping regions and intergenic spacers between genes of the complete mitogenome were determined manually. Finally, the complete annotated mitogenome was submitted to the GenBank using Sequin tool (http://www.ncbi.nlm.nih.gov/Sequin/).

Phylomitogenomic analysis

To determine the phylogenetic position of *S. sikmaiensis*, the phylogenetic analyses were performed on the concatenated protein-coding genes of 49 Nemacheilidae mitogenomes currently available in NCBI database (https://www.ncbi.nlm.nih.gov/refseq/). To root the phylogenetic tree, four species each from the closely related families Psilorhynchidae served as the outgroup. Nucleotide sequences of the 13PCGs were initially aligned with CLC Genomics Workbench 7.5.2. Then, the poorly aligned nucleotides and divergent regions were removed from the nucleotide alignment with Gblocks version 0.91 (http://molevol.cmima.csic.es/castresana/Gblocks_server.htm) using default parameters. The resulting multiple gene alignments were concatenated using CLC Genomics Workbench 7.5.2. The best-fit nucleotide substitution models and partitioning schemes for maximum likelihood (ML) and Bayesian inference (BI) phylogenetic analyses were determined with Partitionfinder-2.1.1 [18] using the Bayesian information criterion (BIC) and a greedy search algorithm with branch lengths linked. The ML analysis for the concatenated nucleotide dataset was performed using the program Iqtree-2.0 [19] based on the partitioned nucleotide alignments, applying the best-fit models as inferred from PartitionFinder2 with 1,000 replicates of ultrafast likelihood bootstrap. Finally, the phylogenetic trees were visualized and edited using Figtree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Results and Discussion

Mitogenome organization, structure and composition

The mitogenome of *S. sikmaiensis* was a closed-circular DNA molecule of 16581bp in length (GenBank: NC_034746.1; Fig. 1, Table 1), which was comparable with other *Schistura* species mitogenome ranging from 16564 bp in *S. balteata* to 16819 bp in *S. geisleri* (Table 1). It contained 13 PCGs, 22 interspersed tRNA genes, two rRNA genes, and two primary non-coding regions (the origin of light strand replication (O_L) as well as the control region; also known as displacement loop region or D-loop). Amongst the 37 genes, nine genes were encoded on the light strand, and the remaining 28 genes were encoded on the heavy strand (Table 2). The gene content and gene order showed resemblance to the typical pattern of other freshwater loach and cyprinid fishes [20, 22]. In addition to O_L and CR, a total of nine intergenic regions, ranging from 1 to10 bp and 11 gene overlaps ranging from 1 to 13 bp were observed at gene junctions (Table 2). These nucleotides overlapping between neighbouring genes are typical features observed in teleost mitogenome and serve to compact the mitogenomes [20]. Further analysis of commonly used mitogenome behaviour indicators such as A + T content, AT-skew, and GC-skew revealed partiality towards A and T in the mitogenome. Similar to other teleost mitogenomes, the most represented base was A, followed by C >T >G [19]. The A + T content was 55.5% in *S. sikmaiensis* and the overall base composition was almost similar to other *Schistura* species (Table 2.)

