PCR-SEQUENCING OF MITOCHONDRIAL *16S rRNA* GENE OF *LABEO ROHITA*

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Abstract

The genetic structure, diversity and molecular identification of *Labeo rohita* (locally known as *rui* fish) is mostly remained unexplored in Bangladesh. Sequence of *16S rRNA* (ribosomal RNA) along with that of some other mitochondrial genes is commonly used in molecular identification of species. No sequence data for *16S rRNA* gene of *L. rohita* of Bangladesh could be retrieved from the GenBank database. Therefore, to sequence a *16S rRNA* fragment of this species, DNA was extracted from a locally collected sample and then the targeted region of the gene was amplified by polymerase chain reaction (PCR) using a set of universal primers designed for this gene. Polymorphic sites among different *Labeo* species and *L. rohita* of other locations were identified by aligning relevant sequences.

***Key Words*:** *Labeo rohita*, *16S rRNA*, PCR-sequencing and polymorphic sites.

INTRODUCTION

Labeo rohita is one of the economically important freshwater fish belonging to the family Cyprinidae. The complete mitochondrial genome of *L. rohita* is 16,611 bp in length (circular DNA) and consists of 13 protein-coding genes, 22 tRNA genes, 2 rRNA genes (*12S rRNA* and *16S rRNA*), and one control region. Majority of the vertebrates have more or less similar gene organisation and order in the mitochondrial DNA (mtDNA) (Bej *et al.* 2012).

The complete and partial mtDNA sequences of several species of vertebrates have been determined and are available in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). *16S rRNA* and *12S rRNA* genes encode for large and small ribosomal subunits, respectively. *16S rRNA* gene of *L. rohita* is about 1692 bp in length. It has several functions. Like the large (23S) ribosomal RNA of nuclear origin, it determines the positions of ribosomal proteins.

For the analysis of evolutionary processes, mtDNA is found highly useful (Brown *et al.* 1979). The sequence of ribosomal RNA molecules has been widely used for phylogenetic studies and sequence diversity in hyper-variable regions is known to help in differentiating strains. There are several studies where *16S rRNA* gene sequence has been used in finding taxonomic relationships among the fishes (Harris and Mayden 2001, Chakraborty and Iwatsuki 2006). Therefore, the *16S rRNA* gene has great potential in exploring divergences among the cyprinid fishes.

Different molecular techniques have been employed to differentiate *Labeo* species so far. Generally, for species identification, differentiation and genetic diversity study, molecular techniques, such as RAPD, RFLP, AFLP, sequencing, microsatellite markers, etc. are utilized. Among different techniques, PCR-sequencing provides the most necessary information. So, PCR-sequencing has been chosen in the present study.

The main objective of the present study is to sequence a *16S rRNA* gene fragment of *L. rohita* from Bangladesh. Genetic diversity at *16S rRNA* gene among the *L. rohita* from various locations, and among different *Labeo* species will also be explored. It will be helpful

in conserving their diversity. Extension of this study could be pivotal in making positive impact towards biodiversity conservation and bioresource management in general.

MATERIAL AND METHODS

Sample Collection

The live specimens of *L. rohita* were collected from a local market (viz. Palashi Bazar of Dhaka city). The sample was placed in ice immediately after collection and transferred into 70% ethanol at 4°C after returning to the Laboratory of Genetics and Molecular Biology, Department of Zoology, University of Dhaka. Approximately 40-65 mg of soft muscle tissue of the sample specimen was used for DNA extraction.

DNA extraction

Total genomic DNA was extracted by phenol: chloroform extraction method (Begum *et al.* 2004) with slight modification. Tissue sample was squashed in 500 µl CTAB buffer. 10 µl proteinase K was added and the sample mixture was inverted to mix properly. An equal volume of phenol:chloroform was added to the sample mixture. The mixture was spun again at 13,000 rpm at room temperature for five minutes and lower phenol-chloroform phase was removed. DNA is found at the upper aqueous phase. Sample was precipitated with one volume of 100% ethanol at room temperature and inverted several times. DNA was pelleted by centrifugation at 13000 rpm for five minutes and washed with 70% alcohol, dried and dissolvedin 50 µl sterilized distilled water for 10 minutes. The extracted DNA was then visualized in 1.5% agarose gel of a low melting point grade (Life Technologies, USA) at 120 V for 20 minutes.

