International Journal of Fauna and Biological Studies
Available online at www.faunajournal.com

E-ISSN 2347-2677
P-ISSN 2394-0522
https://www.faunajournal.com
IJFBS 2025; 12(1): 16-20
Received: 25-10-2024
Accepted: 28-11-2024

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Molecular characterization of Channa species based on cytochrome c oxidase subunit I (COI) gene

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Abstract
Species level fish identification is traditionally based on morphological features. But due to morphological plasticity and high diversity and, in many cases, fish and their diverse developmental stages are difficult to identify by using morphological characteristics alone. For many animal taxa, sequence divergences within the 5′ region of the mitochondrial cytochrome oxidase subunit I (COI) gene are generally much greater between species than within species. This suggests that the approach is extensively applicable among phylogenetically distant animal groups. The present investigation successfully identified *Channa punctata* and *Channa striata* at molecular level using cytochrome oxidase subunit I (COI) gene. The inter- and intra-specific pairwise distance analysis using Neighbor-Joining K2P model have clearly stated prominent range of genetic distance between the *Channa punctata* and *Channa striata* species and demonstrate that both are distinct species.

Keywords: DNA barcoding, mitochondrial genome, phylogenetic analysis, *Channa punctata*, *Channa striata*

Introduction
Fishes are wonderfully diverse forms. This diversity is a result of the ability of ray-finned fishes to adapt to a wide range of environments, and has made them more specious than other of vertebrates. It is easy to dismiss comparisons between distantly related fishes in efforts to understand the biological aspects of a particular fish species like *Channa striata* and *Channa punctata* (Harris *et. al.*, 2014) [6].

The taxonomic position of snakeheads has been creating trouble for taxonomists for long periods. Due to the difficulties emerged during the morphological identification while obtaining the damaged specimens, advanced strategies have shown to exhibit immediate results with accuracy and precision. Existing morphological keys provide insufficient identification for the Channidae and so there is an urgent need to investigate and update the taxonomic status of Channa species (Serrao *et. al.*, 2014) [12].

Morphology based taxonomy has been used traditionally for identification of fishes which might not be reliable. DNA barcoding appears to be precise for taxonomic identification, characterization, and discovery of newer species, facilitating biodiversity analyses. Fish DNA barcoding, based on the sequencing of a uniform area of Cytochrome C Oxidase type I (COI) gene, has been receiving significant interest as an accurate tool for species identification and phylogenetic analysis.

The most commonly used barcode for animal identification marker is mitochondrial cytochrome c oxidase subunit I (COI), which is highly conserved across species using oxidative phosphorylation for metabolism (Hebert *et. al.*, 2003) [7]. Several studies have shown that COI-based DNA barcoding can delimit diverse animal species, showing high rates of sequence change at species level and constraints on intraspecific divergence in COI sequence. According to Kamran *et. al.*, (2020) [9] the efficiency of this approach is well tested and can be considered relatively reliable for both vertebrates and invertebrates.

The current investigation focuses on the identification of snakeheads using molecular technique DNA barcoding. For analysing fresh and well-preserved animal tissues, a full-length barcode such as a 658-bp region of COI gene is recommended as its PCR amplification and sequencing are feasible. A wide range of mini barcodes can be effectively amplified and sequenced from various processed products including TMs provide sufficient sequence information necessary for species identification (Adamson *et. al.*, 2010) [1]. The data gathered from molecular studies can be used to explore the genetic characteristics in terms of genetic

distance and divergence. The genetic diversity is estimated to be declining faster than species diversity under threats. Theory predicts that similar processes should foster congruent spatial patterns of genetic and species diversity (Manel *et. al.*, 2020) [11]. Studies based on these aspects will help in developing essential strategies for the sustainable fisheries development.

Genetically distinct local populations may go extinct before the whole species does, resulting in the loss of genetic diversity and adaptive capabilities for many species (Connell, 1971) [4]. Studies on the key determinants of genetic diversity patterns and their underlying biological processes would help in designing proper conservation schemes which is comprehensive for genetic diversity (Blanchet *et. al.*, 2017) [21].

