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## Short Communication

# Identification of Three Cyprinidae Family Members through Cytochrome Oxidase I

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**Authors' Contributions**
**SS conducted the study. MNK, MEB and MI supervised the research and reviewed the manuscript. IA and MS helped in data collection and execution of experiments. AW, MNS and MSI helped in statistical analysis.**

**Key words**
***Labeo rohita, Cirrhinus mrigala, Catla catla, COI* gene, DNA barcoding.**

## ABSTRACT

Fishes belonging to family Cyprinidae are important for aquaculture and also for food and economy of Pakistan. Among members of Cyprinidae family, Indian major carp species (*Labeo rohita, Cirrhinus mrigala, Catla catla*) are very suitable fish species for culture in Pakistan. However, the identification and phylogeny of these fish species are of much interest today. DNA barcoding is used as a bio-identification tool for the organism globally. The current study was also conducted for the fast and accurate identification of Indian major carp species. The fishes used in this study were captured from four geographically isolated districts of Punjab province. Blood samples of sixty (60) fishes were taken carefully from caudal veins of each captured fish species. Genomic DNA was extracted from blood and short segment *COI* gene (680bp) was amplified by PCR. The sequences were evaluated by using bioinformatics tools. The average K2P conspecific, congenric and confamilial genetic distances were 0.22%, 6.71% and 12.33%, respectively. Findings of the present study revealed that DNA barcoding using *COI* gene is an efficient molecular technique for the exact identification of typical fish species.

Pakistan freshwater fish fauna consists of almost 193 fish species. These fish species comes under kingdom Animalia, phylum Chordata, class Actinopterygii, and sub-class Teleostei. There are six superorders, 13 orders, 30 families and 86 genera that encompass these fishes. Out of these 193 freshwater fish species, 43 freshwater fish species have special importance as they are native to Pakistan and Kashmir. Indian major carps include three commercially important fish species, *Labeo rohita, Cirrhinus mrigala* and *Catla catla*. These fish species are native to Asia and are widely cultured in Pakistan, India, Nepal, Sri Lanka, Malaysia and in some countries of South Asia (Muhammad *et al*., 2017).

Proper identification of species is a burning question now a day. Morphological and anatomical characteristics sometimes make the identification of particular species ambiguous. Keeping in view these issues, molecular markers are one of the best solutions for the proper identification of species (Hebert *et al*., 2003). Mitochondrial DNA is widely used now a day to determine the genetic relationship among species as it evolves rapidly than nuclear DNA (Keskin and Can, 2009). A short segment of mitochondrial DNA also named as DNA barcode is a very valuable tool to find out genetic makeup of species. DNA barcode library provides an efficient way to manage and control the introduced species in a typical environment. A short region of almost 650-700bp of mitochondrial Cytochrome c oxidase subunit I gene (*COI*) is utilized to identify particular species. The use of this short region of mitochondrial Cytochrome c oxidase subunit I gene (*COI*) for species identification is increasing day by day as it is one of the optimized, cheap and convenient tool for the authentication of species (Valentini *et al*., 2009).

Fish Barcode of Life (FISH-BOL) is a sequence database that helps in identification of species by using "barcodes". Fish Barcode of Life (FISH-BOL) is basically a global initiative that provides a uniform reference sequence library for the fishes that is found in all over the world. The barcode sequence of unidentified species is matched with the reference sequence library present in Barcode of life data (BOLD). If the sequence matched then we can identify that specimen easily, otherwise, if the sequence does not match then it leads to a new barcode sequence for the given specimen (Hajibabaei *et al*., 2007).

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This study aims to assess the genetic structure of three species of Indian major carps; *Labeo rohita, Cirrhinus mrigala* and *Catla catla* by amplifying the short segment of mitochondrial Cytochrome c oxidase subunit I gene (*COI*).

*Materials and methods*

The fishes used in this trial were *Labeo rohita, Cirrhinus mrigala* and *Catla catla*. Twenty (20) fishes of each species were selected for blood sampling. These fishes were captured from four different districts located in Punjab province of Pakistan. Blood samples were taken from caudal vein and these samples were further preserved in EDTA coated vials in order to prevent blood clotting. The volume of blood in each vial was almost 1ml. The average length of these experimental fishes was 28±5 cm and average weight was 900±100g. The Morphological identification of all the selected fishes was done with the help of fish identification key provided by Punjab Fisheries Department. The genomic DNA was isolated from whole fish blood by using the standard organic method of DNA extraction (Sambrook and Russell, 2006a). The extracted DNA was dissolved in low TE buffer and was stored at -20ºC. 1% agarose gel was used for the confirmation of DNA bands in the gel documentation system. Further DNA quantification of each sample was carried out with the help of Nanodrop (Thermo Scientific). Universal fish primers used for the amplification of a short segment of mitochondrial DNA (Ward *et al*., 2005) are given as follows: Fish F1: 5-TCAACCAACCACAAAGACATTGGCAC-3' and Fish R1: 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'.