DNA amplification

DNA fragment encoding the *16S rRNA* gene 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 $MgCl_2$, 0.5 µl of 10 mMdNTP, 0.5 µl of *16S rRNA* forward primer, 0.5 µl of *16S rRNA* reverse primer, 0.16 µl of *Taq* DNA polymerase, 2 µl template DNA, and sterilized distilled water is used to make up a total volume of 25 µl. 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 primers used in the amplification of the DNA fragments are as follows, forward primer (5´-CGC CTG TTT AAC AAA AAC AT-3´) and reverse primer (5´-CCG GTT TGA ACT CAG ATC ATG T-3´). The PCR products of interest were then purified using Favor Prep PCR Clean up Mini Kit (Favorgen Biotech Corp.).

DNA Sequencing

Sequence of amplified DNA was determined by Sanger Dideoxy Sequencing method in the Centre for Advanced Research in Sciences (CARS), University of Dhaka, Dhaka.

Sequence retrieval and multiple sequence alignment

The nucleotide sequence of *16S rRNA* gene (partial) obtained from the present study was submitted to the GenBank database (Accession no. KJ425468.1). Two more *16S rRNA* sequences (Accession No: JN412817.1 and AP011201.1) of *L. rohita* were collected from NCBI nucleotide sequence database. Further, *16S rRNA* sequences of different *Labeo* species and one *Catla* species have been collected similarly for the purpose of multiple sequence alignment (MSA). Informative sites were scored. SEAVIEW software (Gouy *et al.* 2010) was used for MSA.

RESULTS AND DISCUSSION

A fragment of mitochondrial *16S rRNA* gene has been amplified by PCR. PCR product has been visualized after gel electrophoresis (Fig. 1).

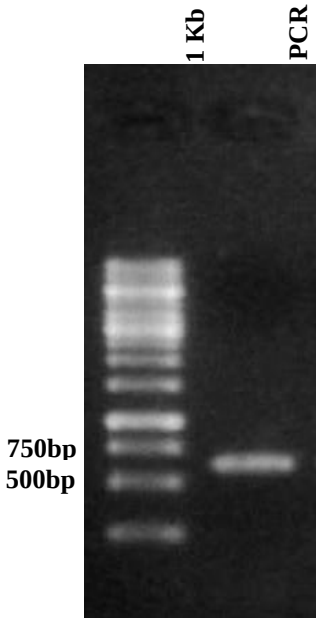

Fig. 1. Agarose gel electrophoresis of a *16S rRNA* gene region amplified by PCR. Genomic DNA extracted from *L. rohita* has been used as template.

The partial nucleotide sequence of the *16S rRNA* gene of *L. rohita* (Bangladesh) obtained from the present study has been submitted to the GenBank database (Accession no. KJ425468.1). A multiple sequence alignment of this sequence (612bp) with five other selected *Labeo* sp. and one *Catla* sp. has been presented in the Figure 2. Two more *L. rohita* from other geographical locations have been included in the analysis. The sequences are highly conserved among themselves with few polymorphic sites. In the sequenced region of *16S rRNA* gene, three informative sites have been detected in *L. rohita* which have nucleotides different from other *Labeo* species. The positions of the sites are 283, 379 and 450 where *L. rohita* possesses nucleotides G, C and G respectively. Between *Labeo* and *Catla* genus, two sites in the position 310 and 379 are informative.

