CHARACTERIZATION OF THE COMPLETE MITOCHONDRIAL GENOME OF *LABEO ROHITA* FROM BANGLADESH

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Hawa Jahan, Moumita Chakraborty, Mohammad Shamimul Alam, and Rowshan Ara Begum*

Department of Zoology, University of Dhaka, Dhaka-1000, Bangladesh

ABSTRACT

Labeo rohita, commonly known as rui/rohu possesses an important role in agricultural and economic aspects in Bangladesh. This study aimed to characterize the mitochondrial genome of this economically important fish species. Primers (24 pairs) were designed to amplify the whole genome and most of the regions contained 700 to 800bp. In this study, nucleotide sequences of 16,607 bp of the mitochondrial genome of *L. rohita* were determined for the first time from Bangladesh, which consists of 13 protein-coding genes, 22 tRNA genes, 2 rRNA genes and one D-loop region. All of the genes were similar in size compared to other fish mitochondrial genomes. The gene arrangements, and intergenic and gene overlapping nucleotides of the present study, were similar to that of other *Labeo* genera so far reported. The phylogenetic tree shows 100% similarity in *L. rohita* mitochondrial genomes collected from two different areas of Bangladesh. This nucleotide sequence data of the mitogenome of *L. rohita* would provide necessary information for further studies, including population genetics of carp fishes.

KEYWORDS: Mitochondrial genome, *Labeo rohita*, protein-coding gene, tRNA, control region.

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CORRESPONDING AUTHOR: Rowshan Ara Begum, Department of Zoology, University of Dhaka, Dhaka-1000, Bangladesh
Email: rowshanbegum@du.ac.bd

Introduction

Labeo rohita possesses essential roles to play in agricultural and economic aspects, mainly in Bangladesh and the South-Asian continent (Choudhury and Dutta 2012). Many species in this family have various uses such as being ornamental, food, used for extracting oil, and medicinal value (Gupta and Banerjee 2015; Bogard et al. 2015; Steffens 2006; Ghelichi et al. 2017; De et al. 2012). This major carp seems to be quite popular in the aquaculture sector in Bangladesh due to its fast-growing nature and taste (Bej et al. 2012; Majumder et al. 2018).

Mitochondrial DNA (mtDNA) sequence and structure are widely used to examine phylogenic relationships and population structure (Nardi et al. 2003). The set of mitochondrial genes is nearly identical throughout vertebrates, specifying 13 protein-coding, 22 tRNA and 2 rRNA genes with one non-coding gene (Arab et al. 2017). Mitochondrial genome sequences are available in large numbers in Genbank and new sequences are being published nowadays at an increasing pace (Arab et al. 2017). The complete mitochondrial genome is considered to provide much more robust phylogenetic information than smaller portions of the mtDNA (Wang et al. 2020).

The complete mitochondrial genome sequences have been reported for different species of *Labeo* from different countries (Bej et al. 2012; Sahoo et al. 2016; Behera et al. 2015; Sahoo et al. 2021). Partial sequence of the mitochondrial genes, namely 16S rRNA, COI genes, etc. have been published from Bangladesh (Alam et al. 2015; Ahmed et al. 2018). With this present study, we have determined the complete mitochondrial genome of *L. rohita* for the first time from Bangladesh, which will provide information for future investigations on molecular taxonomy, population genetics, evolution and molecular phylogeny of *L. rohita* and its relatives.

Materials and Methods

The live specimen of *Labeo rohita* was collected from Daudkandi of Comilla and brought to the Laboratory of Genetics and Molecular Biology, Department of Zoology, University of Dhaka. A morphological study was conducted immediately after returning to the laboratory and tissue sample was then collected for DNA extraction. The rest of the fish was preserved in 95.5% ethanol, labelled with the voucher number GMB CS01.

DNA Extraction, gene amplification, and sequencing

DNA extraction was done by using the modified CTAB (Cetyl trimethylammonium bromide) method (Jahan et al. 2017). Total genomic DNA was prepared by homogenization of muscle tissues underneath the dorsal fin in 600µl extraction buffer (CTAB) containing 10N NaOH, 1M Tris-HCl, 0.5M EDTA. Muscle tissue (0.05g) was mashed in the buffer using a pestle.

