

PARTIAL SEQUENCE ANALYSIS OF MITOCHONDRIAL *CYTOCHROME B* GENE OF *Labeo calbasu* OF BANGLADESH

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Abstract

Labeo calbasu (Family Cyprinidae) was studied at DNA level to know genetic diversity within and between species. The mitochondrial *cytochrome b* (*cyt-b*) gene of *L. calbasu* was sequenced and compared to the corresponding sequences of other *Labeo* species. DNA was isolated from the tissue sample of *L. calbasu* using phenol: chloroform extraction method. Forward and reverse primers were designed to amplify the target region of *cytochrome b* gene. A standard PCR protocol was used for the amplification of the desired region. Then, the forward and reverse sequences obtained were aligned and edited to finalize a length of 510 nucleotides which was submitted to NCBI genbank database. Nucleotide BLAST of this sequence at NCBI resulted 100% sequence similarity with *L. calbasu* sequence of the same region of *cyt-b* gene. Multiple sequence alignment of the sequence with seven more *Labeo* species sequences revealed 120 polymorphic sites, which have been mark of diversity among the species and might be used in molecular identification of the *Labeo* species. A constructed phylogenetic tree has shown relationship among the *Labeo* species. This research demonstrated the usefulness of mitochondrial DNA-based approach in species identification. Further, the data will provide appropriate background for studying genetic diversity within-species of the *Labeo* species in general and of *L. calbasu* in particular.

Key words: Cytochrome *b* gene; Mitochondrial DNA; Nucleotide polymorphism; *Labeo calbasu*.

INTRODUCTION

The freshwater habitats of Bangladesh are very rich. There are about 260 freshwater fish species in Bangladesh (IUCN Bangladesh 2015). *Labeo*, is a genus of fish belonging to the family Cyprinidae of the order Cypriniformes and found in the tropical countries of the world especially in the Indian subcontinent. Cyprinidae is the largest among the vertebrate families and also the largest fish family. Ten *Labeo* species are reported from Bangladesh (IUCN Bangladesh 2015). Among them, *Labeo calbasu* has the highest demand because of its delicacy.

The water bodies of Bangladesh are being polluted by agricultural chemicals and industrial effluents continuously making the fish habitats more stressful. There are evidences that a total of 30 riverine finfish species has already been extinct from Bangladesh (Hossain 2014). Species diversity and genetic diversity (i.e. species number and population size) have significantly reduced in Bangladeshi aquatic habitats (Chaki *et al.* 2014, Joadder *et al.* 2015, Mohsin *et al.* 2014). Similarly, the abundance of *L. calbasu* is affected by the environment. Its number is decreasing day by day in the habitats which might cause loss of genetic diversity within population. Since, small populations are vulnerable to the loss of genetic diversity, the study of *L. calbasu* at genetic level is very important. From this perspective, molecular identification through mitochondrial gene sequence is a starting point toward studying the species more intensively.

Mitochondria is a cell organelle that harbors a circular DNA molecule of around 16KB possessing 37 genes. The sequences of the mitochondrial genes are successfully used in species identification. Among them, the use of *cytochrome oxidase I* (*COI*) is very common (Hebert *et al.* 2003). However, *COI* sequence is not always sufficient to resolve the taxonomic uncertainties of closely related species, especially those of the same genus. So, other genes of the mitochondrial genome, such as *cytochrome b* (*cyt-b*), *16SrRNA*, *12SrRNA*, *ND1*, *ND2* and D-loop region, are often used in molecular identification to

remove any unambiguity. *Cyt-b* gene is a protein coding gene producing cytochrome b that is essential for mitochondrial functioning. It is located in between tRNA^{glu} and tRNA^{thr} in the mitochondrial genome (Fig. 1).

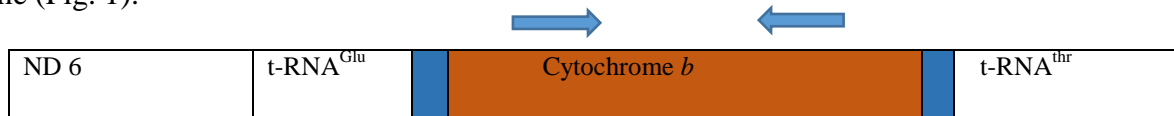


Fig. 1. Schematic diagram of Cytochrome *b* gene showing its position in mitochondrial genome.

