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The Complete Mitochondrial Genome of *Bangana dero* (Cyprinidae: labeoninae) and its Relationship with Other Labeonin Fishes

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ABSTRACT

Background: *Bangana dero* is a cyprinid fish species that is classified under the subfamily Labeoninae. It occurs in the streams of China, India and Nepal near the foot of the Himalayan range. *B. dero's* whole mitochondrial genome has been sequenced and annotated. In this work, the tRNAs, protein-coding genes have been characterized and analyzed the phylogenetic relation of this fish with other cyprinid species.

Methods: Nanopore sequencing was utilized to determine the entire mitochondrial genome of *B. dero*. Utilizing NEBNext Ultra DNA Library Prep Kit, end-repair was performed on the isolated DNA using the. Barcoding adapter ligation (BCA) and cleaned. Sequencing was done. All the long read bases were trimmed based on lowest base quality score, Q<9. Barcode and adapters sequences were also trimmed using a porechop programme and the statistical graph illustrations were generated and genome assembly was done using Canu1.5.

Result: The mitogenome is 16613 bp and contains 13 protein-coding genes, 2 rRNA genes, 22 tRNA genes, as well as a non-coding region. The nucleotide makeup is A=32.5%, T=25.21%, C=26.51% and G=15.77%, G + C = 42.28% and A+T=57.71%. The clover-leaf secondary structure was found in all tRNAs except tRNA-Ser2, which has no dihydrouridine arm. For 12 out of 13 protein-coding genes, the initiation codon was ATG, but COI used GTG. Methionine and tryptophan are unique among amino acids as they are each encoded by a single codon. Origin of light chain replication (OL) could fold into a hairpin secondary structure and carried a sequence 5′-GGCGG-3′ in its stem. A long intergenic spacer of 46 bp was observed between tRNA-Leu1 and ND1. In the phylogenetic study, *B. dero* clustered closely with the *Bangana* species (AP013327) and *Labeo boggut* (NC_029450), a grouping supported by a bootstrap value of 100. This is the first study on the entire mitochondrial DNA analysis of *B. dero*.

Key words: *Bangana dero*, Mitochondrial genome, Phylogenetic analysis, Protein-coding genes, rRNAs, Transfer RNA.

INTRODUCTION

Bangana dero (Hamilton, 1822) is a popular food and game fish from the Labeoninae subfamily of the Cyprinidae family. The fish is usually found in the streams of China, India and Nepal near the foot of the Himalayan range (Vishwanath, 2010). Commonly, it is called as Kalabans but also known as *Ngaton* or *khabak* (in Manipuri), *Khital* (Tangkhul) and *Ngatai* (Myanmar). It is one of the most popular indigenous minor carps and has great demand in the local markets of Manipur. Morphologically, *B. dero* is distinct from other *Bangana* species due to the existence of a small black spot at the caudal fin base along with a black mark above the pectoral fin (Talwar and Jhingaran, 1991; Vishwanth, 2002).

Although morphological features are commonly used for identification of fish species, the ambiguity in the morphological characteristics and overabundance of synonyms make it difficult to correctly identify and determine the phylogenetic relationship with other species. Therefore, species identification based on the molecular markers is widely accepted. Basudha *et al*, (2019) reported that *B. dero* has been molecularly characterized using the 16SrRNA gene nucleotide sequences. However, the determination of a phylogenetic relationship based on a single gene remains unclear. Therefore, the development of suitable molecular markers is required for identification and classification of *Bangana* species. For examining phylogeny, genomics and molecular evolution, the mitochondrial genome is generally considered a suitable model (Yang *et al*., 2018). Furthermore, the mitochondrial

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genome has constant gene content and numerous cell copies making it a simpler system for molecular studies than the nuclear genome. Mitochondrial DNA is a desirable tool for evolutionary biology research due to its rapid evolution rate, maternal inheritance, along with minute size (Wolstenholme, 1992; Moritz *et al*., 1987).

This study involved comprehensive sequencing along with annotation of *B. dero*'s mitochondrial genome. We investigated cyprinids phylogenenetic relationships and defined the tRNAs along with protein-coding genes. Since there have been reports of significant declines in this species in several areas of the north-east states of India, the information derived from this mitogenome will be helpful in genetic conservation, recovery and population enhancement. Further, it will also help investigate lineage evolution and molecular phylogenetics.

MATERIALS AND METHODS

Collection of samples and DNA isolation

From the fish farm of the ICAR Research Complex for NEH Region Manipur Centre, Imphal (24.8287° N, 93.9267° E), the specimens of *Bangana dero* were obtained. Based on the previous morphological descriptions (Talwar and Jhingaran, 1991; Vishwanth, 2002), the specimens were identified. Muscle tissues and fin clips from ten fish samples were removed and each stored in 95% alcohol. The tissue samples were kept at -80°C until they were used for isolation of DNA. Following the manufacturer's instructions, mitochondrial DNA was extracted from 40 mg of tissue samples using the Mitochondrial DNA Isolation Kit (GM 11, GCC Biotech, India).