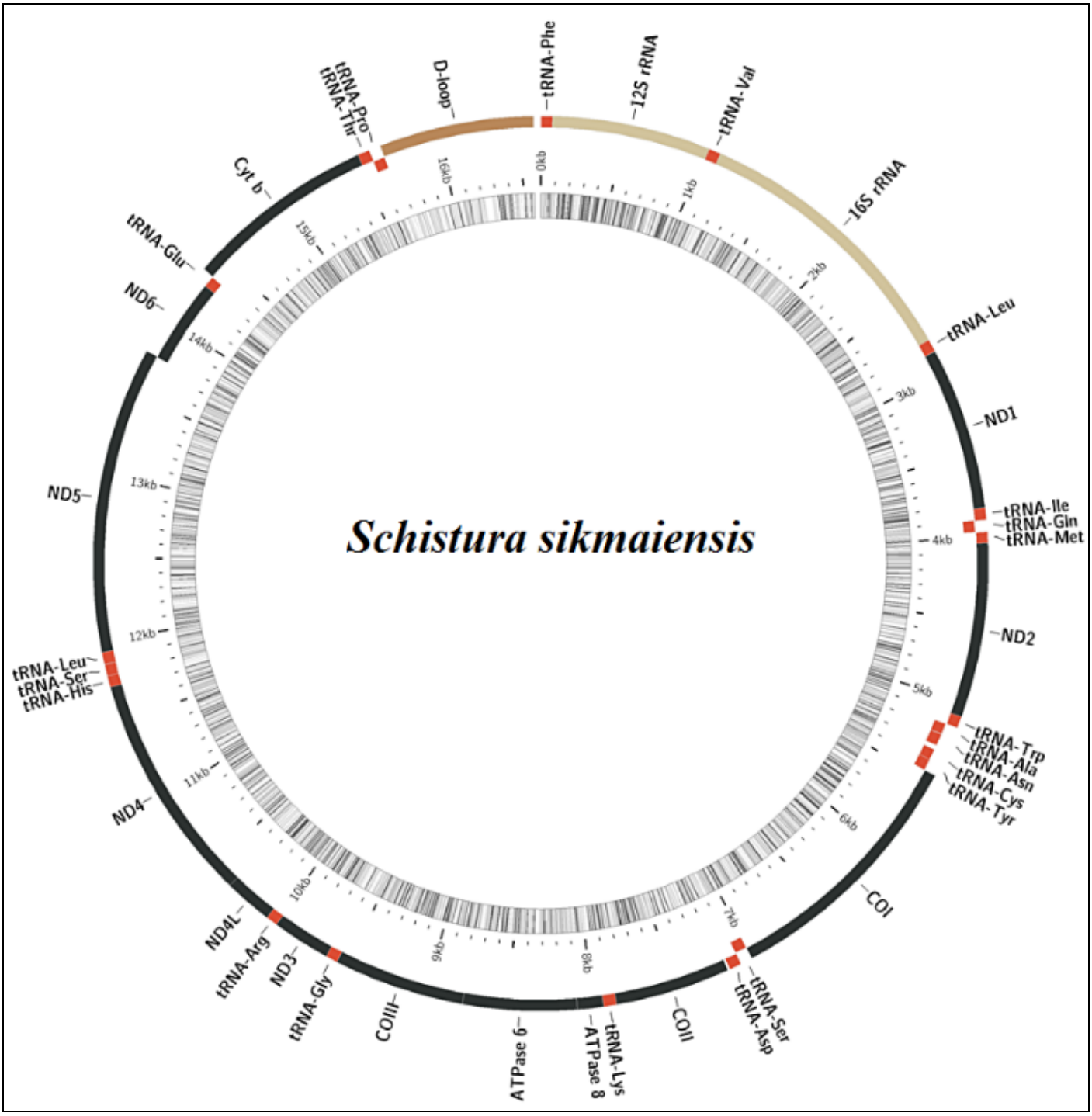

Fig 1. The organization of the mitochondrial genome of *S. sikmaiensis*

Table 1: Nucleotide composition (A + T only) of key components and full-length mitogenomes of the *Schistura* species

Species	Complete		PCGs		12s		16s		CR	
	Length (bp)	AT (%)	Length (bp)	AT (%)	Length (bp)	AT (%)	Length (bp)	AT (%)	Length (bp)	AT (%)
S. balteata	16564	57.6	11423	57.5	950	51.3	1,665	57.5	934	69.3
S. notostigma	16568	55.1	11427	54.6	950	50.4	1,673	54.8	928	67.9
S. corica	16572	56.1	11427	56	951	50.4	1,676	55.4	924	67.9
S. kaysonei	16575	55.4	11427	54.9	952	50.1	1,674	56.2	924	66.9
S. pridii	16576	55.3	11427	54.9	952	50.3	1,672	54.6	935	67.7
S. sikmaiensis	16581	55.5	11433	55.2	950	50.9	1,680	55.5	930	65.4
S. longa	16582	55.4	11428	55.3	951	49.8	1,686	55.0	925	66.2
S. scaturigina	16585	56.6	11427	55.6	957	50.9	1,677	56.1	913	66.9
S. jarutanini	16594	54.8	11427	54.4	951	49.6	1,675	54.6	947	65.9
S. reticulofasciata	16603	55.9	11433	55.4	954	50.6	1,677	56.1	936	67.6
S. geisleri	16819	54.8	11427	54	952	50.3	1,672	55.1	1177	66.3

Table 2. List of annotated mitochondrial genes of *S. sikmaiensis* and its characteristic features [a], Negative value indicates the overlapping sequences between adjacent genes; [b], H: Heavy strand; L: Light strand