The DNA-based barcoding method has proven to be a valuable molecular tool for species identification and it is accessible to non-specialists (Wang *et. al.*, 2018) [14]. Approximately 98% of reported marine fish species can be distinguished by COI barcoding, and this approach has been used to catalogue and record fish in many geographic regions. Convergent and divergent adaptation also lead to changes in the morphological characteristics of fish species, imposing great challenges to morphological taxonomy, in which species identification is mainly based on morphological characteristics, and the classification of many species has thus become confusing. The limitations inherent in morphology-based identification systems and the declining number of taxonomists emphasises the need for molecular approaches for precise species identification (Steinke *et. al.*, 2009) [13].

The traditional morphological species identification requires experienced taxonomists, and the phenotypic plasticity of taxa may lead to misidentifications. DNA barcoding method has been proven to be an effective tool for species identification, particularly with specimens that are damaged, incomplete, or consisting of several morphologically distinct stages. DNA barcoding also has limitations. In some cases, related species may present identical sequences making DNA barcodes useless for species discrimination. Therefore, it can serve as a complementary tool for the identification of species, but it cannot replace morphological taxonomic analyses. However, the accuracy of molecular identification relies on having a complete reference database, as inconsistent genetic marker usage could impede the application of molecular authentication (Chang *et. al.*, 2017) [3].

Hebert *et. al.*, (2004) [8] proposed DNA barcoding technology, in which the mitochondrial cytochrome c oxidase subunit I (COI) gene sequence was used as a barcode for species identification with the expectation of barcoding all species for the purpose of species identification. Intraspecific diversity of the COI gene in animals was significantly lower than the interspecific diversity, using the COI gene as a barcode was effective for classifying and identifying vertebrates and invertebrates, and the COI gene has been widely used in various biological groups (Doña *et. al.*, 2015) [5].

From the review of literature, it was observed that no one has initiated a study concerning the molecular mtDNA analysis of *Channa punctata* and *Channa striata* in context with genetic distance estimation. The present study emphasized on the assessment of Mt DNA sequences analysis, phylogenetics and genetic diversity of fish *Channa punctata* and *Channa striata* collected from Thrissur, Kerala, India. The primary aim of this investigation is to generate comprehensive Mt DNA sequences to distinguish the afore mentioned fresh water fish species and compare conspecific populations.

Methodology

Specimens of snakehead fish were collected from two fresh water bodies of Thrissur (Pullu, Manakkodi, Thrissur), Kerala. Caudal fin tissues were taken from each specimen for subsequent molecular studies. Genomic DNA was isolated from the tissues using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions. Agarose Gel Electrophoresis was done for DNA Quality check.

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). The PCR products were checked using Agarose Gel electrophoresis. It was followed by ExoSAP-IT Treatment and Sequencing using BigDye Terminator v3.1. The cleaned-up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems). The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1.

Results

The present investigation has analysed two fish species *Channa punctata* and *Channa striata* at molecular level. The extensive developments in the molecular biology like mitochondrial DNA analysis allow us to complement the conventional taxonomic methods of species identification. A total of 65 COI nucleotide sequences were analysed in this investigation. Among them 63 nucleotide sequences were retrieved from the NCBI database.

All sequences were aligned using CLUSTAL W and all ambiguous positions were removed for each sequence pair. This investigation successfully identified *Channa punctata* and *Channa striata* at molecular level. Figure 1 depicts the phylogenetic analysis of *Channa punctata* and Fig 2 that of *Channa striata*. The COI gene-based mitochondrial DNA analysis of the aforesaid species such as *Channa punctata* and *Channa striata* revealed distinct clustering of individual fish species within every genus together with strong bootstrap support.

As depicted in Figure 1, the nucleotide sequence named "Channa_punctata_CND1_ cytochrome_oxidase_subunit_1_ (COI)_gene_partial_cds_mitochondrial" was recognized as the isolated sequence of *Channa punctata* while the sequence for *Channa striata* named as "Channa_striata_voucher_CND1_COI"(Fig 2). The clustering patterns for respective samples strongly approved the conventional morphological identification, permitting the differentiation of individual fish species based on mitochondrial DNA analysis (COI sequences).