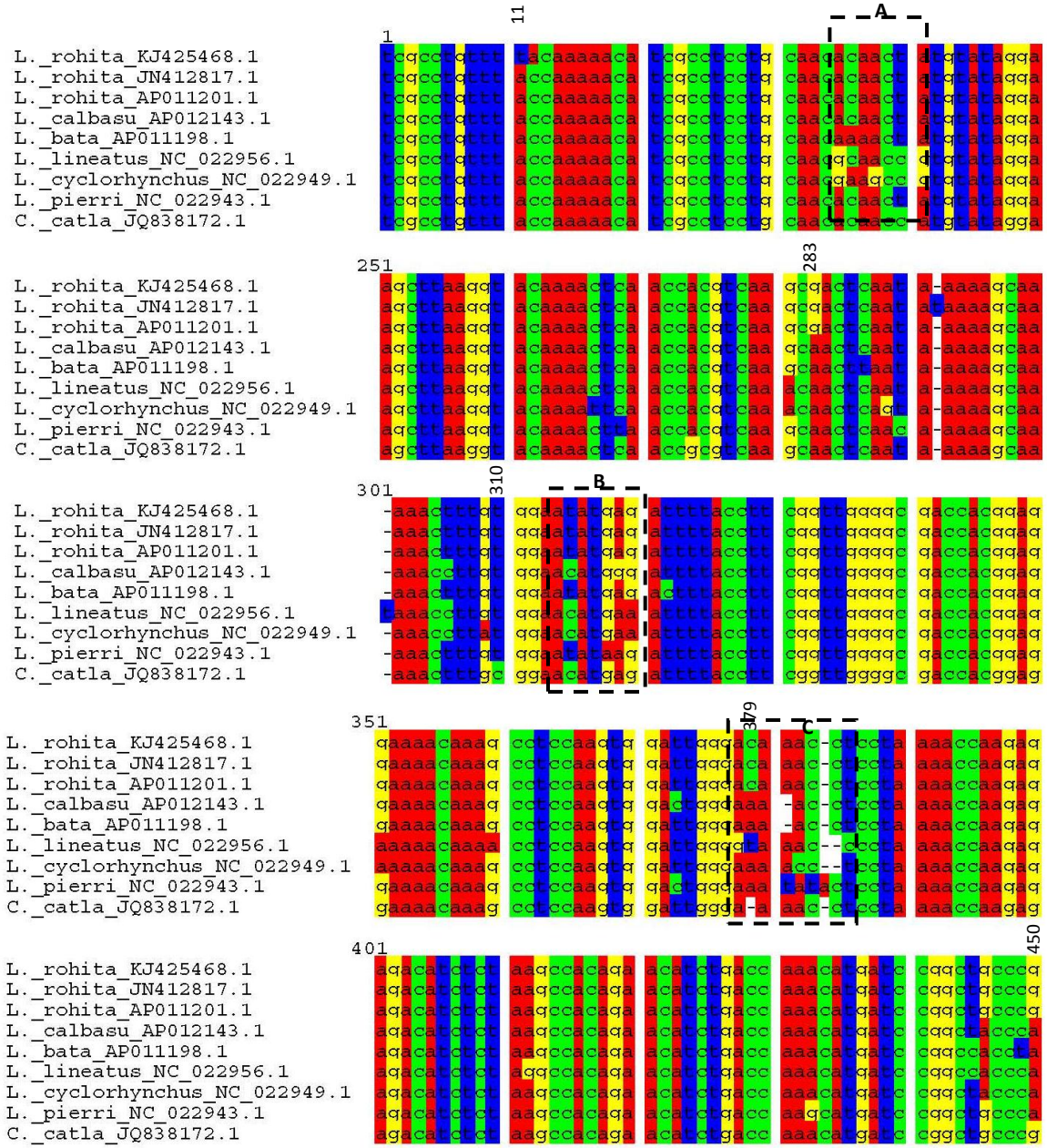

Fig. 2. Multiple Sequence Alignment of *16S rRNA* gene sequences (partial) of some selected *Labeo* species and one *Catla* species to find out informative polymorphic sites. Important (variable) regions (indicated by dashed line above) have been shown in the figure.