Adding 10µl of proteinase K (20 mg/ml), the lysate was incubated at 56°C for 2h. After incubation an equal volume of phenol: chloroform was added, and the mixture was spun again at 13,000 rpm at room temperature for 5 min. Then, the lower phenol-chloroform phase was removed. DNA is found at the upper aqueous phase. The sample was precipitated with isopropanol at room temperature and inverted several times. The sample was spun at 13000 rpm for 5 min and the isopropanol was carefully discarded from the DNA pellet. DNA pellets were washed in 500µl washing buffer (70% ethanol) and centrifuged for 1 min at 13000 rpm. The alcohol was then removed. The remaining DNA pellet was dried in the air, followed by adding 30µl deionized distilled water and incubated at 37°C for 5 min. Isolated DNA was stored at -20°C. The amplification of the mitochondrial genome of *Labeo rohita* was conducted by conventional polymerase chain reaction (PCR) using 24 primer pairs (Supplementary Table 1) for the respective targeted regions in Veriti Thermal Cycler (Thermo Fisher Scientific). Double-stranded PCR products were purified using GeneJet PCR purification kit (Thermo Fisher) and subsequently were sent to Macrogen Inc. (South Korea) for direct cycle sequencing using the automated Sanger sequencing method. The sequence of the respective target regions was determined by using the same set of primers used for PCR.

Sequence analysis

Raw sequence reads obtained from forward and reverse directions were visualized and aligned using the software Chromas (Treves 2010) and Serial cloner (http://serialbasics.free.fr/Serial_Cloner.html). The identity of the sequences was then checked and confirmed using BLAST searches from NCBI. Partial sequences were aligned and compiled manually to get the whole mitochondrial genome sequence of *Labeo rohita*.

Secondary structures for tRNA were obtained using ViennaRNA website and manually adjusted if necessary (Hofacker 2003; Gruber et al. 2008). The circular arrangement of the mitogenome, as well as structural and functional annotations, were performed using Mitoannotator, an online webserver (Sharma et al. 2020). Amino acid sequences were deduced by the codons of vertebrate mitochondrial genome using the computer software program DNASIS max (Hitachi Software Engineering Co. Ltd) and ExPASy (Gasteiger et al. 2003). With MEGAX (Kumar et al. 2018) software, the nucleotide composition of the whole mitochondrial genome was calculated. The method of measuring nucleotide composition bias was AT skew = (A−T)/(A+T) and GC skew = (G−C)/(G+C) (Sharma et al. 2020; Wang et al. 2020).

A phylogenetic tree was constructed using complete mitochondrial genome sequences of different *Labeo sp.* available at GenBank, where *Protopterus annectens* was used as an outgroup. The evolutionary history was inferred from the tree using the Neighbor-Joining method (Saitou and Nei 1987). Evolutionary distances were computed using the number of differences method (Nei and Kumar 2000) and are in the units of the number of amino acid differences per sequence. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

Results and Discussion

Genome organization

The complete mitochondrial genome sequence of *Labeo rohita* from the present study is available in the GenBank database under the accession number MN533986, so far the first reported data from Bangladesh (26th April 2020). Later, three more complete genome sequences of *L. rohita* were published in the NCBI database (accession no. MW557325.1, MT909814.1, OR074507.1) that were sequenced through next-generation sequencing technique unlike ours, which was done by Sanger sequencing method.

According to the findings of the study, the mitochondrial genome of *L. rohita* has a length of 16,607 bp in total, with 2 rRNA, 22 RNA, and 13 protein-coding genes as found in other vertebrates with one non-coding region (Table 1 and Figure 1). The base composition of *L. rohita* mitogenome is A: 32.6%, G: 15.2%, C: 27.8%, T: 24.3%. The nucleotide composition order is A>C>T>G, which is consistent with fish mitochondrial genomes. (Wang et al. 2008). The AT content (56.9%) represents a nucleotide compositional bias over the GC content with a negative (-0.29) value. The AT and GC skews indicate a strand asymmetry in nucleotide composition (Wei et al. 2010).

Gene Arrangement

The gene organization and its order that had been found were similar to other vertebrates (Broughton and Dowling 2001; Sahoo et al. 2021). The gene arrangement was determined as 12S− $tRNA^{Val}$ − 16S− $tRNA^{Leu}$− ND1− $tRNA^{Ile}$ − $tRNA^{Gln}$− $tRNA^{Met}$− ND2− $tRNA^{Trp}$ − $tRNA^{Ala}$ − $tRNAA^{sn}$ − $tRNA^{Cys}$ − $tRNA^{Tyr}$− COI−COII−$tRNA^{Lys}$ −ATP8− ATP6−COIII− $tRNA^{Gly}$ − ND3− $tRNA^{Arg}$ − ND4L− ND4 − $tRNA^{His}$ − $tRNA^{Ser(AGY)}$ − $tRNA^{Leu}$− ND5− ND6− $tRNA^{Glu}$−Cytb−$tRNA^{Thr}$−$tRNA^{Pro}$−D-loop for mitochondrial genome of *Labeo rohita* and are shown in Figure 1.

