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Identification and characterization of selected freshwater fishes based on DNA barcoding

Jonaira Rashid[1*] | Md. Amdadul Haque[1] | Md. Harunor Rashid[1] | Anuradha Bhadra[2]

[1]Bangladesh Fisheries Research Institute, Freshwater Station, Mymensingh-2201, Bangladesh
[2]Bangladesh Fisheries Research Institute, Mymensingh-2201, Bangladesh

Correspondence: Jonaira Rashid (rjonaira@yahoo.com)

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ABSTRACT

DNA barcoding provides an efficient molecular approach for species identification using mitochondrial cytochrome c oxidase subunit I (COI) gene sequences. This study aimed to generate DNA barcodes for endangered freshwater fish species of Bangladesh and evaluate their taxonomic identification. A total of 12 species were successfully identified with 98–100% sequence similarity using GenBank and BOLD databases, confirming the reliability of COI for species-level discrimination. The amplified sequences exceeded 650 base pairs, ensuring high-quality mitochondrial DNA analysis. However, slight discrepancies were observed in certain species, such as *Osteobrama cotio* and *Botia dario*, which showed relatively lower identity scores (~98%). Notably, COI sequences of *Channa striata* and *Glossogobius sexualis* were absent in the BOLD database but were recorded in GenBank, highlighting gaps in global reference databases and contributing new sequence data through this study. The findings also emphasize challenges related to database inaccuracies and taxonomic ambiguities. Phylogenetic analysis further validated species identification and revealed the dominance of Cypriniformes, consistent with regional biodiversity patterns. Additionally, variations in species composition compared to previous studies suggest the influence of environmental changes and sampling strategies. Overall, this study demonstrates the effectiveness of DNA barcoding as a reliable tool for fish identification and biodiversity assessment, contributing valuable genetic resources for conservation and management of freshwater fish species in Bangladesh.

Keywords: DNA barcoding, COI gene, BLAST search, genetic distance

1 | Introduction

Bangladesh is exceptionally rich in fish biodiversity, often regarded as a hotspot for freshwater ichthyofauna. According to IUCN Bangladesh (2015a, b), a total of 253 freshwater fish species has been assessed, of which nearly one-fourth (64 species) are threatened, including 9 Critically Endangered, 30 Endangered, and 25 Vulnerable species. Additionally, 27 species are categorized as Near Threatened, 40 as Data Deficient, and 122 as Least Concern. Despite this richness, there remains a significant lack of readily accessible, species-specific information necessary for sustainable fisheries management. The study of fish diversity and accurate species identification is therefore a critical component of conservation and resource management. However, identification based solely on morphological characteristics is often complex and challenging, particularly for non-specialists.

DNA barcoding has emerged as a reliable taxonomic tool for species identification, utilizing short, standardized genetic markers from an organism's DNA. The mitochondrial cytochrome c

oxidase subunit I (COI) gene has been widely adopted as a universal barcode for animal species (Hebert et al., 2003).

This method provides a consistent and efficient alternative to traditional morphological identification. Numerous studies have demonstrated the effectiveness of DNA barcoding in identifying both marine and freshwater fish species across diverse geographical regions (Griffiths et al., 2013; Knebelsberger et al., 2014; Diaz et al., 2016; Shen et al., 2016). Moreover, it enables accurate identification of different life stages and body parts, such as larvae, eggs, fillets, and fins, which are otherwise difficult to distinguish morphologically (Trivedi et al., 2016).

The COI gene region offers high resolution at the species level in fishes and other aquatic organisms, including crabs, sharks, and oysters (Laskar et al., 2013; Trivedi et al., 2012, 2014). Accurate species identification is essential for maintaining ichthyofaunal biodiversity and supporting conservation planning. However, in Bangladesh, there is still a notable gap in genetic-based studies that employ robust methodologies to assess freshwater fish diversity and evolutionary lineages. This limitation hinders effective conservation and management strategies.

Therefore, integrating DNA barcoding with conventional taxonomy can significantly enhance the accuracy and comprehensiveness of species identification. Both approaches are complementary and equally important in fisheries research. The present study aims to characterize the DNA barcode region and generate COI sequences for morphologically identified endangered freshwater fish species of Bangladesh, thereby contributing to their accurate identification and future genetic conservation.