Mitogenome sequencing and assembly

The isolated DNA was end-repaired by means of the NEBNext Ultra DNA Library Prep Kit of (New England Biolabs, MA, USA) and then cleaned up with with 1xAmPure beads (Beckmann Coulter, USA). BCA (Barcoding adapter ligation) had been done utilizing NEB blunt/TA ligase (New England Biolabs, MA, USA), additionally, cleaning was done with 0.4 x AmPure beads. Utilizing a SpotON flow cell, data was sequenced on a MinION Mk1b (Oxford Nanopore Technologies, UK) over the course of 48 hours using MinKNOW. ONT-Albacore was used for base-calling. The file format was converted from Fastq to Fasta file by Fastx-Toolkit software. The QC of the bases was fixed to the cut of Q>9 Phred scores and all long reads statistics were calculated by NanoStatprogramm. Subsequently, all the long reads were validated and plotted by NanoPlotprogramm. All the long reads bases were trimmed based on the lowest base quality score Q<9. Barcode and adapter sequences from the tail and head were trimmed using the porechop programe of the NanoFilt tool. All the statistical graph illustrations were generated using NanoQC software and genome assembly was conducted by Canu version 1.5.

Mitogenome Annotation and Analysis

The assembled mitogenome was annotated using a pipeline for annotating fish mitogenomic sequence, MitoAnnotator (http://mitofish.aori.u-tokyo.ac.jp/annotation/input/) following published reports (Iwasaki *et al*., 2013; Sato *et al*., 2018; Zhu *et al*., 2023). The tRNA's secondary structures has been determined using tRNA-scan 2.0 and mapped using the Forna web server (Chan_and Lowe, 2019; Kerpedjiev *et al*., 2015). We used the RNA fold website (http://tna.tbi.univ.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) to ascertain the possible light strand (OL) replication's secondary structure. MEGA (Molecular Evolutionary Genetics Analysis) Version 11 was used to analyze the protein-coding genes (PCGs) and identify their nucleotide composition and RSCU (relative synonymous codon uses) (Tamura *et al*., 2021). The nucleotide composition's skew values had been determined using the subsequent formulas (Perna and Kocher, 1995):

$$\text{AT Skew} = \frac{A-T}{A+T}$$

$$\text{GC skew} = \frac{G-C}{G+C}$$

The whole annotated mitogenome was subsequently submitted to the NCBI GenBank.

Phylogenetic analysis

The nucleotide sequence of *B.dero* mitogenome was subjected to basic local alignment search tool (BLAST) to locate the similar sequences available in the NCBI GenBank. Thirty-four (34) sequences were selected from the top 100 similar results and the nucleotide sequences were downloaded for phylogenetic analysis. Another four mitogenome sequences belonging to *Psilorhynchus* species of the closely related family, Psilorhynchidae, were also included as outgroups in the analysis. Using MUSCLE of MEGA 11 a total of 39 sequences, including the *B. dero* mitogenome was aligned (Tamura *et al*., 2021). Using the neighbour-joining method, the phylogenetic tree has been generated using the substitute model (T93+G) (Saitou and Nei, 1987). A bootstrap of 1000 repetitions was used to determine the properties of replicate trees that contained the relevant taxonomic categories (Felsenstein, 1985). Tamura-Nei method was used to depict the evolutionary distances and the tree was constructed with branch lengths that match those in size (Tamura and Nei, 1993). All the positions with less than 95% site coverage were eliminated, allowing less than 5% alignment gaps, missing data, and ambiguous bases.

RESULTS AND DISCUSSION

Gene organization and arrangement

The mitochondrial genome of *B. dero* is a circular molecule with 16,613 base pairs (Fig 1). Vertebrate mitogenomes typically have a length of 16-20 kb with conserved order of

genes (Boore, 1999; Roe *et al*., 1985; Bibb *et al*., 1981). Accession number MK461139 was obtained after submitting the whole nucleotide sequence to NCBI GenBank. The mitogenome of *B. dero* was comparable to that of other Labeoninae fish such as *Bangana decora* (16,607 bp), *Cirrhinus reba* (16,597 bp) and *Parasinilabeo longicorpus* (16596 bp) (Li *et al*., 2016; Islam *et al*., 2020; Pan *et al.,* 2020). The mitogenome's base frequency was A=32.49%, T=25.23%, C=26.53% and G=15.73%, being biased to A+T (57.72%). In the present study, the AT and GC skews were 0.126 and -0.225, respectively. Sharma *et al*. (2020) also found similar skewness in other cyprinid fishes. A typical collection of 37 genes that make up the mitogenome comprises of 1 non-coding control region, genes, 22 tRNA genes, 2 rRNA genes (12S and 16S) and 13 protein-coding genes (PCGs) (Table 1). Among them, 9 genes (ND6 and 8 tRNAs) are encoded on the light strand (L) and the remaining 28 genes are located on the heavy strand (H) similar to that of *B. decora* and other cyprinid fish (Li *et al*., 2016).

In the mitogenome, overlapping nucleotides between the adjacent genes were observed (Table 1). The sequences overlapped between tRNA-Ile and tRNA-Gln (2 bp), ATP8 and ATP6 (7 bp), ND4L and ND4 (7 bp), ND5 and ND6 (4 bp), tRNA-Thr and tRNA-Pro (1 bp) (2 bp) and other locations on the H strand. Their lengths ranged from 1-7 bp. The overlapping nucleotide sequences between adjacent genes helped compact the mitogenome and were also a distinctive feature of the teleost mitogenome (Satoh *et al*., 2016; Yang *et al*., 2018). Fish in the Cyprinidae family often exhibit overlapping sequences between ATP8/ATP6 and ND4L/ND4 (Sharma *et al*., 2020).