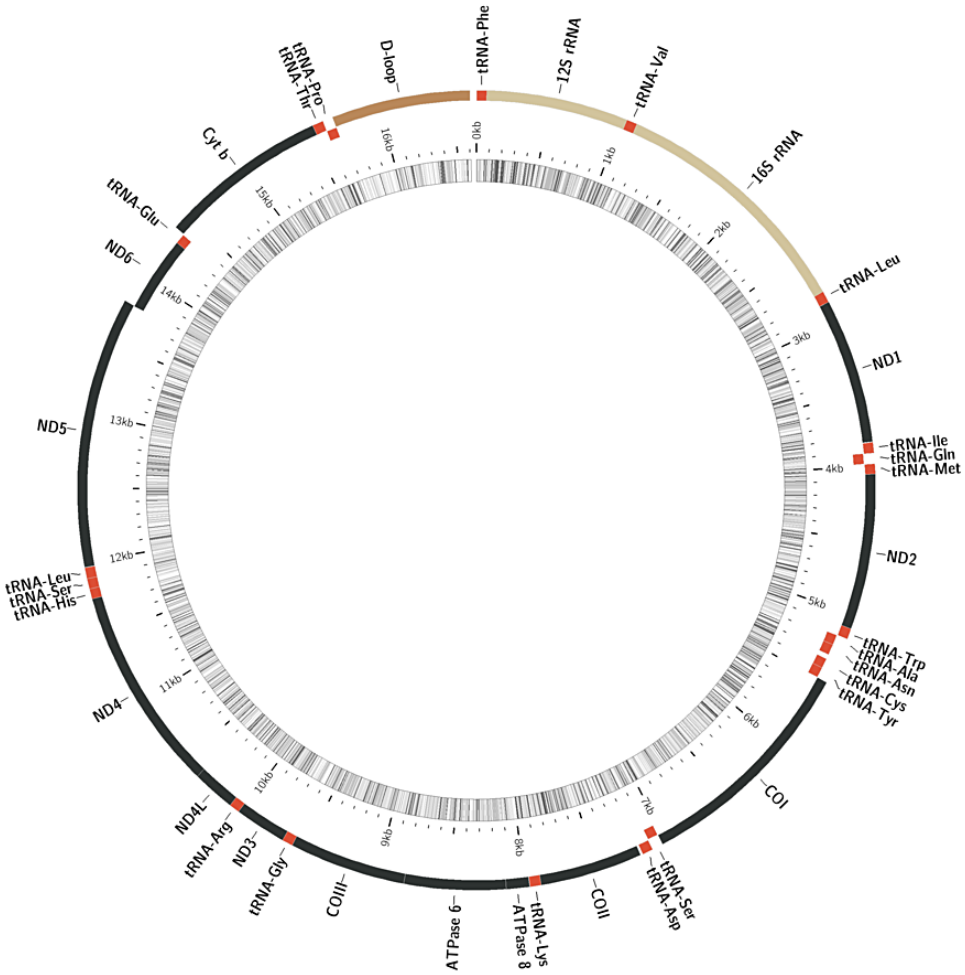

Figure 1. Circular organization of the whole mitochondrial genome of *L. rohita*. The figure shows the positions of 37 genes, including 13 protein-coding genes, 2 rRNA genes, 22 tRNA genes and a putative control region (D-loop). The circular arrangement of the mitogenome, as well as the structural and functional annotations was performed using Mitoannotator, an online webserver (Sharma et al. 2020).

Protein coding genes

In the mitochondrial genome of *L. rohita*, all of the protein-coding genes have the start codon ATG except for COI, which is common for vertebrate mitochondrial DNA (Bej et al. 2012, Zhu et al. 2017). The codon was GTG for COI. The termination codon was TAA for COI, ATP8, COIII, ND1, ND4L, ND5 and TAG for ND6 and are shown in Table 1. However, four of the protein-coding genes, namely ND2, ND3, COII, ND4 terminated with an incomplete codon, T. Similar characteristics for incomplete stop codon have been observed in other teleost species (Peng et al. 2006).

Ribosomal RNA genes

Two genes of ribosomal RNA were found in *L. rohita*, one for a large subunit and the other for a small subunit, as in all other mitochondrial genomes sequenced so far. *L. rohita* 12S ribosomal RNA and the 16S ribosomal RNA lengths in the present study were 956 bp and 1,690 bp, respectively, similar to the finding of Bej et al. (2012). The two ribosomal RNA genes of *L. rohita* 12S rRNA and 16S rRNA are separated by $tRNA^{Val}$, like other vertebrate mitogenomes (Inoue et al. 2000, Peng et al. 2006).