Name	Position		Amino acids	Codons		Space (+)	Strand [b]
	From	To		Start	Stop	overlap (-) [a]	
$tRNA^{Phe}$	1	69				0	H
12s	70	1019				-2	H
$tRNA^{Val}$	1022	1093				0	H
16s	1094	2773				0	H
$tRNA^{Leu}$	2774	2848				-1	H
ND1	2850	3824	325	ATG	TAG	0	H
$tRNA^{ILe}$	3825	3896				2	H
$tRNA^{Gln}$	3895	3965				-2	L
$tRNA^{Met}$	3968	4036				0	H
ND2	4037	5083	349	ATG	TAG	2	H
$tRNA^{Trp}$	5082	5151				-2	H
$tRNA^{Ala}$	5154	5222				-1	L
$tRNA^{Asn}$	5224	5296				0	L
OL	5297	5326				0	H
$tRNA^{Cys}$	5327	5392				0	L
$tRNA^{Tyr}$	5393	5461				-1	L
COX I	5463	7013	517	GTG	TAA	0	H
$tRNA^{Ser}$	7014	7084				-1	L
$tRNA^{Asp}$	7086	7158				-13	H
COX II	7172	7862	231	ATG	T..	0	H
$tRNA^{Lys}$	7863	7937				-1	H
ATP8	7939	8106	56	ATG	TAA	10	H
ATP6	8097	8780	228	ATG	TAA	1	H
COX III	8780	9563	262	ATG	T..	0	H
$tRNA^{Gly}$	9564	9635				0	H
ND3	9636	9986	117	ATG	TAG	2	H
$tRNA^{Arg}$	9985	10054				0	H
ND4L	10055	10351	99	ATG	TAA	7	H
ND4	10345	11727	461	ATG	TAG	1	H
$tRNA^{His}$	11727	11796				0	H
$tRNA^{Ser}$	11797	11864				-1	H
$tRNA^{Leu}$	11866	11938				0	H
ND5	11939	13777	613	ATG	TAA	4	H
ND6	13774	14295	174	ATG	TAG	0	L
$tRNA^{Glu}$	14296	14364				-5	L
Cytb	14370	15510	381	ATG	T..	0	H
$tRNA^{Thr}$	15511	15582				2	H
$tRNA^{Pro}$	15581	15651				0	L
D-Loop	15652	16581				0	H

Comparative analyses of protein-coding genes (PCGs)

Altogether, the PCGs of *S. sikmaiensis* were 11433 bp in length and covered 68.8% of the entire mitogenome sequence of the species. The longest PCG was 1839 bp in length (ND5), whereas the shortest one was 168 bp (ATP8) in size (Table 2). The overall length was similar to *S. reticulofasciata*, but greater than other compared *Schistura* species (Table 1). Most of the PCGs of *S. sikmaiensis* were started by ATG initiation codon; the lone exception is COI, which initiated with GTG (Table 2). The start codon usage in the *Schistura* mitogenome was found to be the same as that of teleost fishes [23]. Out of 13 PCGs, seven genes terminated with TAA and three with TAG stop codon. The start codon usage was found to be the same as that of teleost fishes [21]. The remaining three genes (COII, COIII, and Cytb) had an incomplete stop Codon "T.." which would be presumably completed as the entire stop codon (TAA) via post-transcriptional polyadenylation [24, 25].

A total of 3811 amino acids were encoded in the PCGs of *S. sikmaiensis*. Regarding usage, the most frequent occurrence was observed for leucine (14%) followed by threonine (8.7%). Meanwhile, the least frequent incidence was found for codons encoding cysteine. It should be noted that two amino acids (serine and leucine) were coded by six separate codon formats, whereas the remaining amino acids were coded by either two or four different codons (Fig 2). Codons ending in U and A were the most frequent, which is associated with the high A+T content mitogenome.