It is approximately one thousand base pair long. The structure and function of cytochrome b is well-known (Esposti *et al.* 1993). The nucleotide sequence of the *cyt-b* gene has species-specificity and used in evolutionary studies and forensic investigations (Kocher *et al.* 1989, Smith and Patton 1991). The *cyt-b* gene sequence of *L. calbasu* from India is available in the NCBI genbank database (Accession no. KF574601). But, a complete sequence of *cyt-b* gene of *L. calbasu* of Bangladesh has not been reported yet. Due to geographically separate habitats, there might have differences in the gene sequences of the same species which could be a measure of genetic diversity. So, the *cyt-b* gene of *L. calbasu* from Bangladesh needs to be sequenced. Therefore, the main objective of the present study was to decipher *cyt-b* gene sequence of a local sample of *L. calbasu* from Bangladesh and compare it to that from India. In addition, *cyt-b* sequence of *L. calbasu* was also compared to that of other species available in the genbank database.

MATERIAL AND METHODS

Labeo calbasu sample was collected from local market of Mymensingh city, Bangladesh. The sample was placed in ice immediately after collection and transferred to laboratory for further studies. After morphometric analysis, tissue sample was collected from the dorsal portion of the body. Approximately 45 mg of soft muscle tissue were collected for the extraction of DNA and rest was preserved as voucher specimen. Total genomic DNA was extracted by using phenol: chloroform extraction method with slight modification (Begum *et al.* 2004). The extracted DNA was then visualized in 1.5% agarose gel of a low melting point grade at 120 V for 20 minutes. The extracted DNA samples were run in the agarose gel, along with a loading dye (Bromophenol blue) in a 5:1 ratio.

A region of cytochrome-b gene (target size around 650bp) was amplified by polymerase chain reaction (PCR). The chemicals used for the amplification of mitochondrial DNA were 2 µl of 10X PCR reaction buffer, 1.6 µl of 25 mM MgCl₂, 0.5 µl of 10 mM dNTP, 0.5 µl of *cyt-b* forward primer, 0.5 µl of *cyt-b* reverse primer, 0.16 µl of *Taq* DNA polymerase, 2 µl Template DNA, and sterilized distilled water up to a total volume of 25 µl. The forward and reverse primers designed to amplify a region of the *cyt-b* gene were 5'- GCA ACA TAC ACG CCA ACG G -3' and 5'- TGA GGT GTG TAG GAG GGG TA -3', respectively. Amplifying conditions were 94°C for 60s in denaturation, 53°C for 30s in annealing, and 72°C for 30s in extension for 30 cycles with a final polymerization step at 72°C for five minutes. PCR products were visualized using agarose gel electrophoresis. The PCR products of interest were then purified using Favor Prep PCR Clean up Mini Kit (Favorgen Biotech Corp.). After purification, the PCR product was sequenced in both directions by a sequencing service provider (Macrogen Inc., Korea). The nucleotide sequence of *cyt-b* gene obtained from the present study was subjected to NCBI BLASTn and then submitted to the GenBank database. The *cyt-b* gene sequences of eight selected *Labeo* species were collected from NCBI nucleotide database for intraspecific and interspecific comparison. The accession numbers of the sequences used were AP011329 (*L. angra*), KF574952 (*L. bata*), NC_026217 (*L. boggut*), MG010391 (*L. calbasu* of the present study), KF574601 (*L. calbasu* India), NC_026217 (*L. fimbriatus*), KT001152 (*L. gonius*), AP013340 (*L. pangusia*), KF574619 (*L. rohita*) and NC_001887 (*Balanoglossus carnosus* as outgroup). Multiple sequence alignment and neighbor-joining phylogenetic tree were constructed using SEAVIEW software (Gouy *et al.* 2010). The multiple sequence alignment

allowed to study the number of polymorphic sites, and thus the phylogenetic relationships among different *Labeo* species based on the *cyt-b* gene sequence was investigated.

RESULTS AND DISCUSSION

The image of the extracted DNA ran in 1% agarose gel has been presented in Fig. 2a. The isolated DNA has appeared to be of around 10kb size as it occurs in such cases. The PCR product sent for sequencing checked by running in gel has been presented in Fig. 2b.

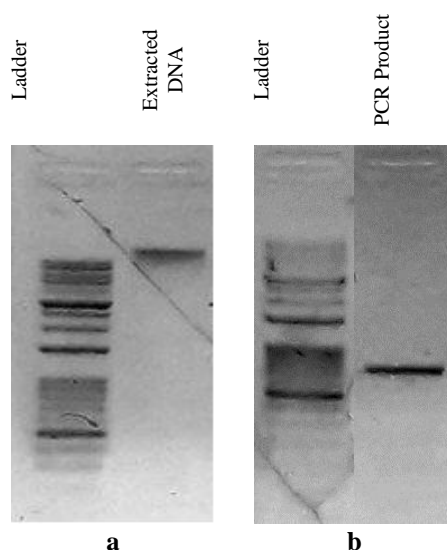


Fig. 2. **a.** The images of agarose gel electrophoresis for the extracted DNA of *Labeo calbasu*; and **b.** Cytochrome *b* primer based PCR product.