As stated in the above section, the sequences that are isolated in this study such as "Channa_punctata_CND1_cytochrome_oxidase_subunit_1_(COI)_gene_partial_cds_mitochondrial" and "Channa_striata_voucher_CND1_COI" have clustered with those of other related species from various regions reported in the NCBI databases. Altogether, this investigation has identified the two fish species such as *Channa punctata* and *Channa striata* using COI sequences. The Neighbour-joining (NJ) phylogenetic tree constructed using the COI sequences of *Channa punctata* and *Channa striata* and was shown in the figure 3. The NJ tree for *Channa* species formed 32 distinct clades with strongly supported bootstrap values, each representing an individual species.

Inter- and intra-specific pairwise distances

Intra specific pairwise distances between the identified *Channa punctata* and previously reported *Channa punctata*

The present study has also found out the genetic distance between the identified two species along with the determination of genetic distance within the same by species. The matrix output of intra specific pair wise distance of *Channa punctata* was prepared and it revealed that the candidate sequence which is isolated in this study (Channa_punctata_CND1_cytochrome_oxidase_subunit_1_(COI)_gene_partial_cds_mitochondrial) have formed genetically different clusters.

Intra specific pairwise distances between the identified *Channa striata* and previously reported *Channa striata*

This study also determined the genetic distance between the identified *Channa striata* with other reported species from worldwide and revealing extensive genetic distance between them. The matrix output of intra specific pair wise distance of *Channa striata was prepared* and it revealed that the candidate sequence which is isolated in this study "Channa_striata_voucher_CND1_COI" have formed genetically different clusters with the following nucleotides such as KP842439.1, KP842438.1 and KP842437.1. As mentioned in the former section, the average value for the inter-specific genetic divergence was 0.100.

Inter specific pairwise distances between the identified *Channa punctata*, *Channa striata* and previously reported sequences

NJ-K2P method for calculating inters specific pairwise distances between the identified *Channa punctata*, *Channa striata* and previously reported sequences have revealed that extensive genetic distance between them.

As enlisted in the table 3, *Channa punctata* have formed divergent clusters with the following nucleotide sequences with an average value of 0.200. Likewise, *Channa striata* has also formed divergent clusters with other nucleotide sequences representing respective individual species with an average value ranging from 0.100 to 0.200. All the fish specimens analysed in this study can, therefore, be identified to species level based on their COI sequences, which offers 100% compatibility between taxonomic and molecular identification and reveals that COI gene based phylogenetic analysis is a convenient approach to complement conventional taxonomy.

The inter- and intra-specific pairwise distances analysis using Neighbor-Joining K2P model have clearly validated prominent range of genetic distance between the *Channa punctata* and *Channa striata* species.

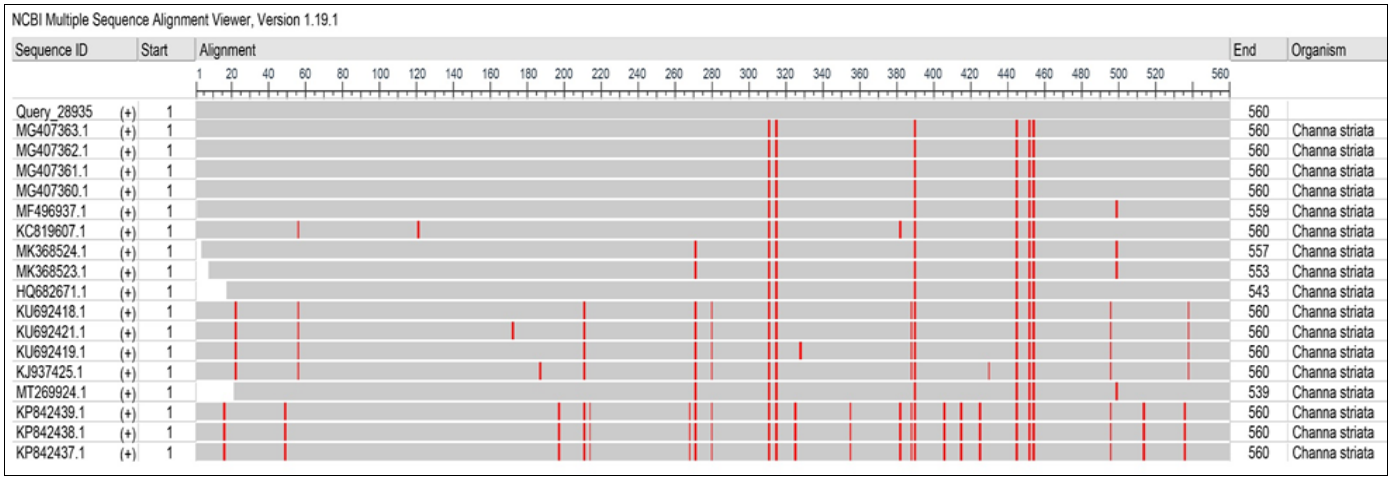

Fig 1: NCBI Multiple Sequence Alignment For Query_8481 (*Channa punctata*)