The polymerase chain reaction (PCR) was accomplished using Thermocycler T100 BioRad (Ozcelik *et al*., 2012). For this PCR reaction, a reaction mixture of 25 ul was made in PCR tubes. This reaction mixture comprised of 2ul DNA template, 1ul dNTPs, 3ul Mgcl2, 3ul buffer, 0.4ul primer forward, 0.4 ul primer reverse, 0.4ul Taq polymerase enzyme, and 14.8ul deionized water. The PCR conditions for this typical reaction were; initial denaturation for 5 min at 95ºC, denaturation for 30 sec at 95ºC, annealing at 55ºC for 1 min, extension at 72 ºC for 1 min and final extension for 10 min at 72ºC. The samples obtained after PCR, amplification was run on 1.5% agarose gel. The gel was then placed in a gel documentation system for the confirmation of PCR bands of required size. The most suitable and clearly identified amplified products were sent for sequencing.

*Results*

A total number of 13 *COI* barcodes were obtained from three fish species of Indian major carps (*Labeo rohita, Cirrhinus mrigala*, and *Catla catla*) that belong to family Cyprinidae. Universal fish DNA barcoding primers FI and RI were applied for the sequencing of the short region of mitochondrial DNA. The obtained barcode sequences were then blasted on NCBI and also checked through BOLD (barcode of life data) system. The species identified after sequencing of the *COI* gene along with their assigned Genbank numbers are listed in Supplementary Table I. The average length of the selected region was 680bp. The polymorphic sites were 119 and Parsimony informative sites were 88. Singleton variable sites were 15 and site positions were 24, 27, 35, 39, 43, 44, 47, 48, 49, 55, 58, 59, 61, 62 and 206. Analyzed sequences did not contain any insertions/deletions or pseudogenes (short sequences with stop codons) or contaminant sequences (*e.g.,* from bacteria) that completely ensured that all the amplified sequences belong to functional mitochondrial *COI* sequences.

The mean K2P genetic distances, as well as mean nucleotide diversity, was calculated by using Kimura 2-parameter (K2P) distance model. All these values are presented in a tabular form in Supplementary Table II.

The phylogenetic analysis was done through the Neighbor-Joining tree method by using MEGA6 software. The nucleotide sequences computed for this analysis were 30. All the sites having missing data and gaps were removed. All the sequences of three above mentioned species were assessed with bootstrap values (1500 replicates). The species having maximum resemblance were clustered under the same node inside the tree. The suggested sequences were separated from sequences taken from NCBI and BOLD by black circles (Fig. 1).

*Discussion*

The objective of the current study was to identify the three members of Indian major carp species (*Labeo rohita, Cirrhinus mrigala*, and *Catla catla)* by sequencing short region of *COI* gene.

Species recognition on the molecular level with the help of DNA barcoding technique is commonly used all over the world. A typical fragment of mtDNA plays a pivotal role in the precise identification of those animal species which are still to be recognized correctly (Dawnay *et al*., 2007). A short segment of mitochondrial Cytochrome C oxidase 1 gene (*COI)* is employed for the better confirmation of unidentified species (Lohman *et al*., 2009). The study of the *COI* gene is very helpful in species authentication as variation in amino acid sequence occurs at a slow rate than another mitochondrial gene (Hebert *et al*., 2003). The main purpose of barcode studies is to define the species boundaries at *COI* gene locus for the recognition of unknown species but sometimes it leads to the discovery of unnoticed species and unusual species diversity (Kerr *et al*., 2009; Meyer and Paulay, 2005).