Protein coding genes

The PCGs in the *B. dero* mitogenome have NADH dehydrogenase's 7 subunits of (ND1-6 and ND4L), cytochrome c oxidase's 3 subunits (COI, COII, along with COIII), 2 subunits of ATP synthase (ATP8 and ATP6), as well as 1 subunit of cytochrome b oxidase (CYT B). The 13 PCGs length varied from 165 bp of ATP8 to 1824 bp for ND5, paralleling those of *Cyprinion semiplotum* (Sharma *et al*., 2020). However, the length of ATP8 is different from that of *B. decora,* which is 168 bp (Li *et al*., 2016). ATP8 and ND5 being the shortest and longest genes, respectively, have been found in most fishes classified as cyprinid (Gutiérrez *et al*., 2015; Satoh *et al*., 2016). PCGs had an average A+T content of 57.74%; COIII had the lowest average (53.69%), while ATP8 had the highest average

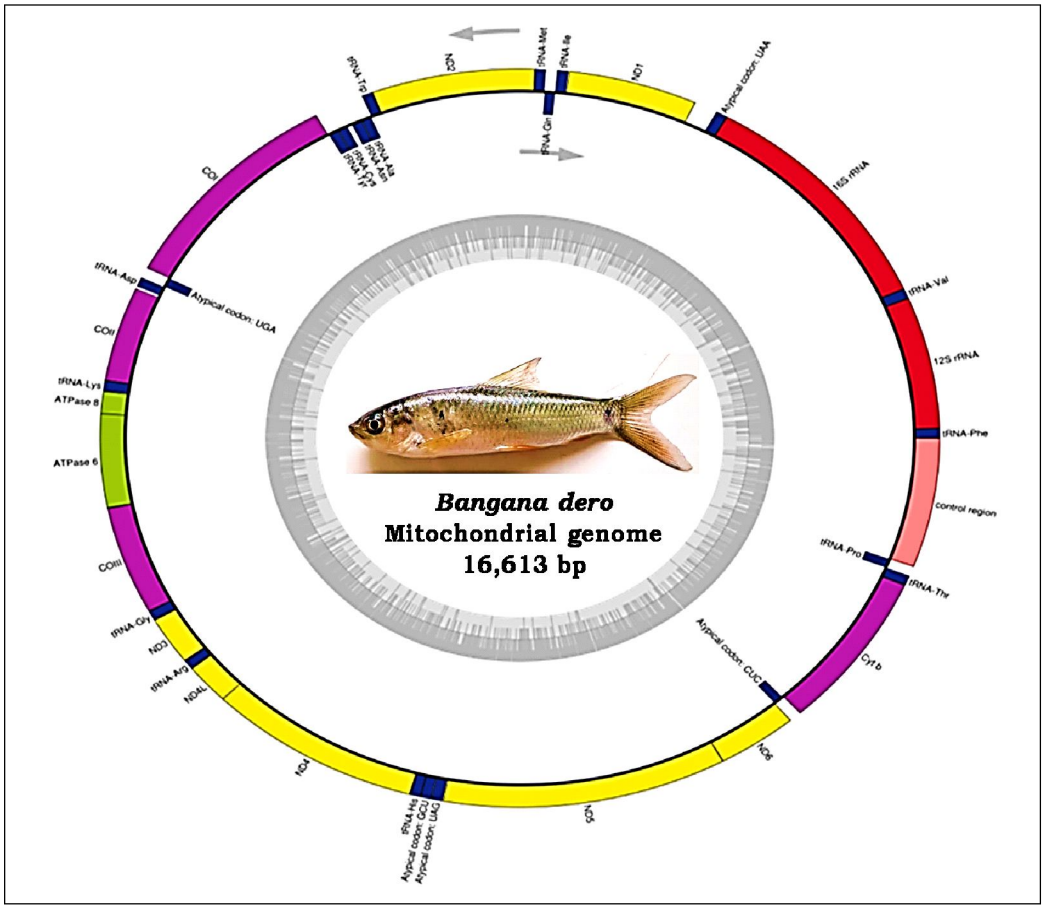


Fig 1: Organization of the genes in the mitogenome of *B.dero*.

(63.03%). Fig 2 display the skew values of AT and GC of all 13 PCGs and illustrated the trend. All the PCGs except ND6 showed negative GC skewness which is in corroboration with other teleost mitogenome (Lu *et al*., 2019; Li *et al*., 2019). While 12 PCGs used ATG as the start codon, like other cyprinid mitogenomes, COI used GTG, which is frequently seen in fish mitogenomes (Siva *et al*., 2018). Eight PCGs' stop codons contained the typical termination codons (TAA as well as TAG), whereas the stop codons of the other five PCGs had truncated codons (TA and T), which may have indicated post-transcriptional polyadenylation (Siva *et al*., 2018; Yu *et al*., 2019). Fig 3 shows the graphic representation of PCGs RSCU (relative synonymous codon usage). Serine and leucine are

Table 1: List of annotated genes in the mitogenome of *B. dero* and its characteristic features.