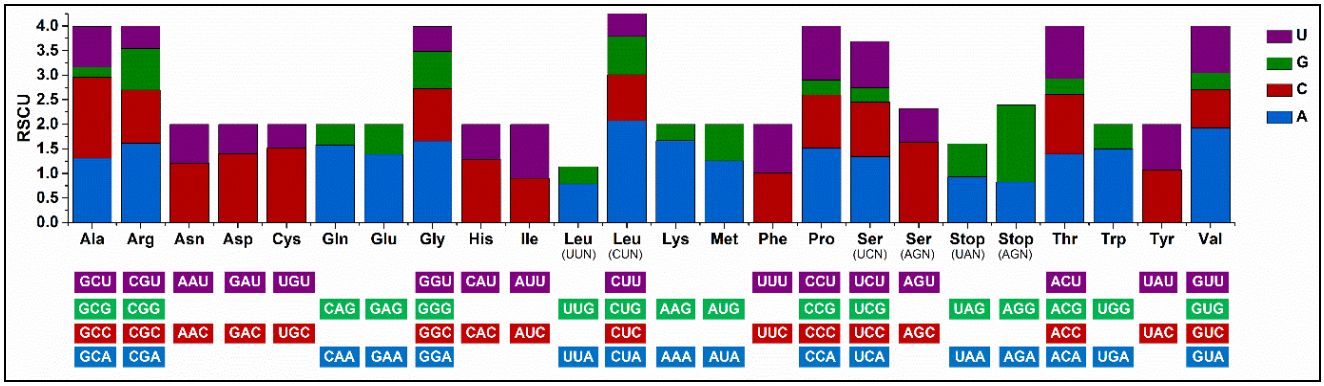

Fig 2: The relative synonymous codon usage (RSCU) of the mitochondrial protein-coding genes of *S. sikmaiensis*

Transfer RNA (tRNAs) and ribosomal RNA (rRNAs)

Sequence analyses revealed that *S. Sikmaiensis* mitogenome contained two rRNA subunits and twenty-two tRNAs typical of vertebrate mitochondrial genomes. The two rRNA subunits, 12S and 16S genes of *S. sikmaiensis* were 950 and 1680 nucleotides long. As in other vertebrates, they were located between $tRNA^{Phe}$ and $tRNA^{Leu}$ (UUR) genes and separated by the $tRNA^{Phy}$ (Fig 3).

The tRNAs were interspersed between the rRNA and protein-coding genes and ranged from 66 bp to 76 bp with a total length of 1559 bp in *S. sikmaiensis*. Of these 22 tRNAs, two were determined to be for serine (TGA and GCT), and other two were for leucine (TAA and GTT) in *S. sikmaiensis* genome. Amongst all the tRNA genes, fourteen genes were located in the H-strand while the remaining genes were encoded on the L-strand. Altogether, tRNAs could be folded into canonical cloverleaf secondary structures except tRNAser (GCT) which lacked a discernible dihydrouridine (DHU) stem (Fig 3).