The size of the amplified product appeared to be between 600 and 700bp indicating proper priming during polymerase chain reaction process. The nucleotide sequence of the amplified region of the *cyt-b* gene of *L. calbasu* has been presented in Fig. 3. Though the size of the targeted PCR product was around 650bp, small regions of the two ends were omitted from further analysis due to common sequencing errors clearly detectable from chromatograms. Thus, a corrected sequence of 510bp was finalized for further study and submitted to genbank database. The accession number given to the sequence submitted to NCBI database is MG010391.1.

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1  tcgcccgagg cctatactat ggatcatact tatataaaga aacctgaaac attggcgtag
61  tactcttact attagtcata ataacagcct ttgttggcta cgtccttcca tgaggacaaa
121 tgtctttctg aggtgccaca gtaatcacia acctattatc cgctgtaccc tacataggag
181 atatactagt acaatgaatc tgagggtggct ttcagtaga caatgcaaca ctaacacggt
241 tcttcgcatt ccacttccta ctaccattta ttatcgccgc cgcaaccatt atccacctgc
301 tattcctcca cgagacagga tctaacaacc caatcggact aaactcggac gcagataaaa
361 tttccttcca tccatacttt acatacaaag acctccttgg attcgtaatt atactgctag
421 ctcttacact actagcgtta ttctcccaaa acctactagg agaccagaaa aacttcaccc
481 ccgcaaacc cttagtaacc ccaccacaca

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Fig. 3. The partial sequence (510bp) of cytochrome *b* gene of *L. calbasu* from Bangladesh

The BLAST results of the sequenced region brought *L. calbasu* as topmost hit with 100% similarity confirmed the taxonomy of the organism (Fig. 4). The available *cyt-b* sequence of *L. calbasu* from India had 100% identity with the *cyt-b* sequenced regions of the present study. In other words, no polymorphic

site was detected when corresponding regions of the *cyt-b* gene of *L. calbasu* from Bangladesh and India were aligned. Thus, this result indicated high sequence conservation in between two individuals of the same species studied from different geographical locations.

Labeo calbasu voucher NBFGRK-1101 cytochrome b (cytb) gene, partial cds; mitochondrial
Sequence ID: [KF574601.1](#) Length: 1141 Number of Matches: 1

Range 1: 293 to 802		GenBank	Graphics	▼ Next Match	▲ Previous Match
Score	Expect	Identities	Gaps	Strand	
942 bits(510)	0.0	510/510(100%)	0/510(0%)	Plus/Plus	
Query	1	TCGCCCGAGGCTATACTATGGATCATACTTATATAAAGAAACCTGAAACATTGGCGTAG			60
Sbjct	293	TCGCCCGAGGCTATACTATGGATCATACTTATATAAAGAAACCTGAAACATTGGCGTAG			352
Query	61	TACTCTTACTATTAGTCATAATAACAGCCTTTGTTGGCTACGTCCTTCCATGAGGACAAA			120
Sbjct	353	TACTCTTACTATTAGTCATAATAACAGCCTTTGTTGGCTACGTCCTTCCATGAGGACAAA			412
Query	121	TGTCCTTCTGAGGTGCCACAGTAATCACAAACCTATTATCCGCTGTACCCCTACATAGGAG			180
Sbjct	413	TGTCCTTCTGAGGTGCCACAGTAATCACAAACCTATTATCCGCTGTACCCCTACATAGGAG			472
Query	181	ATATACTAGTACAATGAATCTGAGGTGGCTTCTCAGTAGACAATGCAACACTAACACGTT			240
Sbjct	473	ATATACTAGTACAATGAATCTGAGGTGGCTTCTCAGTAGACAATGCAACACTAACACGTT			532
Query	241	TCTTCGCATTCCACTTCCACTACCATTATTATCGCCGCCGCAACCATTATCCACCTGC			300
Sbjct	533	TCTTCGCATTCCACTTCCACTACCATTATTATCGCCGCCGCAACCATTATCCACCTGC			592
Query	301	TATTCCTCCACGAGACAGGATCTAACAACCCAAATCGGACTAAACTCGGACGCAGATAAAA			360
Sbjct	593	TATTCCTCCACGAGACAGGATCTAACAACCCAAATCGGACTAAACTCGGACGCAGATAAAA			652
Query	361	TTTCCTTCCATCCATACTTTACATACAAGACCTCCTTGGATTTCGTAATTATACTGCTAG			420
Sbjct	653	TTTCCTTCCATCCATACTTTACATACAAGACCTCCTTGGATTTCGTAATTATACTGCTAG			712
Query	421	CTCTTACACTACTAGCGTTATTCTCCCAAACCTACTAGGAGACCCAGAAAACCTCACCC			480
Sbjct	713	CTCTTACACTACTAGCGTTATTCTCCCAAACCTACTAGGAGACCCAGAAAACCTCACCC			772
Query	481	CCGCAAACCCCTTAGTAACCCACACACA	510		
Sbjct	773	CCGCAAACCCCTTAGTAACCCACACACA	802		