Name	Position		Size (bp)	Start codon	Stop codon	Anticodon	Space (+)	Overlap (-)[a]	Strand[b]
	From	To							
tRNA- *Phe*	1	69	69				GAA	0	H
12SrRNA	70	1026	957					0	H
tRNA- *Val*	1027	1098	72				TAC	0	H
16SrRNA	1099	2789	1691					0	H
tRNA- *Leu1*	2790	2865	76				TAA	46	H
ND1	3012	3842	831	ATG	TAA			4	H
tRNA- *Ile*	3847	3918	72				GAT	-2	H
tRNA- *Gln*	3917	3987	71				TTG	1	L
tRNA- *Met*	3989	4057	69				CAT	0	H
NAD2	4058	5102	1045	ATG	T-*			0	H
tRNA -*Trp*	5103	5173	71				TCA	2	H
tRNA- *Ala*	5176	5244	69				TGC	1	L
tRNA- *Asn*	5246	5318	73				GTT	0	L
OL	5319	5351	33					0	-
tRNA- *Cys*	5352	5418	67				GCA	1	L
tRNA- *Tyr*	5420	5490	71				GTA	1	L
COI	5492	7042	1551	GTG	TAA			0	H
tRNA- *Ser1*	7043	7113	71				TGA	3	L
tRNA- *Asp*	7117	7188	72				GTC	13	H
COII	7202	7892	691	ATG	TAA			0	H
tRNA- *Lys*	7893	7968	76				TTT	1	H
ATPase8	7970	8134	165	ATG	TAG			-7	H
ATPase6	8128	8810	683	ATG	TA*			0	H
COIII	8811	9596	786	ATG	TAA			0	H
tRNA- *Gly*	9597	9668	72				TCC	0	H
ND3	9669	10017	349	ATG	T-*			0	H
tRNA- *Arg*	10018	10087	70				TCG	0	H
ND4L	10088	10384	297	ATG	TAA			-7	H
ND4	10378	11758	1381	ATG	T-*			0	H
tRNA- *His*	11759	11827	69				GTG	0	H
tRNA- *Ser2*	11828	11896	69				GCT	1	H
tRNA- *Leu2*	11898	11970	73				TAG	3	H
ND5	11974	13797	1824	ATG	TAA			-4	H
ND6	13794	14315	522	ATG	TAG			0	L
tRNA- *Glu*	14316	14384	69				CTC	5	L
Cyt b	14390	15530	1141	ATG	T-*			0	H
tRNA- *Thr*	15531	15602	72				TGT	-1	H
tRNA- *Pro*	15602	15671	70				TGG	0	L
D-loop	15672	16613	942						H

[a]Negative value indicates the overlapping sequences between adjacent genes.

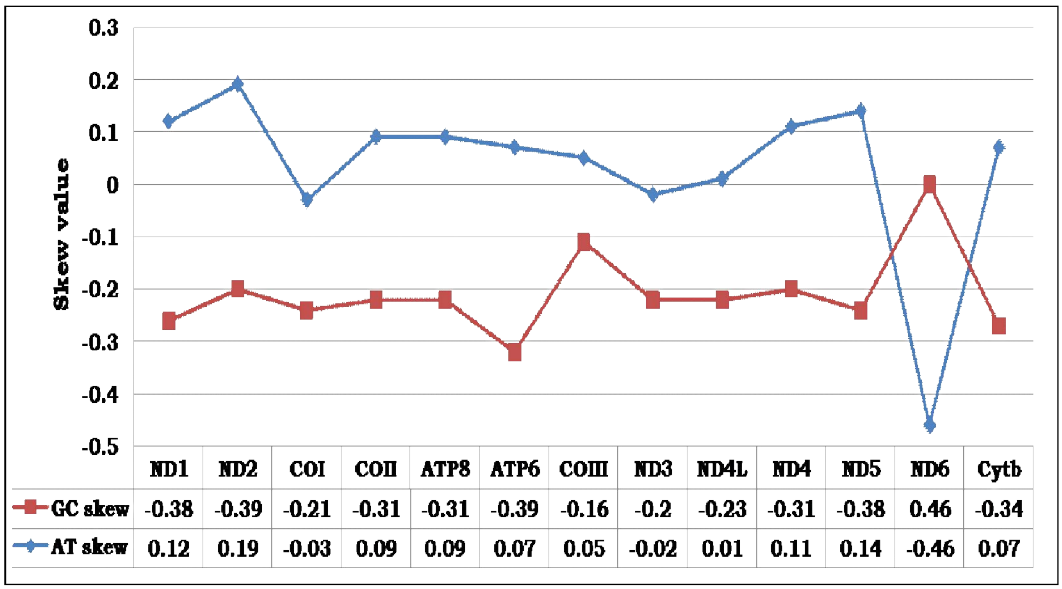
[b]H: heavy strand; L: light strand.

*Incomplete stop codon, Might indicate subsequent polyadenylation.

represented by six codons in contrast to other cyprinid fish, while tryptophan and methionine are encoded by a single codon (Zhang *et al*., 2022). Two or four codons are employed to encode the remaining amino acids. The top utilized codon was CGA (Arginine), followed by CUA (Leucine), CCA (proline), UCA (Serine), GGA (Glycine), GUA (Valine) and ACA (Threonine). It was observed that the codons most used are biased towards A in its third position, which is consistent with other Cyprinid fishes (Sharma *et al*., 2020).

