



FISH IDENTIFICATION USING SEQUENCE ANALYSIS OF THE AMPLIFIED COI GENE

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ABSTRACT

Accurate identification of the study animal is of utmost importance. In case of the fish, the use of only morphological characteristics can lead to an error as these tend to overlap and vary based on the life stage and the habitat. The use of COI gene sequence for species identification is becoming popular since last few years. An attempt was made to use this amplified gene sequence to identify a locally collected freshwater fish from Maharashtra.

KEYWORDS: Fish, COI, freshwater, DNA barcoding, BLAST, phylogeny.

INTRODUCTION

DNA barcoding refers to the technique of sequencing a short fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene, the "DNA barcode," from a taxonomically unknown specimen and performing comparisons with a reference library of barcodes of known species origin to establish a species-level identification.^[1] Mitochondrial COI gene sequence is considered suitable for animal identification because its mutation rate is often fast enough to distinguish closely related species.

A perusal of literature indicates that sequence analysis of COI gene has been successfully used for the identification of a number of animal species such as insects^[2,3,4], birds^[5], shellfish^[6] and fish.^[7,8,9] However, there is dearth of information available on its use for identifying the local fish fauna of Maharashtra state. Therefore, the present investigation was carried out.

MATERIALS AND METHODS

Sample collection

A commonly available, local fish was collected from the Krishna river near Satara, Maharashtra. After collection, it was identified as a fish belonging to the family of Glass-fish based on its morphological characteristics. The collected fish samples (total ten) were immediately put in separate sterile containers having 70% ethanol for preservation. After that, the samples were brought to the laboratory in Mumbai for further analysis following the methodology by Gomes *et al.*, (1999).^[10]

Extraction of DNA

DNA Extraction was carried out using Genelute Mammalian Genomic DNA extraction kit (Sigma, G1N70-1KT). 25mg of tissue was minced and transferred to 1.5ml microcentrifuge tube. 180 μ l of Lysis solution T and 20 μ l of proteinase K were added. The samples were mixed and incubated at 55⁰C to digest the tissue completely. 20 μ l of RNase A solution was added and incubated at room temperature for 2min. Then 200 μ l of lysis solution C was added and incubated at 70⁰C for 10 min. The column was prepared for binding by adding 500 μ l of Column preparation solution to each pre-assembled GenElute Miniprep Binding Column and centrifuge at 12,000 rpm for 1 min. 200 μ l of ethanol was added to the lysate and mixed by vortexing. The entire lysate was transferred into the treated binding column and centrifuge at 10,000 rpm for 1 min. The binding column was then placed in fresh 2 ml collection tube. 500 μ l of Wash solution was added to the binding column and centrifuge at 10,000 rpm for 3min. This step was repeated twice. The column was again transferred to a new tube. 200 μ l of elution buffer was added directly into the centre of the binding column and centrifuge at 10,000 rpm for 1min. The concentration of DNA was determined using UV-1800 spectrophotometer (Schimadzu Corporation A11454806498). The DNA was stored at -20⁰C for further use.

Gene amplification by using PCR

The DNA isolated from Fish were taken further for PCR amplification by using Biometra thermal cycler (T-Personal 48). The PCR reaction mix contained 2.5 μ l of 10X buffer, 1 μ l of each primer, 2.5 μ l of 2.5mM of each dNTP, 2.5 Units of Taq DNA polymerase and 1 μ l Template DNA and 8.5 μ l nuclease free water. The PCR amplification cycle consisted of a cycle of 5 min at 94⁰C; 35 cycles of 1min at 94⁰C, 1 min at 55⁰C, 2 min at 72⁰C; and additionally 1 cycle of 7 min at 72⁰C. The reagents used were procured from GeNei. Primers

for COI gene amplification were used as suggested by Lakra *et al.*, 2011 shown in the table 1.^[11]

Table 1: Primers used for COI gene amplification of fish samples.

Primers	Primer Sequence (5'-3')
Fish F1 (Forward)	TCAACCAACCACAAAGACATTGGCAC
Fish R1 (Reverse)	TAGACTTCTGGGTGGCCAAAGAATCA

Gel electrophoresis

Gel electrophoresis was performed using 1.0% agarose (Seakem, 50004L) to analyze the size of amplified PCR product. The size obtained was approximately 500 bp for COI region.

DNA sequencing

The PCR product was purified using AxyPrep PCR Clean up kit (Axygen, AP-PCR-50). 100 µl of PCR-A buffer was added to the 25 µl of reaction. The sample was mixed and transferred to column placed in 2 ml collection tube and centrifuge at 10,000 rpm for 1 min. the filtrate was discarded. 700 µl of W2 buffer was added to the column and centrifuged at 10,000 rpm for 2 min.