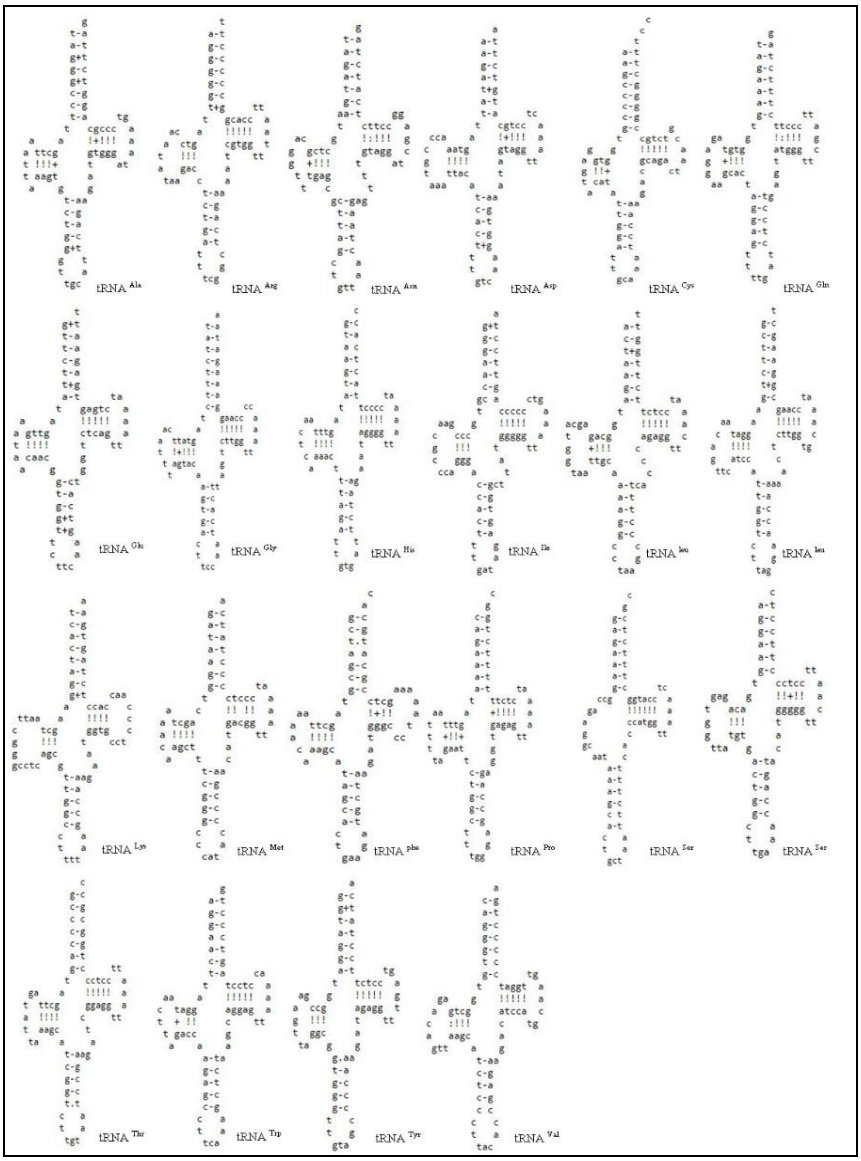

Fig 3: Putative secondary structures for 22 tRNA genes in mitochondrial genome of *S. sikmaiensis*

Control region (CR)

In *S. sikmaiensis*, the origin of L-strand replication, with 30 bp, was located in a cluster of five tRNA genes (WANCY region) between $tRNA^{Asn}$ and $tRNA^{Cys}$. The position of O_L in mitogenome was consistent with the findings of previous reports on other teleosts. Further, the 930 bp long intergenic region assumed to be the D-loop/CR is located between the tRNA ^{pro} and $tRNA^{phe}$ genes (Fig.1). The region was A + T rich with an overall AT composition of 65.4% (Table 1). Similar to other teleost fishes, the central conserved sequence block domain (CSB-A, B, C, F and F) and conserved sequence block domains (CSB-I, CSB-II, and CSB -III) were depicted in the conserved domains of *S. sikmaiensis* CR (Fig. 4). However, CSB-E, which is commonly found in fishes, was not found in the control region of *S. Sikmaiensis* [25, 26]. It suggest that the regulative mechanisms of transcription m vary between species, and could provide significant diagnostic characters.

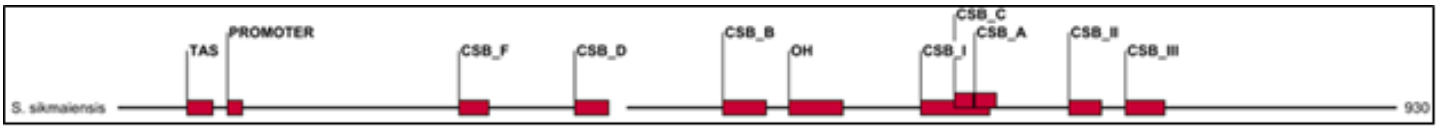

Fig 4: Conserved features present in the control regions of the *S. Sikmaiensis* mitogenome.

Phylogeny

To identify the phylogenetic position of *S. sikmaiensis* and study the relationships among the Nemacheilidae species, a dataset of 53 species containing the concatenated nucleic acid and amino acid sequences of 13 PCGs was used to generate phylogenetic relationships (Fig. 5). The ML tree placed *S. sikmaiensis* as a sister group to *S. reticulofasciata* with maximum bootstrap support. In the resulting phylogenetic tree, species of *Schistura* grouped in three different clades within Nemacheilidae. Majority of the *Schistura* species including *S. sikmaiensis* were found in Clade 1, which contains species from *Mesonoemacheilus, Oxynoemacheilus* and *Nemacheilus.* However, two other *Schistura* species namely *S. kaysonei and S. dabryi* clustered more closely to *Homatula* and *Triplophysa* species respectively. This grouping pattern of *Schistura* species was broadly consistent with earlier study and confirmed the consensus that the genus *Schistura* is not monophyletic [1]. However, further mitogenome data from remaining Nemacheilidae fishes would be useful for a better understanding of the phylogenetic and evolutionary relationships among *Schistura* species and other Nemacheilids. Finally, the present study recommends that the systematics of *Schistura* species needs a comprehensive integrated taxonomic study including morphological as well as molecular examination for proper conservation and management.