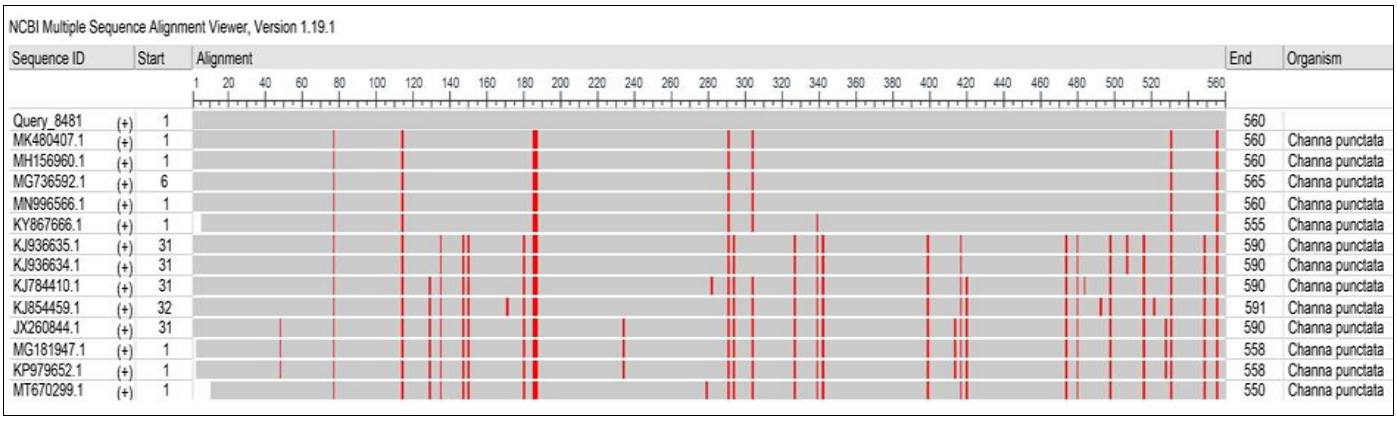

Fig 2: NCBI Multiple Sequence Alignment for Query_28395 (*Channa striata*)