Fig. 4. The *cytochrome b* gene sequence (partial, 510bp) of *Labeo calbasu* from Bangladesh showing 100% similar identity with that of the same species from India. Image taken from NCBI BLAST search result.

Multiple sequence alignment involving total eight *Labeo* species (viz. *L. angra*, *L. bata*, *L. boggut*, *L. calbasu*, *L. fimbriatus*, *L. gonius*, *L. pangusia* and *L. rohita*) showed variations in the corresponding regions among the species (Fig. 5). Out of 510 nucleotides in the sequence, there were total 120 polymorphic sites. These polymorphic sites could be used as the markers of *Labeo* species identification through PCR-sequencing. In molecular identification, mitochondrial *COI*, *16SrRNA*, *12SrRNA* and *cyt-b* are used frequently. Jahan *et al.* (2017) have studied *16SrRNA* gene sequence of three *Labeo* species viz., *L. rohita*, *L. bata* and *L. gonius* and found 23 polymorphic sites (5.8%) in 396 nucleotide long sequence. The polymorphic site variations (23.5%) are more frequent than that reported by Jahan *et al.* (2017) because of larger size sequences, higher number of species (eight) and use of different region of mitochondrial genome (part of *cyt-b*) in the present study.

The GC content of the sequenced region of *L. calbasu* is 43.33%. Similarly, the GC contents of the corresponding *cyt-b* gene fragment of the available *Labeo* species are 43.33% in *L. angra*, 43.73% in *L. bata*, 42.75% in *L. boggut*, 44.51% in *L. fimbriatus*, 44.51% in *L. gonius*, 42.35% in *L. pangusia*, and 44.31% in *L. rohita*. Though the GC content of the *cyt-b* gene region of *L. calbasu* does not differ with that of *L. angra*, there are variations in the nucleotide sequence among these two species (Fig. 5). On the other hand, GC contents in *L. fimbriatus* and *L. gonius* do not differ and their nucleotide sequences also do not differ which could be a sign of very close relationship among these two species.