Transfer RNA genes

The arrangement and structure of 22 tRNA genes in the mitochondrial genome from *L. rohita* followed the typical vertebrate mitogenome. The lengths of obtained tRNA genes of *L. rohita* ranged from 69 to 76 bp so all were large enough to fold into the cloverleaf structure, characteristics of tRNA, except $tRNA^{Ser(AGY)}$ (Table 1). The $tRNA^{Ser(AGY)}$ lacked dihydrouridine arm and there was a large loop instead of the conserved stem-and-loop structure, which is common in other vertebrate mitogenome (Bej et al. 2012, Zhu et al. 2017). The primary and secondary structures of the tRNA genes were confirmed by comparing them with those of other vertebrate species. Mispairing was observed in the secondary structure of the tRNA genes, and among them, G-T mispairing was the most common type of base mispairings. Each tRNA was observed to have at least one G-T mismatch, except for $tRNA^{Arg}$, in their respective secondary structures, which formed a weak bond (Zhu et al. 2017). Most of the tRNA genes are encoded in the H-strand except for $tRNA^{Gln}$, $tRNA^{Met}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, $tRNA^{Tyr}$, $tRNA^{Ser(UCN)}$ and $tRNA^{His}$. Mentioned eight tRNA genes are encoded in the L-strand (Table 1).

Phylogenetic analysis

The phylogenetic tree shows that there is 100% similarity in *L. rohita* mitochondrial genomes collected from two different areas of Bangladesh and the sequence showed the closest distance with *L. rohita* sequence of Odisha, India (Figure 2). Similarly, *L. fimbriatus* from Odissa, India has the closest distance based on mitochondrial genome, indicating the closest identity between Bangladeshi *L. rohita* with Indian *L. fimbriatus* among the analysed sequences (Figure 3).

Table 1. Characterization of the mitochondrial genome of *Labeo rohita*

Gene	Position number		IGN	Size(bp)	Codon		Anticodon	Strand
	From	To			Start	Stop		
$tRNA^{Phe}$	1	69	0	69			GAA	H
12S rRNA	70	1025	0	956				H
$tRNA^{Val}$	1026	1097	0	71			TAC	H
16S rRNA	1098	2785	0	1688				H
$tRNA^{Leu(L\text{-}UUR)}$	2786	2863	0	78			TAA	H
ND1	2864	3838	4	975	ATG	TAA		H
$tRNA^{Ile}$	3842	3915	-1	74			GAT	H
$tRNA^{Gln}$	3914	3983	2	70			TTG	L
$tRNA^{Met}$	3986	4054	0	69			CAT	L
ND2	4055	5100	0	1046	ATG	TA^{-}		H
$tRNA^{Trp}$	5101	5171	2	71			TCA	H
$tRNA^{Ala}$	5174	5242	2	69			TGC	L
$tRNA^{Asn}$	5245	5317	31	73			GTT	L
$tRNA^{Cys}$	5349	5419	-1	71			GCA	L
$tRNA^{Tyr}$	5418	5488	1	71			GTA	L
COI	5490	7040	0	1551	GTG	TAA		H
$tRNA^{Ser(UCN)}$	7041	7111	3	71			TGA	L
$tRNA^{Asp}$	7115	7186	15	72			GTC	H
COII	7202	7896	-4	695	ATG	T^{--}		H
$tRNA^{Lys}$	7893	7968	1	76			TTT	H
ATP8	7970	8134	-7	165	ATG	TAA		H
ATP6	8128	8811	-1	684	ATG	TAA		H
COIII	8811	9596	0	786	ATG	TAA		H
$tRNA^{Gly}$	9597	9668	0	72			TCC	H
ND3	9669	10017	0	349	ATG	T^{--}		H
$tRNA^{Arg}$	10018	10087	0	70			TCG	H
ND4L	10088	10384	-7	297	ATG	TAA		H
ND4	10378	11758	0	1381	ATG	T^{--}		H
$tRNA^{His}$	11759	11827	0	69			GTG	L
$tRNA^{Ser(S\text{-}AGY)}$	11828	11896	1	69			AGC	H
$tRNA^{Leu(L\text{-}CUN)}$	11897	11970	3	74			TAG	L
ND5	11974	13797	-4	1824	ATG	TAA		H
ND6	13794	14315	0	522	ATG	TAG		L
$tRNA^{Glu}$	14316	14384	5	69			TTC	L
Cyt b	14390	15530	0	1141	ATG	T^{--}		H
$tRNA^{Thr}$	15531	15602	-1	72			ATC	H
$tRNA^{Pro}$	15602	15671	0	70			TGG	L
D-loop	15672	16607	0	936				