This step was repeated twice. The column was transferred to a new tube. 25 µl of Eluent was added into the column and incubated at room temperature for 2 min. Then centrifuge at 10,000 rpm for 5 min. It was further sequenced using Applied Biosystems 3730xl DNA Analyzer USA and chromatogram was obtained. For sequencing of PCR product Fish F1- 5' (TCAACCAACCACAAAGACATTGGCAC 3') sequencing primer was used.

BLAST analysis

The DNA sequences were analyzed using online BLASTn (nucleotide Basic Local Alignment Search Tool) facility of National Center for Biotechnology Information (NCBI). The BLAST results were tabulated.

RESULTS

COI gene sequence of fish from Krishna River

Genomic DNA extracted from the fish collected from the Krishna River, Satara when targeted to amplify its COI region by PCR produced a sequence of 466 bp as shown in the Fig.1.

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> Partial COI gene sequence> Fish sample< Maharashtra>
AGGCGACGATCAAATCTATAACGTCATCGTTACAGCACATGCTTTTGTAAATGATT
TTCTTCATGGTCATACCTGTAATAATTGGAGGCTTTGGAAATTGACTGGTCCCTC
TTATAATTGGAGCCCCAACATAGCCTTCCCTCGAATAAATAATATGAGCTTCTG
GCTCCTTCCCCCCTCATTCTTCTCCTCCTTGCCTCTTCCGGCGTAAAAGCCGG
GGCCGGAAGTGGCTGAACTGTCTACCCTCCGCTGGCTGGTAACCTACCCCATG
CAGGACCATCCGTTGACTTACCAATCTTCTCCCTTCATCTAGCCGGTGTCTCCTC
AATTCTAGGGGCAATTAACCTTATTACTACAATTATTAACATGAAACCCCCAGCC
ATTACTCATTACCAAACACCCCTTTTTGTATGACCTGTGTTAATTACGGCCGTTCT
CCAGCTCTTCTCTCTCCATTCTTGCA

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Figure 1: COI gene sequence for the unknown fish.

Results for BLAST Analysis

The amplified COI gene sequence of the fish sample collected from the Krishna River was compared with the existing sequences in the nucleotide database library and by this analysis, it was identified as *Chanda nama* as it showed 100% sequence similarity with the already deposited COI gene sequences of the isolate of *Chanda nama* (Table 2). Other phylogenetic neighbour of the study sample was found to be *Pseudambassis ranga* which showed 94% sequence similarity.

Table 2: Phylogenetic neighbours of the unknown fish sample by BLAST analysis.

Description	Max score	Identity	Accession
<i>Chanda nama</i> isolate Fish5 cytochrome oxidase subunit I (COI) gene	861	100%	KJ588168.1
<i>Pseudambassis ranga</i> voucher NF 221 COI gene	717	94%	JQ667560.1
<i>Pseudambassis ranga</i> voucher PR-1001 COI gene	712	94%	KJ936704.1
<i>Pseudambassis ranga</i> voucher DOF13 COI gene	699	94%	JN815276.1
<i>Pseudambassis ranga</i> voucher DOF23 COI gene	693	94%	JN815283.1

DISCUSSION

So using the amplified COI gene sequences, the unknown fish sample collected from the Krishna River was identified as *Chanda nama*. It is better known as Elongate Glass Perchlet. It is a fish endemic to Indian subcontinent and has been reported from Indus, Ganges, Mahanadi, Krishna and other river basins in India. It is classified into Kingdom: Animalia; Phylum: Chordata; Class: Actinopterygii; Order: Perciformes and Family: Ambassidae. So

based on the morphological characteristics, even though family was identified, the actual identification was made easier with the aid of gene sequence of COI.

Chanda nama is a low priced fish and is available in plenty in rainy season so it preferred as a good source of protein rich food by many. It is important to mention that this species has been suggested for its effective use in the control of guinea worms and also for malarial control.^[12]

BLAST analysis indicated that the closest phylogenetic neighbour of the *Chanda nama* was *Pseudambassis ranga* based on the sequence similarity. *Pseudambassis ranga* also belongs to the family of Glass-fish and is better known as Indian Glass perch and is morphologically quite similar to *Chanda nama*. However, COI gene sequences successfully showed the sequence diversity between the collected sample and the existing sequence of *Pseudambassis ranga* indicating though they both belong to the same Family of Ambassidae, COI gene sequences can actually help in delineating them.

More studies are ongoing to use COI gene sequences to characterise and identify more local fish from the state of Maharashtra.

CONCLUSION

We can therefore conclude that amplified COI gene sequence can be used for barcoding animals such as fishes to study and identify them in a quicker, accurate and cost effective way.

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