Transfer and ribosomal RNAs

The *B. dero* mitogenome has 22 tRNAs, ranging in size from 67 bp (tRNA-Cys) to 76 bp (tRNA-Leu1 and tRNA-Lys). Eight of the 22 tRNAs (tRNA-Gln, tRNA-Ala, tRNA-Asn, tRNA-Cys, tRNA-Tyr, tRNA-Ser1, tRNA-Glu and tRNA-Pro) are encoded on L strand, whereas the remaining 14 tRNA genes have been encoded by H strand. Anticodons for GTC (tRNA-His) and CTC (tRNA-Glu) was different from GTG (tRNA-Glu) and TTC (tRNA-Glu) (Sharma *et al*., 2020; Li *et al*., 2022). There were two anticodons for serine (UGA and GCU) and leucine (UAA and UAG). A typical characteristic of fish mitogenomes is the presence of multiple tRNAs with distinct anticodons for a single amino acid (Cui *et al*., 2017; Villela *et al*., 2017). Fig 4 shows the secondary structures predicted by tRNA scan 2. All tRNAs except tRNA Ser (GCT), which lacks dihydrouridine arm, exhibit the conventional clover leaf shape. A similar case was found in *B. decora* (Li *et al.,* 2016).



	ND1	ND2	COI	COII	ATP8	ATP6	COIII	ND3	ND4L	ND4	ND5	ND6	Cytb
GC skew	-0.38	-0.39	-0.21	-0.31	-0.31	-0.39	-0.16	-0.2	-0.23	-0.31	-0.38	0.46	-0.34
AT skew	0.12	0.19	-0.03	0.09	0.09	0.07	0.05	-0.02	0.01	0.11	0.14	-0.46	0.07

Fig 2: Graphical presentation of AT and GC-skew in 13 protein-coding genes (PCGs) of *B. dero* mitogenome.

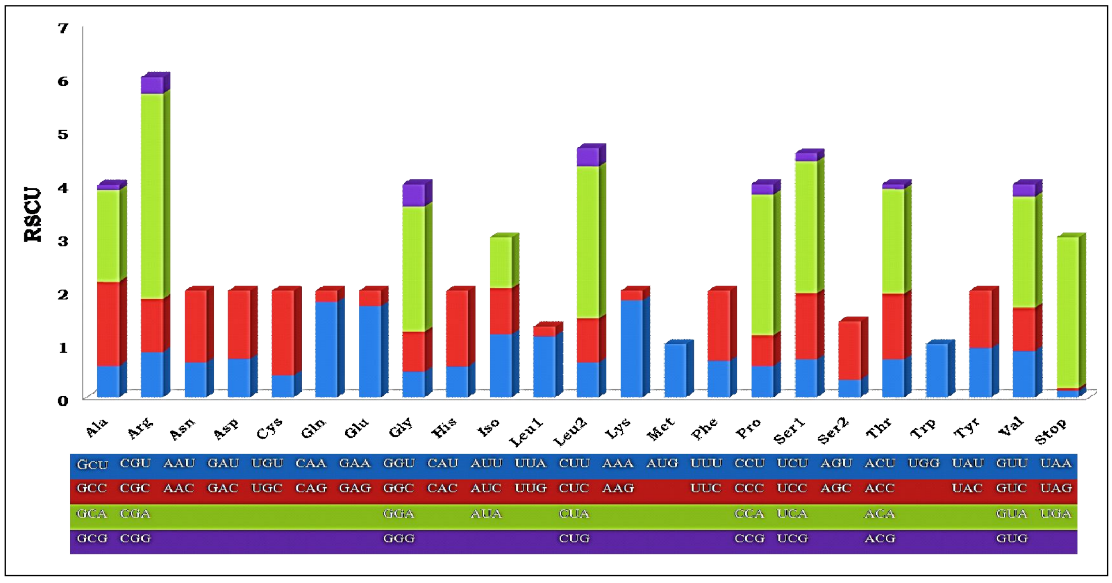


Fig 3: The relative synonymous codon usage (RSCU) of the protein-coding genes of *B. dero* mitogenome.

Much as in other vertebrates, the ribosomal RNAs (12S rRNA and 16S rRNA) were found on the H strand, divided by tRNA-Val (Yang *et al*., 2018; Zhang *et al*., 2019). The 12S rRNA gene showed a distance of 957 bp between tRNA-Phe and tRNA-Val. Positioned between tRNA and tRNA-Leu1, the 16S rRNA gene measured 1691 bp in length. In comparison to other fish, the length of rRNA gene in the *B. dero* mitogenome was observed to be longer (Sam *et al*., 2021; Zhang *et al*., 2023).

Non coding regions

The origin of light chain replication (OL) was found between tRNA-Asn and tRNA-Cys. The OL was 33 bp long and forms a hairpin structure similar to *B. decora* (Li *et al*., 2016). A stem with 19 bases and a loop with 14 bases make up the secondary structure of OL (Fig 5a). The stem of the hairpin structure had a sequence of 52 -GGCGG-3, similar to *Garra* species but different from *Scomber* species, which has 52 -GCCGG-32 (Zhang *et al*., 2022: Catanese *et al*, 2010.

