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Molecular identification and phylogenetic analysis of the Indian major carp *Catla catla*

Rose Gregoria P.J.[1] and Moses Inbaraj R[2].

Endocrinology Unit, Department of Zoology, Madras Christian College, Tambaram, Chennai 600 059, India

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ABSTRACT

DNA barcoding is one means of establishing a rapid, accurate, and cost-effective system for the identification of species. It involves the use of short, standard gene targets to create sequence profiles of known species against sequences of unknowns that can be matched and subsequently identified. The *cytochrome oxidase subunit-1* gene fragments were then sequenced from the samples in accordance with the standard DNA barcoding protocols. *Catla catla* is a member of the genus Catla, of the carp family Cyprinidae and order Cypriniformes. This work aims at barcoding the freshwater species *C. catla*, known as the Indian major carp and its phylogenetic assessment. Significance of this study is to check the mutation rate in the specimen from the southern part of India by comparing many barcode results from the northern part. This is the first study of *C. catla* barcoding from the southern part reported to NCBI. As the global market for fisheries and aquaculture products expands, mislabeling of these products has become a growing concern in the food safety arena. Molecular species identification techniques hold the potential for rapid, accurate assessment of proper labeling.

Key words *: Barcoding, CO1, Sequencing, Cyprinidae, Mutation, Phylogenetic assessment.*

Introduction

The three Indian major carps, catla (*Catla catla*), rohu (*Labeo rohita)* and mrigala (*Cirrhinus mrigala) are* the main components in composite fish farming in India. Of the three, catla is the fastest growing, attaining a maximum size of 63 kg (Jhingran, 1968). Intergeneric hybrids of catla with Indian and other carp species have been produced (Tripathi, 1992) of which the rohu female x catla male hybrid combines the advantages of the deep body of the catla and the small head rohu. Naturally occurring catla x rohu hybrids have been found in the Rihand reservoir. But insufficient scientific study has been carried out on this amazing freshwater species *C. catla* or Indian Carp.

Fish identification was traditionally based on morphological features. However, due to high diversity and morphological plasticity, in many cases, fish and their diverse developmental stages are difficult to identify by using morphological characteristics alone (Victor *et al*., 2009).

Hebert *et al*. 2003 proposed that a single gene sequence would be sufficient to differentiate all, or at least the vast majority of animal species, and proposed the use of the mitochondrial DNA gene *cytochrome oxidase subunit1* (*COI*) as a global bioidentification system for animals. The sequence was likened to a barcode, with species being delineated by a particular sequence or by a tight cluster of very similar sequences.

For many animal taxa, sequence divergences

within the 5' region of the mitochondrial *cytochrome oxidase subunit1* (*COI*) gene are generally much greater between species than within species. This in turn suggests that the approach is extensively applicable among phylogenetically distant animal groups (Hajibabaei *et al*., 2006; Neigel *et al*., 2007). Another investigation has shown that intraspecific variation of *CO1* barcodes is generally pretty small and clearly discriminable from interspecific variation (Wong *et al*., 2011). High efficiency of species identification was demonstrated in DNA barcoding by Xing Bingpeng, *et al*., 2018. Recently Bryan *et al*., 2019 achieved objective of designing a single pair of universal COI primers that is capable of generating high quality barcode sequences for the various species of Characiform fishes in Brazil. Mizanur Rahman *et al*., 2019 propose the new term Operational Barcode Unit (OBU) to simplify references to would-be DNA barcode sequences and sequence clusters. Dutrudi Panprommin *et al*., 2019 indicated that DNA barcodes are an effective approach to identify fish species for diversity studies.

Fish and fish products are important contributors to human food security. Accurate and unambiguous identification of fish and fish products, from eggs to adults, is important in many areas. It would enable retail substitutions of species to be detected, assist in managing fisheries for long-term sustainability, and improve ecosystem research and conservation. Present work aims at barcoding the freshwater species *C. catla*, known as the Indian major carp and its phylogenetic assessment. Significance of this study is to ensure the mutation rate in the specimen from the southern part of India by comparing many barcode results from the northern part. This is the first study of *C. catla* barcoding from the southern part reported to NCBI.