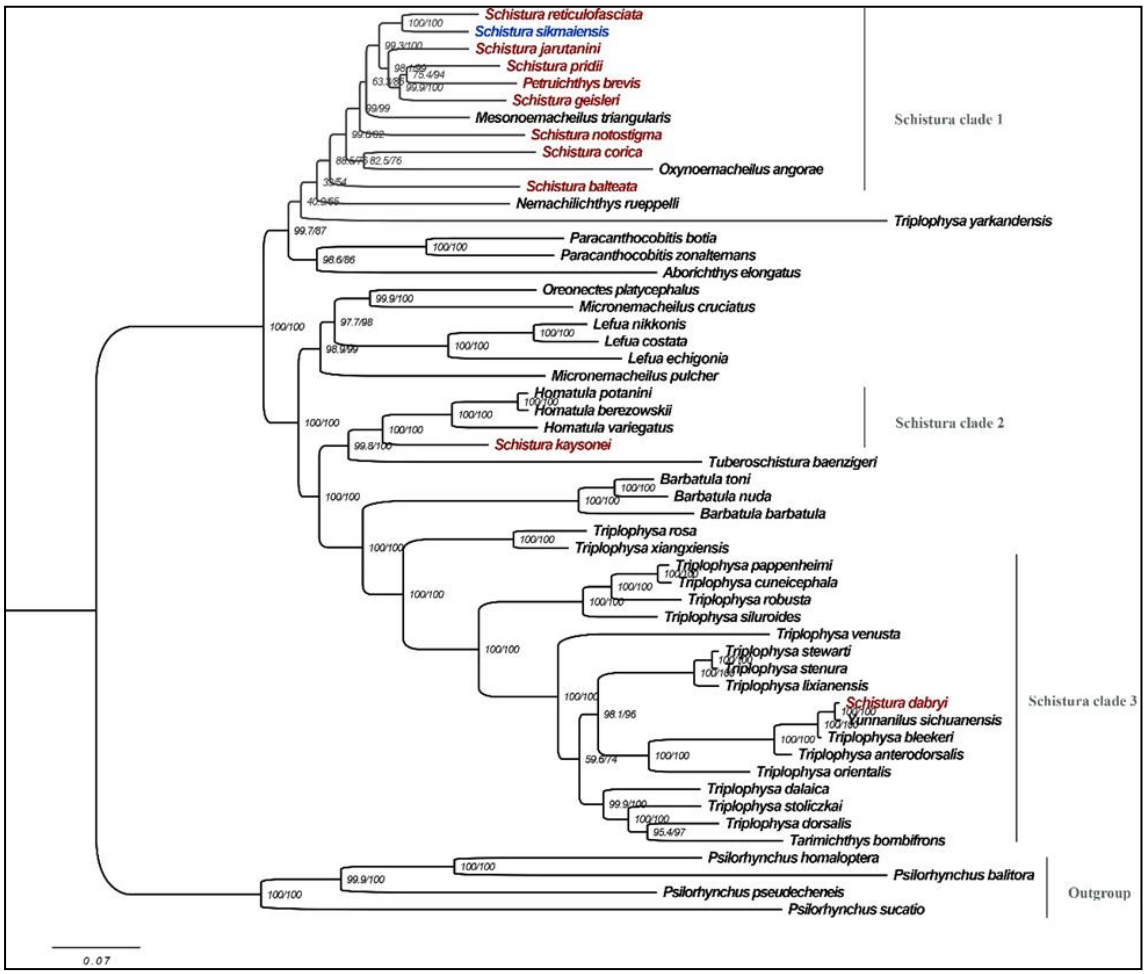

Fig 5: Phylogenetic tree inferred from nucleotide sequences of 13 PCGs of the mitogenome using ML analysis. The numbers along branches indicate bootstrap support values

Conclusion

We characterised the first complete mitogenome of *S. sikmaiensis* using next-generation sequence technology.

Consistent with typical teleost mitochondrial genome, the sequences of *S. sikmaiensis* mitogenome was highly conserved in terms of gene content, gene size, gene order, base composition, codon usage of PCGs, and RNA secondary structures. The ML analysis confirmed the consensus that the genus *Schistura* is not monophyletic. Therefore, the systematics of *Schistura* species require additional comprehensive integrated taxonomic studies including morphological as well as mitogenome data for proper conservation and management.

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