The mitochondrial genome of *B. dero* contains a CR region that is 942 bp long, situated between the genes of tRNA-Pro and tRNA-Phe. The origins of transcription and replication are found in the longest noncoding region (CR) of the vertebrate mitogenome (Bronstein *et al*., 2018). The A+T content, AT skew and GC skew in CR were 67.83%, 0.045 and - 0.198 respectively which were consistent with other Cyprinid fishes (Sharma *et al*., 2020). The conserved sequenced blocks (CSBs), ETAS (extended terminal associated sequences) and CD (central domain) were identified within the CR (Fig 5b). The control domain was arranged in different boxes (CSB-F, CSB-E and CSB-D) and there were three regions (CSB1, CSB2 and CSB3) in the CSB domain similar to arrangement in the mitogenome of many fishes (Satoh *et al*., 2016).

These arrangements were similar to those found in the mitogenome of many fishes. In addition to OL and CR, there were non-coding intergenic spacers ranging in sizes from 1 to 46 bp (Table 1). Presence of long noncoding sequences has been reported in other fishes which may serve as a mechanism of gene rearrangement supporting the tandem duplication-random loss (TDRL) model (Satoh *et al*., 2016).

Phylogenetic analysis

Phylogenetic analysis was performed in the current work using 39 mitogenome sequences from various species, including *B. dero*. The phylogenetic tree was rooted with *Psilorhynchus* species as an outgroup (Fig 6). In the tree,

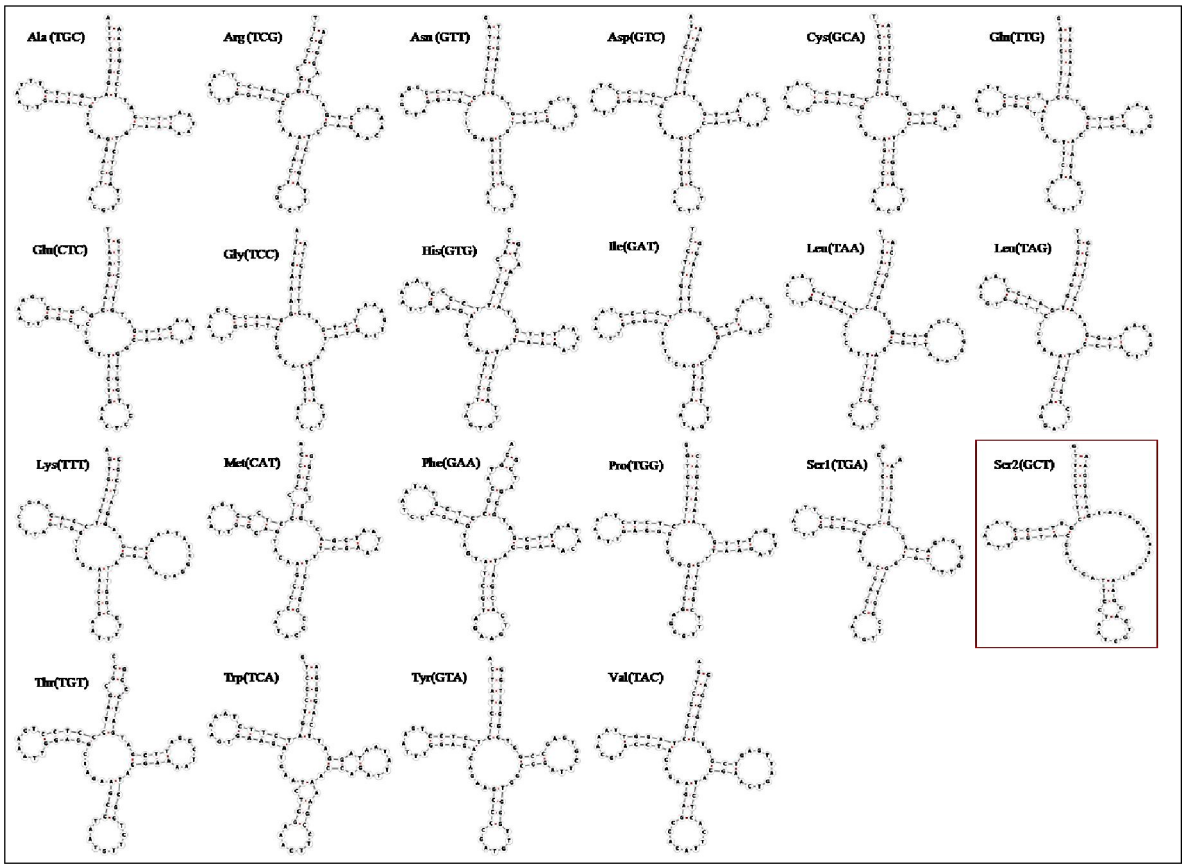
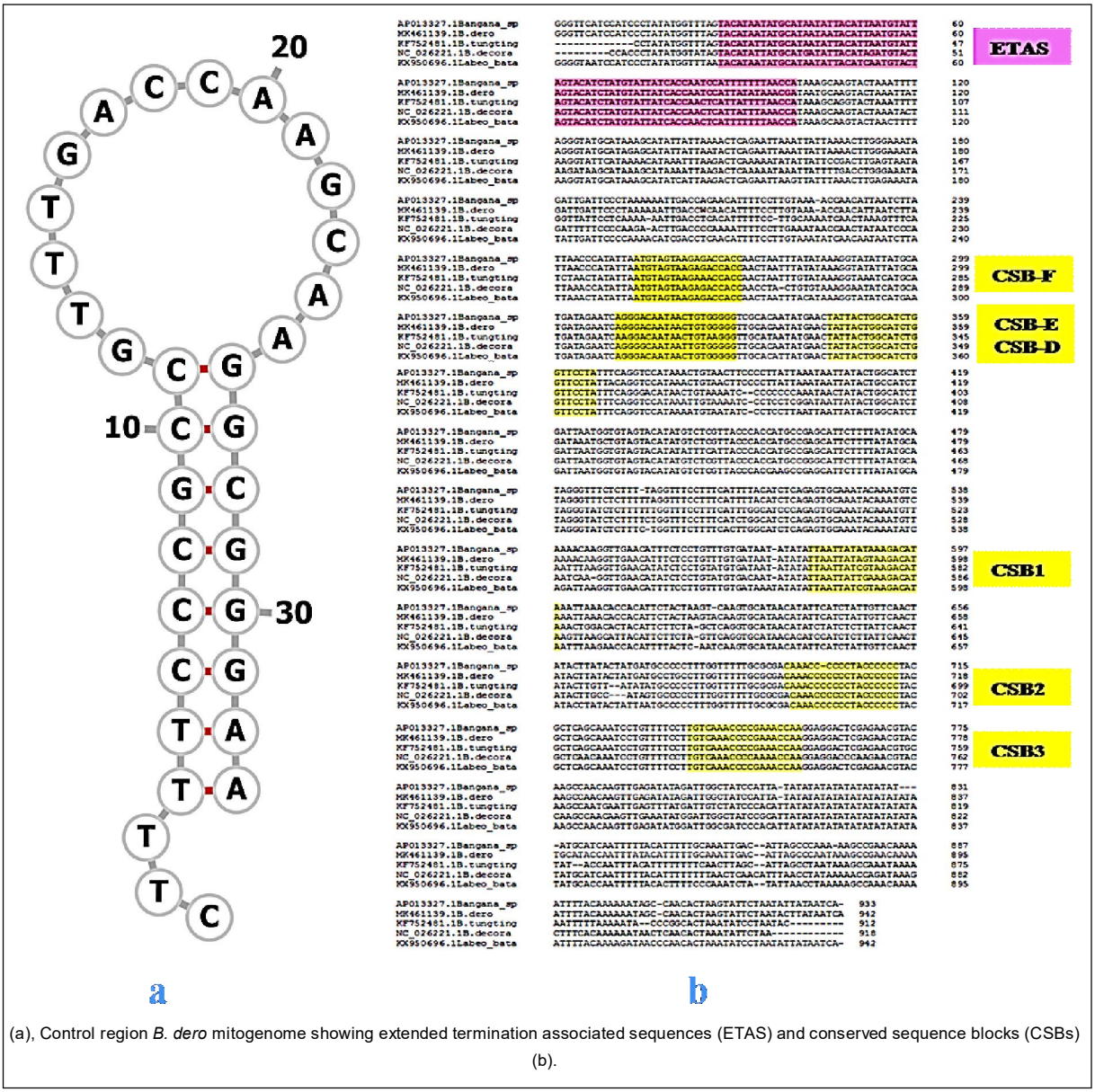