Materials and Methods

Sample Collection

The fish samples of the Indian major carp, *C. catla* were collected from the Tamil Nadu Fisheries Department Corporation (TNFDC), Sathanoor Dam, situated about 200km south of Chennai. Gill samples are collected from the live fishes in eppendorf tubes and kept in 95% ethanol.

Experimental Method

Genomic DNA was isolated from the samples provided using Sigma Aldrich DNA extraction Kit. Its quality was evaluated on 0.8% Agarose Gel electrophoresis. A single band of high molecular weight DNA was observed. CO1 gene fragment was amplified by PCR from the above isolated genomic DNA. A single discrete band was observed when resolved on Agarose Gel. The PCR amplicon was purified by column purification in order to remove contaminants.

The concentration of the purified DNA was determined and was subjected to automated DNA sequencing on ABI3730xl Genetic Analyzer. To avoid errors in sequencing, PCR amplification of samples were sequenced on both strands. Each nucleic acid sequence was edited manually to correct falsely identified bases and trimmed to remove unreadable sequence at the 3' and 5' ends (considering peak and Quality Values for each base) using the sequence analysis tools. The edited sequences (*CO1* gene) were then used for similarity searches using BLAST (Basic Local Alignment Search Tool) programme in the NCBI Gen Bank DNA database for identifying the sample. Multiple sequence alignments were created using the Clustal W Sequence Alignment program. The software used for phylogenetic analysis was Molecular Evolution Genetics Analysis software4 (MEGA4). Phylogenetic trees were constructed and evolution history was analysed by using the Neighbor-Joining method. Totally 55 sequences were selected from the NCBI GenBank which belongs to cyprinidae family and other families of freshwater species of India.

Results

The length of the Consensus Sequence Data from the forward primer was 631bp and the length of the Consensus Sequence Data from the riverse primer was 636 bp. The sequences were subjected to run in Blast and the results showed 100% similarity with *Catla catla.* Contiguous Assembly of DNA Sequence of CO1 Gene length was 631 bp and the sequence data is published in NCBI GenBank. The accession number is KJ406530.

Multiple sequences Alignment of 55 selected Sequences was done by using ClustalW. The sequences were selected from NCBI GenBank, which includes both closely related and distant related freshwater fishes in India. The Accession numbers of these selected sequences are KJ406530 (*Catla catla*), HM776362 (*Ctenopharyngodon idella*),

HM776365 (*Hypophthalmithys nobilis*), HQ536319 (*Carassius cuvieri*), HQ536352 (*Chanodichthys erythropterus*), HQ961090 (*Carassius gibelio*), KC242342 (*Henicorhynchus siamensis*), JX074182 (*Labeo coubie*), KF430008 (*Labeo fimbriatus*), HM536924 (*Cirrhinus microlepis*), HM536911 (*Catlocarpio siamensis*), JX260850 (*Cirrhinus cirrhosus*), JX074222 (*Labeo vulgaris*), KF429922 (*Catlacatla*), KF558297 (*Carassius auratus*), JX983251 (*Channa punctata*), HM117206 (*Channa striata*), JX983244 (*Channam arulius*), JX024343 (*Clarias batrachus*), JX983311 (*Heteropnneustes fossilis*), JF280832 (*Clarias macrocephalus*), JQ667532 (*Danio rerio*), HM536921 (*Oreichthys cosuatis*), JX983443 (*Puntius amphibious*), JQ713852 (*Puntius chola*), EF452891 (*Chela cachius*), JN673955 (*Esomusdanricus*), JX983247 (*Channa orientalis*), KF738188 (*Botiastriata*), JN628918 (*Rita rita*), JQ842475 (*Gambusia affinis*), JX983514 (*Xenentodon cancila*), JX983239 (*Chanda nama*), JX983365 (*Mastacembelus armatus*), JX983309 (*Glossogobius giuris*), JX887591 (*Anguilla bengalensis*), JX983214 (*Anabas testudineus*), KF516682 (*Etroplus suratensis*), JX983509 (*Wallago attu*), JX983422 (*Oreochromis mossambicus*), JQ866902 (*Salmo trutta*), JX983505 (*Tor tor*), JX401294 (*Tor khudree*), EU716112 (*Tincatinca*), JX983369 (*Mugil cephalus*), KF929723 (*Chanos chanos*), EU189379 (*Lates calcarifer*), JX983339 (*Labeocal basu*), KF430004 (*Labeo bata*), JX946419 (*Labeo gonius*), JX074167 (*Cirrhinus cirrhosus*), and JQ435844 (*Puntius chelynoides*).

Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.,* 2007). The evolutionary history was inferred using the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) (Fig. 1). From the method of phylogenetic tree it is clear that the relationship of each species is also based on the similarities of mitochondrial *CO1*sequence data. It confirm the similarity of the specimen subjected to barcoding with the species *C. catla* since both comes under the same clade. Another important point noticed was that all the species named as Indian major carps comes verymuch close to the *C. catla* and secondly all the species comes under cyprinidae family comes together with the Indian major carps. In the phylogenetic tree of Neighbor-Joining method *C. catla* from bottom to *Chela cachius* comes under the same family called cyprinidae except one species *Botia striata* belongs to botidae family. Even then cyprinidae and botidae belongs to the same order cypriniformes. So this finding can be concluded as *Botia striata* can be a common ancestor for cyprinidae family or it may be originated from cyprinidae family. And when we analyse other top part of the tree it is clear that families belong to the same order comes together and orders belong to the same super order comes together. These results can be concluded as that the phylogenetic analysis also predicts the accuracy of barcoding.

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 4.54691885 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary

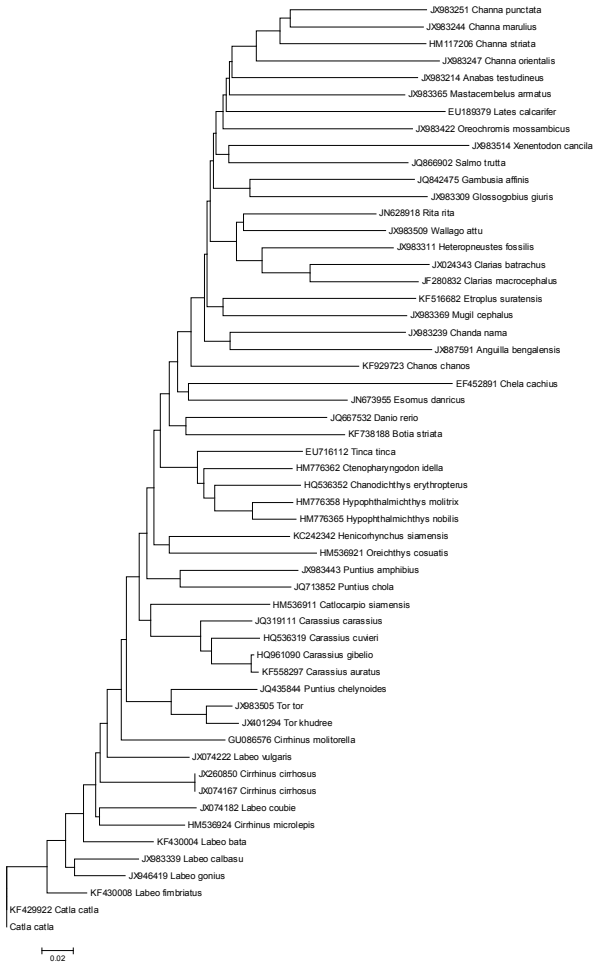

Fig. 1. Evolutionary relationships of 55 taxa towards C. catla (Neighbor-Joining method)

distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 503 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al*., 2007).

Discussion

DNA barcoding has been proposed as a universal method to identify species and uncover biological diversity. As noted Hebert (2004) demonstrated that sequence divergences for 5' region of the mitochondrial DNA gene *cytochrome oxidase subunit I* (*COI*) were generally much greater between species than within them for many species and in turn suggests that the approach is widely applicable across phylogenetically distant animal groups. Because of this reason the sequence obtained in the present study shows 100% similarity with previous sequences of the specimen *C. catla*.