2 | Materials and Methods

2.1 Sample collection and DNA isolation

Fish samples were collected from different freshwater habitats, fish landing centers, fish markets or from the local fishermen. Approximately 50 mg of fin tissue from each specimen were preserved in 95% ethanol for genomic DNA isolation. The concentration and purity of isolated samples were determined with the help of nano-drop spectrophotometer and stored at −20°C for further use.

2.2 COI Amplification and purification

For DNA barcoding, partial 5′ region of COI gene was amplified in a final volume of 50 µl with final concentration of 1X reaction buffer (10 mM Tris-HCl,50 mM KCl,), 2.0 mM $MgCl_2$, 0.2 mM of dNTP mix, 10 pmol of forward and reverse primer, 2U of Taq DNA polymerase and 100 ng of template DNA. The primers used for amplification of COI gene were 5′-TCAACCAACCACAAAGACATTGGCAC-3′ and 5′ TAGACTTCTGGGTGGCCA AAGAATCA-3′ (Ward et al., 2005). Each reaction included a negative control (no template DNA) and were carried out in a 96 well thermal cycler under following thermal cycling conditions: initial denaturation at 95 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 35 sec, primer annealing at 52 °C for 30 sec and primer extension at 72 °C for 40 sec and final extension for 5 min at 72 °C. Amplified products of COI were separated on 1.5% agarose gel. In the final step, purified products were eluted in 1X TE buffer. The PCR products were purified using PureLink™ PCR purification kit and sequenced from Apical Scientific Sdn Bhd**,** Malaysia. Sequences were checked and aligned using Sequencher v5.4, and the datasets were compared to the NCBI NT database using BLASTn search for GenBank with referred accession numbers (Table 6).

2.3 Nucleotide Sequencing of COI gene

The purified products (free of salts and protein impurities) of COI were directly sequenced in Applied Biosystem 3500 genetic analyzer using BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems) as per manufacturer's

instructions sequenced from Apical Scientific Sdn Bhd**,** Malaysia. The sequencing (dye labeling) reaction will be optimized to a final volume of 10 µl, using 50 ng template DNA, 33 µM primer, 1X BigDye Terminator reaction mix, followed by thermal cycling at initial denaturation at 96 °C for 4 min, 25 cycles of denaturation at 94 °C for 10 sec, annealing at 50 °C for 10 sec and extension at 60 °C for 4 min. The samples were denatured at 95 °C for 5 min and chilled on ice for 5 min to prevent the renaturation. The denatured samples were transferred into 96 well sequencing plate with rubber closure and placed into ABI 3500 genetic analyzer for nucleotide sequencing at a DNA sequencing facility of molecular biology laboratory at Apical Scientific Sdn Bhd, Malaysia.

2.4 Bioinformatic and statistical analyses

The obtained good consensus sequences from Sanger sequencing were selected for analysis based on chromatogram peak clarities with the help of Chromas Lit. Bioinformatic analyses of the sequences were performed using CLC Workbench, Mega and ClustalW. Base compositions were analyzed using CLC Workbench and Megav5.05. Genetic distance was calculated using Phylogenetic analysis pipeline, ClustalW. Alignment and phylogenetic reconstructions were performed using the function "build" of ETE3 v3.1.1 (Huerta-Cepas et al., 2016) as implemented on the GenomeNet (https://www.genome.jp/tools/ete/). The tree was constructed using FastTree v2.1.8 with default parameters (Price et al., 2009). A phylogenetic tree generated by multiple sequence alignment software, ClustalW alignment of CAD amino acid sequences from fish species using MEGA. The bootstrap values, expressed as percentages of 500 replications, are shown at branch points. GenBank accession numbers are displayed within the tree.

3 | Results

All the fish samples were successfully amplified with two primers using PCR (Figure 1). The lengths of COI sequences were more than 650bp (Table 1). The genetic distances within species ranged from 0.056 to 0.130 (Table 1). For the identification of organisms at the species level, a total of 60 COI barcode sequences representing 12 different species were used (2 to 5 samples per species). GenBank and BOLD databases were used for species identification (Table 1). GenBank based identification of all species ranged from 98 to 100% identities. However, *T. tor* was a 99% identity value, *Puntius sarana* with a 100% identity value and *Clupisoma prateri* was identified as *Clupisoma garua* with a 99% identity value.