All the *Labeo* species have T in the position 310 whereas *Catla* species has C in the same position. Among three *L. rohita* sequences, one can be differentiated from other two in positions 11 and 609, though these small number variations could be attributed to within species variations at population level. At position 11, only *L. rohita* from Bangladesh possess T instead of A in other species as well as the same species of different locations under analysis. Similarly at position 609, *L. rohita* from Bangladesh possess A instead of G in all other samples under analysis. Since, these two polymorphisms are near the two ends of the 612bp sequence, both forward and reverse sequencing have been done twice in the present study and the result was the same in both times. In a similar work, *16S rRNA* gene of three

catfishes from Bangladesh has been sequenced. Analysis of the sequences revealed informative sites useful for species differentiation (Alam *et al*. 2015).

Within the sequenced DNA fragment, there are some regions highly variable among the selected *Labeo* species as marked by dashed boxes (Fig. 2 A, B & C). These highly variable regions show the genetic diversity among the species at these loci and could be utilized in molecular differentiation. The polymorphic sites which are variable in more than two species among the selected species have been presented in the Table 1.

Table 1. Selected polymorphic sites and nucleotide diversity among the *Labeo* species and *Catla catla 16S rRNA* regions. Sites with variations among more than two species are presented here. '-' means deletion gap.

Positions/ Species	**45**	**46**	**50**	**51**	**306**	**315**	**320**	**351**	**373**	**379**	**381**	**385**	**445**	**446**	**450**
L. rohita (BD)	A	C	T	A	T	T	G	G	T	C	A	C	T	G	G
L. calbasu	A	C	T	A	C	C	G	G	C	A	-	C	T	A	A
L. bata	A	A	T	A	T	T	G	G	T	A	-	C	C	A	A
L. lineatus	G	C	C	G	C	C	A	A	T	T	A	-	C	A	A
L. cyclorhynchus	G	A	C	G	C	C	A	A	T	A	A	-	T	A	A
L. pierri	A	C	T	A	T	T	G	G	C	A	T	C	T	G	A
Catla catla	A	C	C	A	T	C	G	G	T	-	A	C	T	G	G

Among the selected *Labeo* species, *L. rohita, L. calbasu and L. bata* are found in Bangladesh. Among these three *Labeo* species, nucleotides at positions 46, 306, 315, 373, 379, 381, 445 and 450 could be useful in differentiating them. *L. rohita* is different from other two Bangladeshi species at positions 379, 381, 446 and 450. *L. calbasu* is different from *L. rohita* and *L. bata* at positions 306, 315 and 373 (possessing 'C' instead of T in all three positions). On the other hand, *L. bata* can be identified based on the nucleotides at positions 46 and 445 when the Bangladeshi *Labeo* species are considered. Similarly, other *Labeo* species and *Catla* species could be differentiated based on the diversity in *16S rRNA* sequences.

Other techniques, such as RAPD has been used to differentiate six *Labeo* species in a previous study (Das *et al*. 2005). RAPD technique has also been used to study genetic diversity in wild and farmed *L. calbasu* from Bangladesh (Mostafa *et al.* 2009). Genetic diversity of an Indian *L. fimbriatus* population has been studied using microsatellite markers (Swain *et al.* 2013). Allozyme markers have been used in analyzing genetic diversity of *L. gonius* (Tewari *et al.* 2013), though *16S rRNA* sequence of *L. gonius* is not found in the GenBank database. Yang *et al.* (2012) studied molecular phylogenetic relationship and genetic diversity among cyprinid fishes by sequencing four nuclear and five mitochondrial genes. The partial sequence of *16S rRNA* gene obtained in the present study has made it possible to analyze genetic diversity among selected *Labeo* species and *Catla* species. Thus, based on the nucleotide diversity, molecular identification of species is possible, though more than one gene from both nuclear and mitochondrial genome will provide more confidence.

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