The present study is more relevant in the sense that this is the gateway of barcoding the Indian major carp, *C. catla* from the southern part of India even though many barcodings were done from the northern part. Accession numbers of previous findings of *C. catla* barcoding results were JX983238.1, JX983237.1, JX887594.1, JX887593.1, JX887592.1, JX260838.1, KC757327.1, KC757326.1, KC757325.1, KC757324.1, KC757323.1, KC757322.1, KC757321.1, KC757320.1, KC757319.1, KC757318.1, KC757317.1, KC757316.1, KC757315.1, KC757314.1, KC757313.1, KC757312.1, KC757311.1, KC757310.1, JQ801755.1, JQ236669.1, HM026494.1, KF429922.1, KF429921.1, KF429920.1, KF429919.1, KF429918.1, KF429917.1, KF429916.1, KF429915.1, KF429914.1, FJ459463.1, FJ459462.1, FJ459461.1, FJ459460.1, GU195124.1, GU195065.1, GU195064.1, GU195063.1, GU195062.1, GU195061.1, GU195060.1, GU195059.1, GU195058.1, GU195057.1, GU195056.1, GU195055.1, GU195054.1, GU195053.1, GU195052.1, GU195051.1, GU195050.1, EU847510.1. In all these findings sequence data was above 600bp and the blast results shows 100% similarity with these sequences.

As revealed the similarity in the sequences of same species in the present study the effectiveness of barcoding has been demonstrated in diverse taxa, including spiders (Barrett and Hebert, 2005), butterflies (Hebert *et al*., 2003; Hajibabaei *et al*., 2006), flies (Smith *et al*., 2007), fishes (Ward *et al*., 2005), birds (Kerr *et al*., 2009) and mammals (Borisenko 7*et al*., 2008).The Fish Barcode of Life campaign (FISH-BOL) seeks to establish a standard reference sequence library for the molecular identification of fishes worldwide (Steinke and Hanner, 2010). The identification process using *COI* sequence data for fishes is promising, as supported by recent examples of its application. DNA barcoding surveys of 207 Australian marine fish species (Ward *et al*., 2005) and 210 Australasian shark and ray species (Ward *et al*., 2008) have concluded that DNA barcoding can be used for both teleost and chondricthyan species identification. Hubert *et al*. (2008) were able to distinguish 93% of 190 Canadian freshwaters fishes using the mitochondrial DNA *COI* gene. Steinke *et al*. (2009) demonstrated that sequence variability in the barcode region permitted discrimination of 98% of 201 fish species from the Canadian Pacific. In addition, barcodes were subsequently used to identify marine fish larvae from Australia (Victor, 2007) and Antarctic (Webb *et al*., 2006) waters. The study of Wazir Lakra *et al*.,2015 strongly validated the efficiency of COI as an ideal marker for DNA barcoding of Indian freshwater fishes.The findings of Sophia *et al*., 2017 confirm that DNA barcoding is a robust tool for fish species identification, and that mini-barcoding has high potential for useas a complement to full barcoding.

The current finding points out the import of phylogenetic analysis based on the barcoding sequences. It obviously indicates the nearness and also distance between different species. So the mitochondrial DNA (mtDNA) has become one of the most widely used molecular markers for diversity and phylogenetic studies in animals because of its size, maternal mode of inheritance, high rate of mutation, and simple genomic structure. Although mtDNA sequences have proved valuable in determining phylogenetic relationships, the choice of a gene as a molecular marker and clock in phylogeny is also important (Naylor *et al*., 2012). Recent phylogenetic studies in different taxa suggest that full-length mitochondrial genomic sequences provide an improved resolution for reconstructing a robust phylogeny and for molecular dating of divergence events within a phylogeny (Inoue *et al*., 2010). This work also reveals the relevance and accuracy of DNA barcoding in taxonomy and also the impor-

tance of phylogenetic tree construction to identify the similarities and differences of sequences in between different taxa.

In summary, most of the freshwater fish species in India analyses here through phylogenetic tree construction based on the barcoding sequence of the Indian major carp *C. catla*, exhibit a similar pattern of genetic diversity at *COI*, each species being a single cluster of tightly related mtDNA sequences distinct from all other species. Therefore, the present investigation supports the view that the use of *COI* barcodes is a powerful tool for species identification. Using this method would clearly allow the identification of individually isolated freshwater fish eggs, larvae, fillets and fins, hence providing many new tools useful for the practice of conservation and forensics genetic in these freshwater fishes. And also this stuff provides a precise molecular basis for the identification of the species *C. catla*.

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