BOLD-based identification of 12 fish species ranged from 98-100% identities. No match was found for *Gagata sexualis*, and it lacked the COI sequence of *C. striata*. In addition, *Osteobrama cotio*, *Systomus sarana*, *Ompok bimaculatus, Botia dario* and *Rhinomugil corsula* were identified with high similarities (100%). From the similarity search, a total of 40 fish samples consisting of 12 species, 7 families, and 4 orders were used (Table 1).

Order Cypriniformes contained the largest number of species (6 species, 50.00%). However, these species were grouped into only one family, Cyprinidae. Furthermore, the order Siluriformes, Anabantiformes and Mugiliformes contained 3 species (25.00%), 2 species (16.67%), and 1 species (8.33%), respectively (Figure 2).

Of the 12 species of fishes collected from Mymensingh region, only *T. tor* was found to be in the IUCN category of critically endangered (*CR*). *Channa marulius*, *Clupisoma prateri, Ompok bimaculatus* and *Botia dario* were categorized as having endangered (EN) status. The remaining majority were grouped into the least concern (*LC*) and near threatened (*NT*) status (Table 2).

The phylogenetic tree based on multiple sequence alignment software, ClastalW showed that the taxa were closely related and formed sister groups (Figure 3). Multiple sequence alignment based on ClastalW revealed an identical phylogenetic relationship among the species. Similar species were found clustered together. Fish samples were

separated according to their order and family. Siluriform formed distinct clusters based on their characters. Perciformes formed a distinct cluster based on their group (Figure 2).

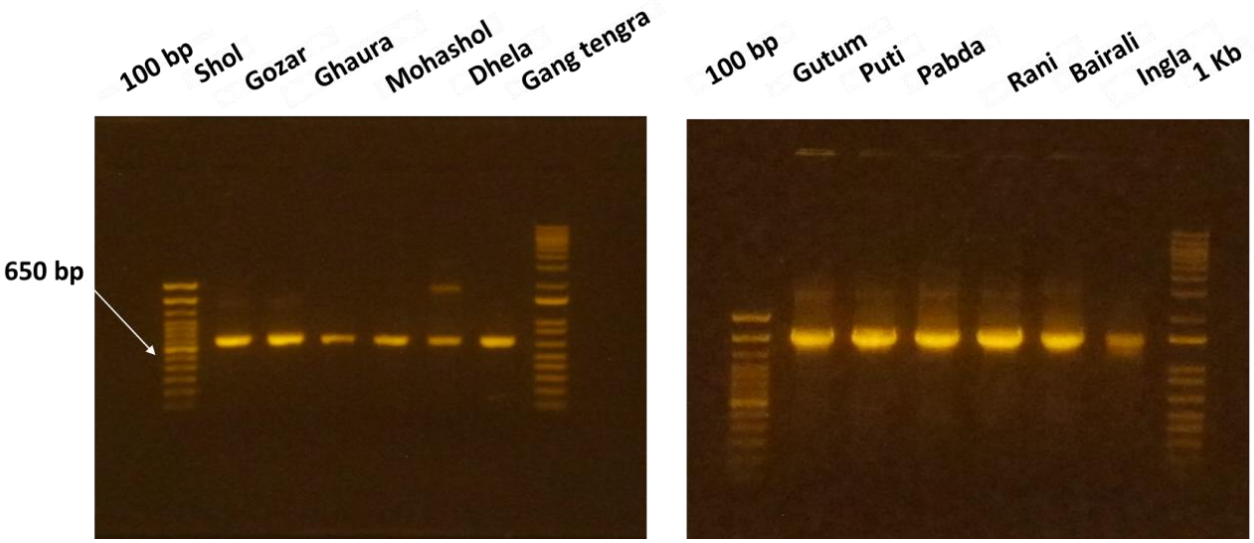

Figure 1 | PCR amplifications of 650 bp amplified product of mt-COI gene of selected fish specie M: Molecular weight marker (1Kb and 100 bp DNA ladder)