Fig 4: Putative secondary structures for 22 tRNA genes in Mitochondrial genome of *B. dero*. tRNA-Ser2 (in red box) lack a D arm.

two major clades were observed, which further split into subclades. In Clade I, the genera present were *Labeo, Bangana, Cirrhinus, Incisilabeo and Schismatorhynchos*. Clade II consists of *Garra, Discocheilus, Discogobio, Labiobarbus, Lobocheilos, Parasinilabeo, Prolixicheilus, Ptychidio, Semilabeo and Sinocrossocheilus*. A bootstrap value of 100 supports the close grouping that of *B. dero* formed with *Bangana* species (AP013327) and Labeo boggut (NC_029450) in sub clade I of Clade I. Based on the mitochondrial 16S rRNA sequence of *B. dero*, a similar outcome was found in an earlier investigation (Basudha *et al*., 2019). The same subclade also contains *Bangana tungting* (KF752481). However, *Bangana decora* was present in clade II along with *Garra* and other species, similar to the previous report (Zhang *et al*., 2022). They have found that all *Garra* species, with the exception of *G. pingi pingi* and *G. imberba*, are grouped within a single clade, while the two species are clustered alongside other Labeoninae species which also encompasses *B. decora*. They suggested that the alteration of mitochondrial rearrangement in one group of *Garra* has caused a distant relationship with another group that has not undergone similar changes. The occurrence of various species from the same genus in different clades of a phylogenetic tree can be attributed to the non-monophyletic nature of the genera. It has been reported that the genus *Labeo, Garra, Bangana, Cirrhinus* and *Crossocheilus* are non monophyletic (Yang *et al*., 2012). It suggests that certain



(a), Control region *B. dero* mitogenome showing extended termination associated sequences (ETAS) and conserved sequence blocks (CSBs) (b).

Fig 5: Secondary structures of the OL of *B. dero* mitogenome.