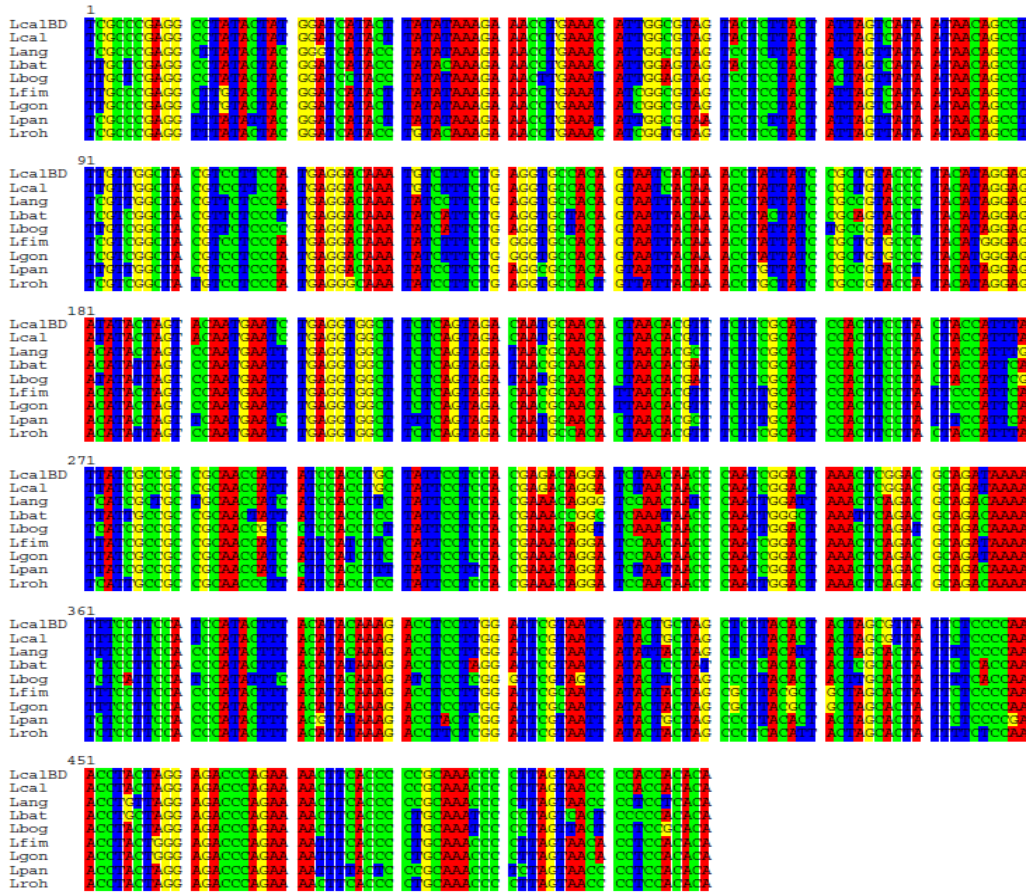


Fig. 5. The multiple sequence alignment of the orthologous regions of cytochrome b gene showing sequence diversity among different species under the genus *Labeo*. Total eight *Labeo* species have been compared. These are *L. calbasu* (LcalBD represents present study and Lcal, India), *L. angra* (Lang), *L. bata* (Lbat), *L. boggut* (Lbog), *L. fimbriatus* (Lfim), *L. gonius* (Lgon), *L. pangusia* (Lpan) and *L. rohita* (Lroh).

The neighbor-joining phylogenetic tree constructed with 100 bootstrap values have been presented in Fig. 6. A hemichordate, *Balanoglossus carnosus*, was chosen as out group.

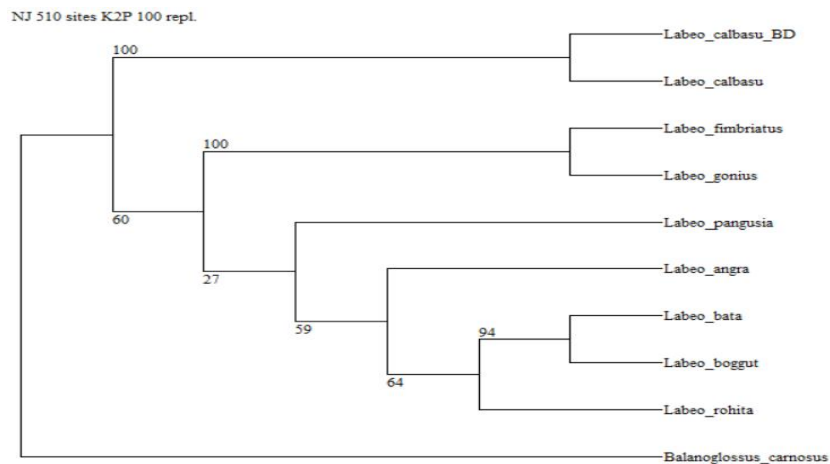


Fig. 6. Relationship among *Labeo* species based on *cyt-b* gene sequence. Phylogenetic tree has been constructed using neighbor-joining method with 100 bootstrap values. *L. calbasu* Bangladesh represents the *cyt-b* gene sequence of the present study. A hemichordate *Balanoglossus carnosus* has been selected as outgroup.

In the tree, two sequences of *L. calbasu* (Bangladesh and India) formed a separate group indicating distinctiveness of this species from all other *Labeo* species of present study. On the other hand, *L. fimbriatus* and *L. gonius* were in the same group. Though these are two different species, they showed monophyly due to highly conserved sequences of *cyt-b* gene regions. Again, *L. bata* and *L. boggut* created a monophyletic group with the same origin indicating their close relationship. The similar relationship of *L. bata* and *L. boggut* have been reported by Yang *et al.* (2012) who studied the molecular phylogeny of the cyprinid tribe Labeonini extensively. *L. rohita* and *L. calbasu* formed separate clade as presented by Yang *et al.* (2012) that also conforms the result of the present study.

The sequence variations reflected in multiple sequence alignment and phylogenetic tree have significance in understanding the relationships among the *Labeo* species and molecular identification of the species through PCR-sequencing or RFLP (restriction fragment length polymorphism) analysis. Besides, species-specific primers can be designed from the sequence analysis to identify the species effectively with less cost. Furthermore, the *cyt-b* gene of other *Labeo* species of Bangladesh also needs to be sequenced like *L. calbasu* to have data of the local species.

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