4 | Discussion

DNA barcoding aims to provide an efficient molecular method for species-specific identification using the mitochondrial COI gene sequence (Pugedo et al., 2016). DNA barcoding has discriminated against freshwater fish species from Australia, Canada, India, and Cuba (Ward et al., 2005; Hubert et al., 2008; Lara et al., 2010; Lakra et al., 2016). Here, we have profiled the barcode of freshwater endangered fishes collected from Bangladesh. Moreover, the average length of the amplified sequences was greater than 650 bp, the limit typically observed for nuclear DNA sequences originating from mtDNA (Gunbin et al., 2017). From the GenBank database, 12 fish species could be identified with 98-100% identities based on the species-level identification methods of Wong and Hanner (2008). Except for low identities, 98% were presented to *Osteobrama cotio* and *Botia dario*, respectively, whereas high identities (approximately 98-100%) were matched using the BOLD database. In the high-throughput sequencing era, there is a high likelihood that tentative, incorrect, or low-quality sequences will be submitted to databases (Soe-been et al., 2008; Wong et al., 2011). The COI sequences of two fish species, *C. striata and G. sexualis*, have been recorded in the GenBank database. In contrast, the sequences of those two species were not found in the BOLD database. Thus, this study increased the COI gene sequences of these fish species in both databases. Furthermore, the fish species identified in this study would help address the problem of unidentified species from previous research, namely *Oreochromis* sp., a juvenile fish species found in the tributaries of Kwan Phayao (Tuncharoen et al. 2018). In www.iucnredlist.org, *G. youssoufi* is described as a junior synonym of *G. sexualis;* however, in the Interagency Taxonomic Information System (www.itis.gov), *G. youssoufi* is a valid name, and *G. sexualis* is a separate species. The species was evaluated as Not Threatened (IUCN Bangladesh 2000). The fish is found in Bangladesh, India, and Myanmar (Rahman, 2005). It is found in the Old Brahmaputra River near Mymensingh, the Sangu River near Bandarban, Shashikar Beel near Shariatpur, and the Meghna River near Chadpur (Rahman, 2005). The fish was

Table 1 | Identification and characterization of selected freshwater fishes based on DNA barcoding data

Local name	NCBI GenBank Scientific name	Identity (%)	BOLD Scientific name	Identity (%)	No of samples	Length of COI (bp)
Shol	*Channa striata*	99-100	*Channa striata*	N/A	5	685
Gozar	*Channa marulius*	99	*Channa marulius*	99.85	4	682
Ghaura	*Clupisoma prateri*	99-100	*Clupisoma prateri*	99.85	2	682
Mohashol	*Tor barakae*	99	*Tor barakae*	99.84	2	681
Dhela	*Osteobrama cotio*	98	*Osteobrama cotio*	100	5	681
Gang tengra	*Gagata sexualis*	99	*Gagata sexualis*	N/A	3	682
Gutum	*Lepidocephalichthys guntea*	99	*Lepidocephalichthys guntea*	99.84	4	685
Sharputi	*Puntius sarana*	99	*Puntius sarana*	100	4	683
Pabda	*Ompok bimaculatus*	99	*Ompok bimaculatus*	100	5	681
Rani	*Botia dario*	98	*Botia dario*	100	3	683
Boirali	*Barilius bendelisis*	99	*Barilius bendelisis*	99.84	2	682
Ingla/ Khorsula	*Rhinomugil corsula*	99	*Rhinomugil corsula*	100	2	681

also reported from the Jamuna River near Kazipur Upazila, and from 3-7% of the study sites studied in the fish catch monitoring program. *R. corsula* is a widespread species with no known major threats and is assessed as Least Concern. It is found in Bangladesh, India, Myanmar, and Nepal (Dahanukar, 2010). It is found throughout Bangladesh's rivers and estuaries (Rahman, 2005). It is reported from the river Padma (Rahman *et al.,* 2012), the River Choto Jamuna (in the northwest region) (Galib *et al.,* 2013), and the Meghna River (Rahman, 2005).