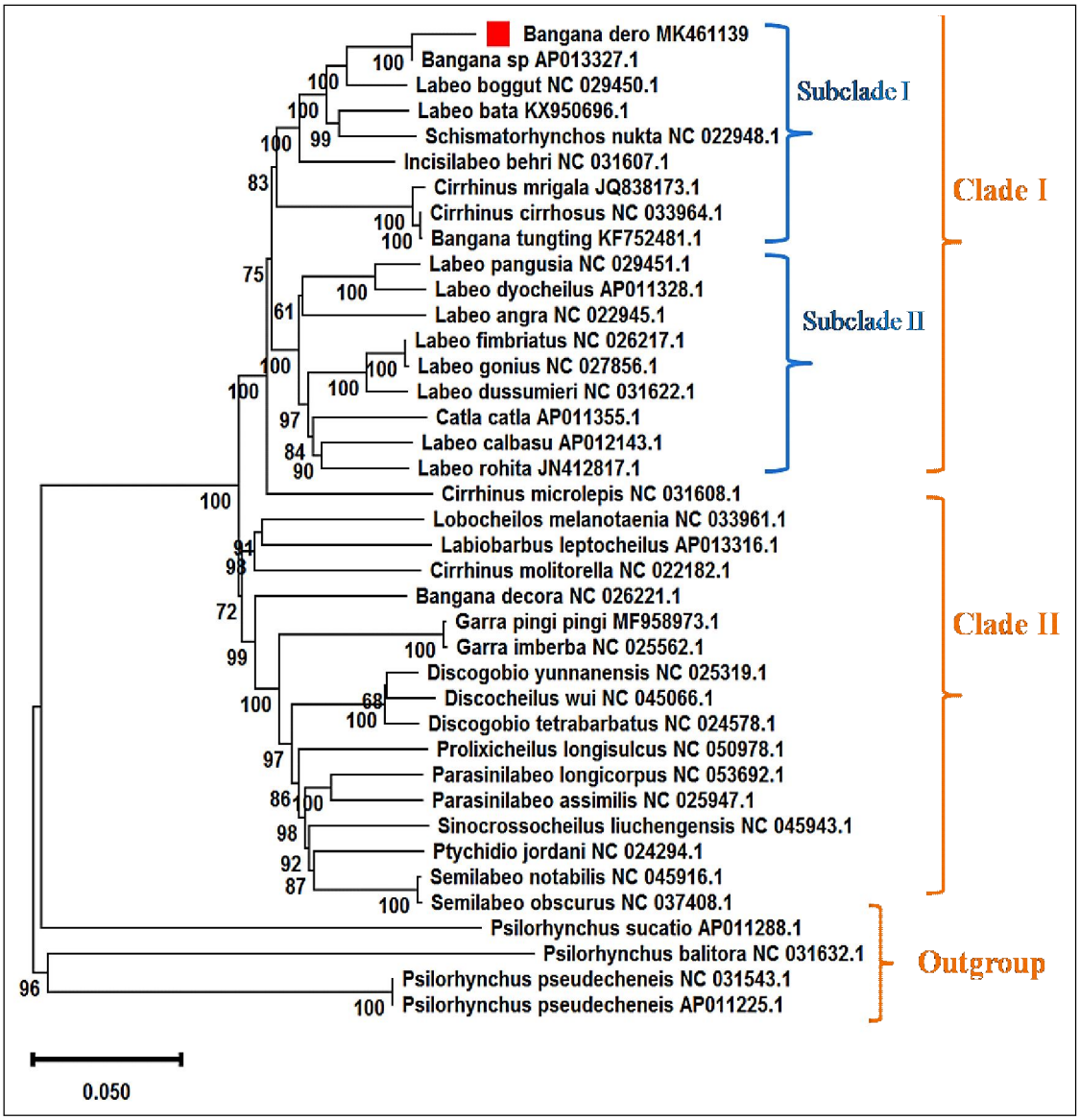


Fig 6: Phylogenetic tree inferred from nucleotide sequences of complete mitogenome using Neighbour Joining method. *B. dero* of the present study is indicated by a red box. Boostrap values are indicated along the branches.

species within a particular genus may be more closely related to species of other genus.

CONCLUSION

The complete mitochondrial genome of *B. dero* is a circular molecule with 16,613 base pairs. The mitogenome is made up of a single non-coding control region along with 37 genes, consisting of 22 tRNA genes, two rRNA genes (12S and 16S) and 13 protein-coding genes (PCGs). It was discovered that the sizes of the mitogenome, nucleotide composition, organization of the gene, as well as usage of the codon were comparable to those of fish. However, some differences observed include a lack of dihydrouridine arm in tRNA-Ser2, presence of a long intergenic spacer of 46 bp in between tRNA-Leu1 and ND1 genes. In the phylogenetic tree, *B. dero* was grouped with other species of *Bangana*, whereas *B. decora* was located in a separate clade, suggesting that the genus *Bangana* is not monophyletic. The information generated in this study will be useful in reconstructing evolutionary relationships and phylogenetic tree to elucidate the patterns of diversification and speciation in *Bangana* species. Further, mtDNA can serve as a marker for analyzing the historical development of populations, particularly in relation to migration patterns, periods of isolation and the effects of environmental barriers. Most importantly, mtDNA can be used for assessing genetic diversity and inbreeding levels, which are essential for evaluating the health and sustainability of fish populations, especially in the realm of conservation.

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Conflict of interest

The authors declare no conflict of interest.

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