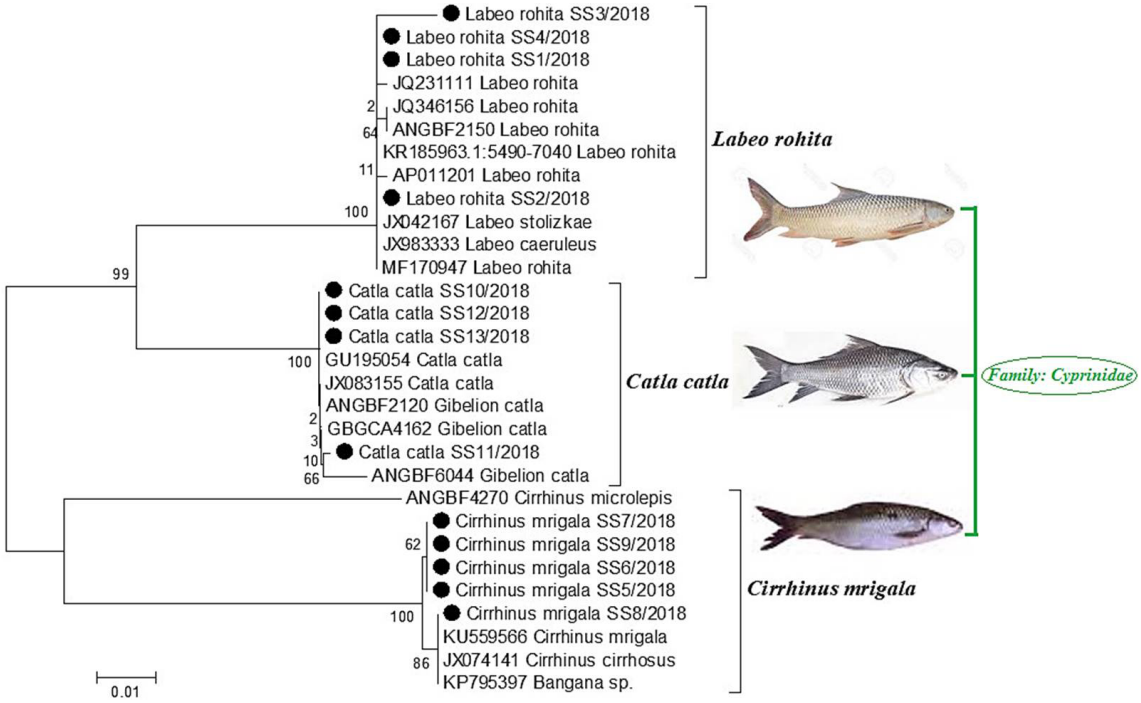


Fig. 1. Neighbour joining tree of Indian Major Carps constructed on the basis of K2P genetic distance.

In this study universal fish primers, F1 and R1 having average read length of 680bp were applied for the recovery of DNA barcodes of three members of Indian major carp species. The polymorphic sites were 119 and Parsimony informative sites were 88. Singleton variable sites were 15 and site positions were 24, 27, 35, 39, 43, 44, 47, 48, 49, 55, 58, 59, 61, 62 and 206. Analyzed sequences did not contain any insertions/deletions or pseudogenes (short sequences with stop codons) or contaminant sequences (e.g., from bacteria) that completely ensured that all the amplified sequences belong to functional mitochondrial *COI* sequences. The absence of stop codons in average read length of 680bp shows that NUMT (nuclear DNA sequence that originate from mitochondrial DNA sequence and are typically less than 700bp in length) were not sequenced and this result was in parallel with the already presented results (Lakra *et al*., 2011; Ward *et al*., 2005).

The generated barcode sequences were blasted on NCBI and BOLD to obtained the related *COI* gene sequences for the assessment of nucleotide diversity and genetic divergence analysis of barcode sequences. A comprehensive analysis of all the barcode sequences showed a clear-cut identification and differentiation of all the species taken in this research work. The average K2P genetic distance within the species was 0.22% whereas the estimated genetic distance within genera was 6.71%. These results indicated that the average nucleotide diversity between same species is far less than that of mean nucleotide diversity between different fish species as indicated in Supplementary Table II. Furthermore, the intraspecific average nucleotide diversity at subfamily and the family level was 11.62% and 12.33%, respectively which showed a slight increase in nucleotide divergence when we advanced from sub family to family level. These results when compared with results concluded from DNA barcoding of Indonesian fresh water fishes, showed that there was a strong consistency of our results with Indonesian fresh water fishes having intraspecific (0.15%), intrageneric (2.53%) and intrafamilial (5.63%) K2P genetic distances (Muchlisin *et al*., 2013).

The average conspecific, congeneric and confamilial K2P distances of our fishes were also in parallel with Australian marine fishes (0.39%, 9.93%, and 15.46%, respectively) (Ward *et al*., 2005) as well as Canadian freshwater fishes (0.27%, 8.37%, and 15.38%, respectively) (Hubert *et al*., 2008) (Supplementary Table II). It was observed that there was a gradual increase in genetic distances with the rise of the taxonomic level, so a prominent change in the genetic distances was noticed when we move from species to higher taxonomic level. The variation was more among congeneric individuals which supports the arguments in the previous research papers that with the rise in taxonomic level the rate of nucleotide divergence increases (Lakra *et al*., 2011; Jaafar

*et al*., 2012; Ward *et al*., 2005).

The neighbor-joining tree analysis of 30 sequences was done that comprised of 13 resultant sequences and 17 additional sequences from closely related species showing 95-98% similarity in NCBI GenBank database. The neighbor-joining tree was generated by aligning the sequences with the help of Muscle (Multiple Sequence Comparison by Log-Expectation) alignments. These results revealed that the nucleotide divergence among the same species was low. However, it was high between different species of the same family. These results and tree presentation was almost parallel with the previous studies on fish barcoding (Karim *et al*., 2015; Lakra *et al*., 2011).

*Conclusion*

To study species identification and species diversity exactly is the main interest of many scientists all over the globe. DNA barcoding is an emerging tool for the precise recognition of typical species as well as proper discrimination among different species. It is a beneficial method for certification of many diverse species as old methods for species identification have some restrictions. DNA barcoding is done with the help of a short segment of *COI* gene of mitochondrial DNA as this segment has a lot of variations among species which is critical for species identification. This technique is also helpful in finding out the nucleotide divergence among organisms having closely related taxa. The current study involved the identification of Indian major carp species (*Labeo rohita, Cirrhinus mrigala, Catla catla)* through the amplification of a small sequence of *COI* gene of mitochondrial DNA. The results of this study indicated that *COI* gene can be effectively utilized for species discrimination and is also nominated as standard region for DNA barcoding.

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*Supplementary material*

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/2020.52.1.sc13

*Statement of conflict of interest*

The authors declare no conflict of interest.

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