Fish in the order Cypriniformes were found to be the most numerous because this order has the greatest diversity in Southeast Asia (Nelson et al., 2016). In addition, this study's fish diversity differed from previous studies. Forty-five fish species from 17 families were found from June 2003 to September 2005, and 44 species from 17 families were identified from December 2009 to August 2010 (Rattanadaeng et al., 2015). Some fish species were not found in this study, including *Rasbora tornieri*, *Rasbora palustris*, *Macrognathus taeniagaster*, and *Helostoma temminckii*, possibly indicating that ecosystem changes from restoration and dredging may have affected their behavior, growth, and survival. Different fish sampling methods, sampling periods, and sampling frequencies may also affect the number of fish species (Rattanadaeng et al., 2015). Moreover, some fish species were found only in this study, including *Coptodon rendalli*, *C. zillii*, *Pterygoplichthys pardalis*, and *Pterygoplichthys anisitsi*. These fish species were found in Kwan Phayao because they were released there, and they are not native species. Thus, these fish are introduced or alien species in Thailand (Vidthayanon, 2005). Ward et al. (2006) bar-coded a 650-base-pair region of the mitochondrial cytochrome c oxidase I gene in Asian sea bass (*Lates calcarifer*) from Australia and Myanmar, suggesting that the two are distinct species. However, he recommended further examination on the genetic and morphological level for confirmation. This study shed light on errors in fish identification. COI genes were successfully amplified from Shol Gozar (*C. marulius*), Ghaura

(*C. garua*), Mohashol (*T. tor*), Dhela, Gang tengra, Gutum, Deshi puti, Pabda, Rani, Bairali and Ingla fishes. A total of 12 species were successfully identified at the species level, and a phylogenetic tree was reconstructed based on DNA barcoding data. The present study confirms the effectiveness of the COI gene in identifying freshwater fish species.

Table 2 | Detailed information of collected fish species and its conservation status

Sample ID	Local name	Order	Family	English name	Highest BLASTn match with fish species	Conservation status
1.	Shol	Perciformes	Channidae	Snakehead Murrel, Stripped or banded Snakehead, Asian Snakehead	*Channa striata*	*LC*
2.	Gozar	Perciformes	Channidae	Giant Snakehead, Great Snakehead	*Channa marulius*	*EN*
3.	Ghaura	Siluriformes	Ailiidae Schilbeidae	Garua Bacha, Gagra	*Clupisoma garua*	*EN*
4.	Mohashol	Cypriniformes	Cyprinidae	Mohashol, Mahsheer	*Tor tor*	*CR*
5.	Dhela	Cypriniformes	Cyprinidae	Cotio	*Osteobrama cotio*	*NT*
6.	Gang tengra	Siluriformes	Sisoridae	Gangetic gagata, Indian gagata, Clown catfish	*Gagata sexualis*	*NT*
7.	Gutum	Cypriniformes		Peppered Loach, Guntea Loach	*Lepidocephalichthys guntea*	*LC*
8.	Sharputi	Cypriniformes	Cyprinidae	Olive barb, Peninsular Olive Barb	*Puntius sarana*	*NT*
9.	Pabda	Siluriformes	Siluridae	Butter Catfish, Two Spot Glass Catfish	*Ompok bimaculatus*	*EN*
10.	Rani	Cypriniformes	Cobitidae	Loach, Queen, Loach, Bengal Loach, Rani Mach, Bou Mach	*Botia dario*	*EN*
11.	Boirali	Cypriniformes	Cyprinidae	Hamilton's Baril, Hill Trout	*Barilius bendelisis*	*EN*
12.	Ingla/ Khorsula	Mugiliformes	Mugilidae	Corsula, Kakunda, Corsula Mullet	*Rhinomugil corsula*	*LC*