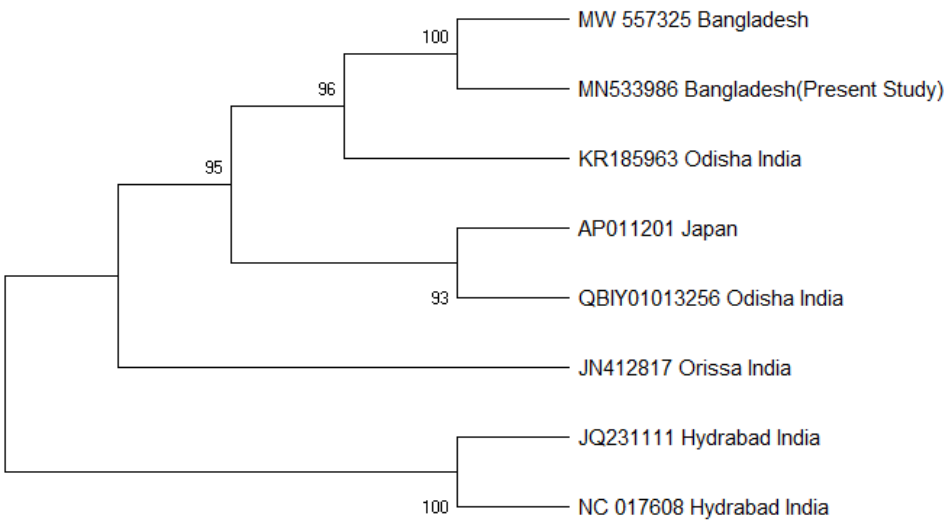

Figure 2. Phylogenetic relationships of *Labeo rohita* based on the available mitochondrial genome sequence in the GenBank database. The phylogenetic history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 71.50000000 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches.

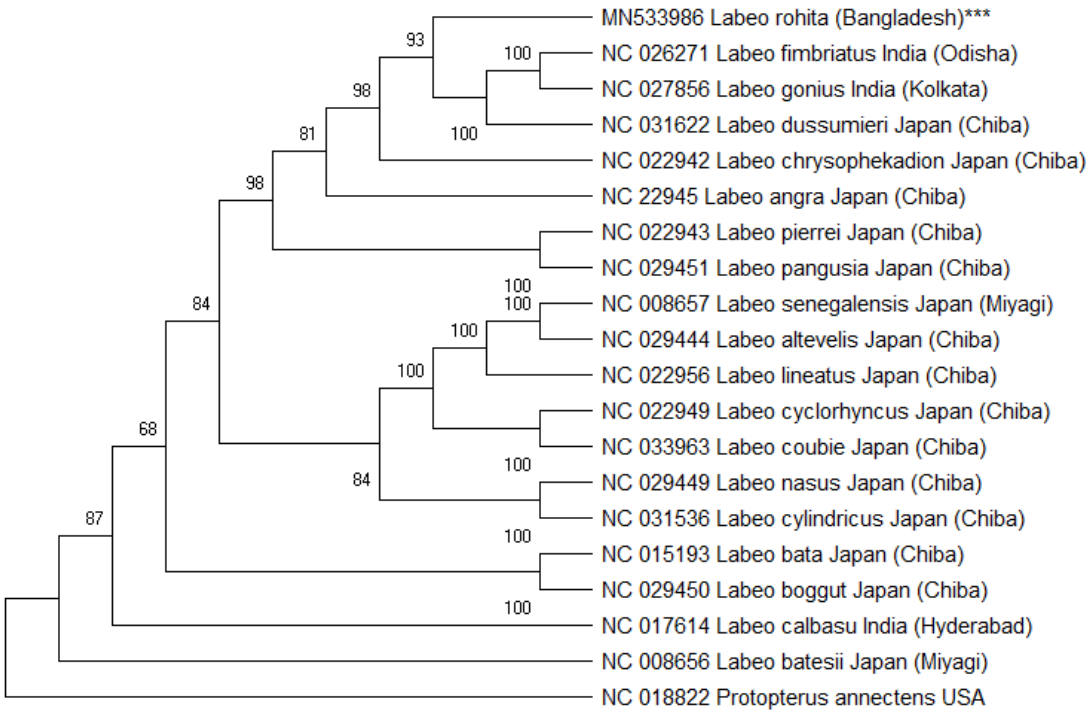

Figure 3. Phylogenetic relationships of different species under genus *Labeo* based on available mitochondrial genome sequence in the GenBank database. The phylogenetic history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 12746.63378906 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches.

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