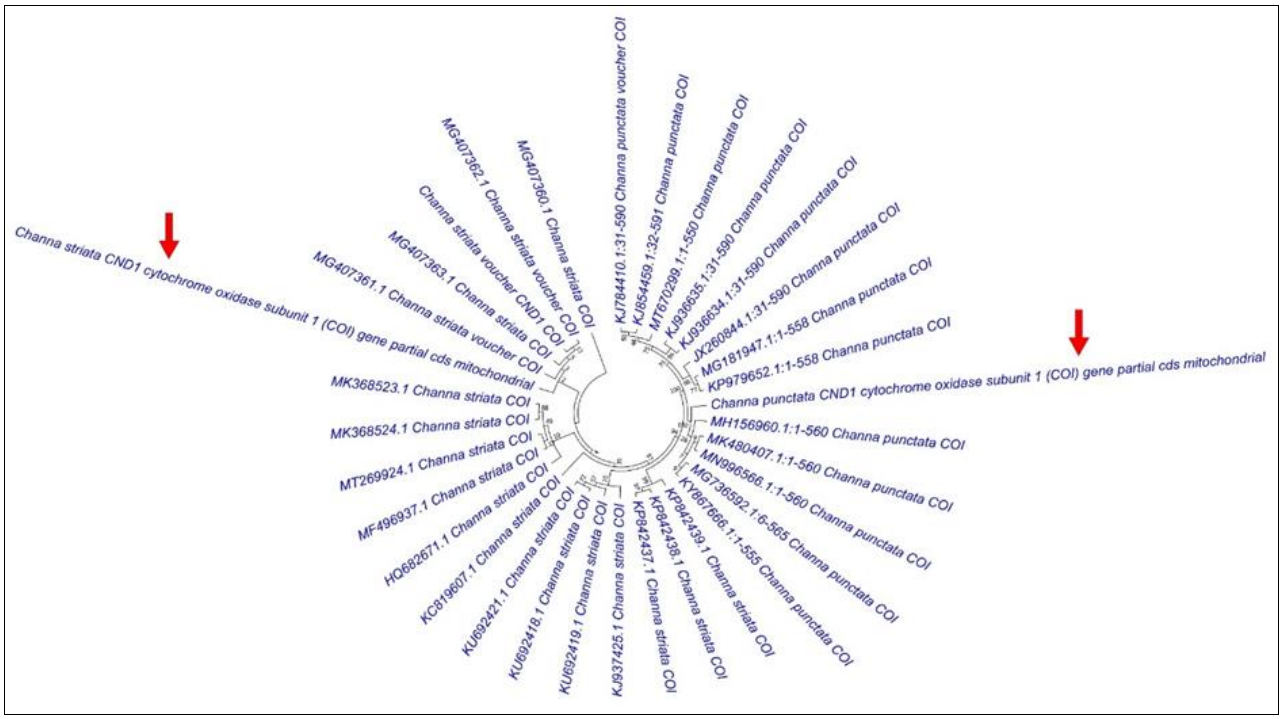

Fig 3: *Channa* sp. Evolutionary relationships of taxa.

Discussion

Apart from being a major component of biodiversity, fish also possess direct economic value and serves as important animal protein sources for human beings. The classification and identification of fish is not only the subject of taxonomy studies but also the key to fishery investigations. The identification of fish species also mainly relies on morphometric and meristic characters. Convergent and divergent adaptation also lead to changes in the morphological characteristics of fish species, imposing great challenges to morphological taxonomy, in which species identification is mainly based on morphological characteristics, and the classification of many species has thus also been confusing. All these problems could be solved to a great extent by using DNA barcoding technique.

The limitations inherent in morphology-based identification systems and the declining number of taxonomists call for a molecular approach to identify species. DNA barcoding based on a fragment of the cytochrome c oxidase subunit I (COI) gene in the mitochondrial genome is widely applied in species identification and biodiversity studies.

However, the accuracy of molecular identification relies on having a reliable and complete reference database, as inconsistent genetic marker usage could impede the application of molecular authentication. In 2003, Hebert *et. al.*, proposed DNA barcoding technology, in which the mitochondrial cytochrome c oxidase subunit I (COI) gene sequence was used as a barcode for species identification with the expectation of barcoding all species for the purpose of species identification.

The present investigation offers molecular characterization of two fish species belonging to the family Channidae from the selected site of Kerala. In the present investigation the mitochondrial COI gene was used to confirm the uniqueness of morphologically identified fish species. Neighbour joining tree constructed using the COI sequences is the most widely accepted approach in taxonomy.

The results from the COI gene-based species identification through the NJ phylogenetic tree analysis strongly approved the result of morphological identification. Based on the similarity of COI sequences, the fishes were identified with strong bootstrap values in the NJ phylogenetic tree approving the results of conventional morphological identification.

It was found that the intraspecific diversity of the COI gene in animals was significantly lower than the interspecific diversity, using the COI gene as a barcode was effective for classifying and identifying vertebrates and invertebrates, and the COI gene has been widely used in various biological groups. The results show that intraspecific similarity ought to be lower than interspecific similarity. The isolated sequences in this study "*Channa punctata* CND1 cytochrome c oxidase subunit I(COI) gene partial cds mitochondria"l and "*Channa striata* voucher CNDI COI" have clustered with those of other related species from various regions reported in the NCBI databases. The amount of variation in mitochondrial DNA observed in this study can lead to demographic changes in fish populations. The mean K2P distance increased gently within the higher taxonomic ranks of families and species classes (Keskin *et. al.*, 2013). In the present the K2P neighbour-joining trees constructed based on the sequence generally clustered species is in accordance with their taxonomic classifications. So the study ensures the generation of comprehensive mt DNA sequences to distinguish fresh water species and also demonstrate its value for conservation.

Acknowledgements

The authors are thankful to the Principal, Sree Kerala Varma College, Thrissur, India for permitting to carry out the study in the Zoology Department.

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