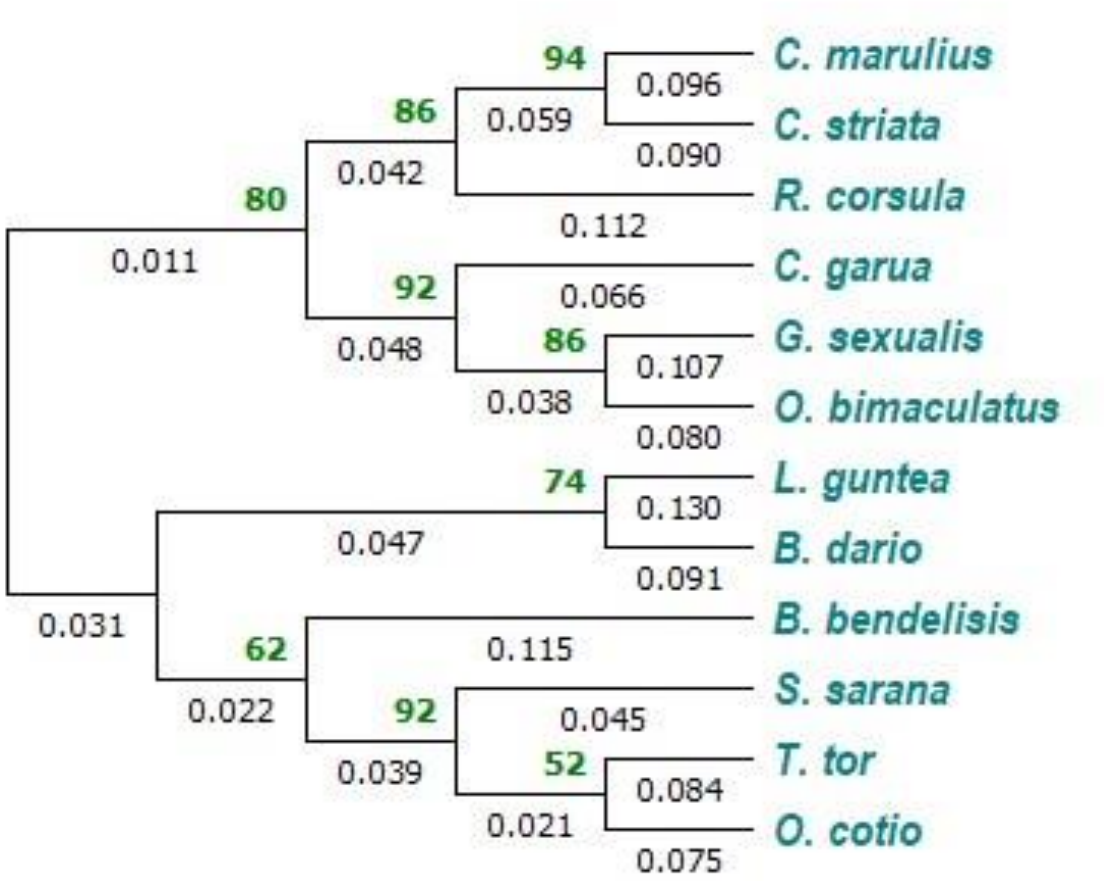

Figure 2 | Maximum likelihood phylogenetic tree of 12 fish species based on CO1gene

5 | Conclusion

Overexploitation, habitat degradation, and climate change threaten fish diversity in Bangladesh. With the significant decline in biodiversity, species extinction enhances the need to conserve fish biodiversity. Our results reveal that DNA barcoding successfully identified most fish species. Identification supported by DNA barcoding could be used to evaluate fish biodiversity, monitor fish conservation, and manage fisheries. This technique will guide future studies of fish species that need to be barcoded. Once a fish DNA barcode database has been established, fish barcoding's scientific and practical benefits are diverse. DNA barcoding can discriminate all fish species and identify the eggs, larvae, and carcass fragments of these species. The results will provide more information on fish diversity to the fisheries managers and ecologists who craft the policies for the conservation and sustainable use of fishing resources. DNA barcoding demonstrated a high efficiency of species identification in the present study, and we conclude that COI sequencing can be used to identify fish species.

Author Contributions

Jonaira Rashid: Conceptualization, data collection, data analysis, interpretation, manuscript writing and editing. Md. Amdadul Haque: Conceptualization, data collection, data analysis, fund acquisition, manuscript writing and editing. Md. Harunor Rashid: Conceptualization, data collection, data analysis, supervision. Anuradha Bhadra: Conceptualization, data collection, data analysis, supervision, manuscript writing and editing. All authors have read and approved the final published article.

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Conflicts of Interest
The authors declare no conflicts of interest.

Data Availability Statement
All data generated and analyzed during this study are included within the manuscript. No additional datasets are available.

Ethics Statement
Not applicable.

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