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First record of COI Gene-Based DNA Barcoding of *Terapon jarbua* and *Terapon puta* in Pakistan

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Abstract

The Terapontidae fish family is an important coastal fish assemblage in Pakistan that contributes to local fisheries. Despite its economic and ecological importance, no previous genetic studies of terapontid species have been reported from Pakistani waters. DNA barcoding, particularly using mitochondrial cytochrome c oxidase subunit I (COI) gene sequences, is a relatively novel method for species identification. COI gene sequences can be effectively used to confirm species identity and investigate phylogenetic relationships among species. The aim of this study was to generate mitochondrial COI gene-based DNA barcodes for *Terapon jarbua* and *Terapon puta* fishes collected from Pakistan. DNA was extracted using the CTAB method, and PCR reactions were carried out using Fish F1/R1 and F2/R2 primer pairs. A total of 13 sequences of Terapontidae family (*Terapon jarbua*, *Terapon theraps*, *Terapon puta*, *Pelates quadrilineatus*) were downloaded from NCBI database and aligned with the sequences generated in this study using multiple sequence alignment tools. The interspecific divergence and intraspecific variation were estimated using the Kimura 2-parameter model in MEGA X, while phylogenetic relationships were reconstructed using the Maximum Likelihood method. Intra-specific divergence was very low (0.007) compared with the inter-species divergence (0.687). Phylogenetic analysis indicated a close evolutionary relationship between *T. jarbua* and *P. quadrilineatus*, while *T. puta* showed a distant relationship. This study provides the first DNA barcodes for *T. jarbua* and *T. puta* (Family: Terapontidae) from Pakistani waters. The use of DNA barcodes is discussed as an essential tool for identification of existing species within Terapontidae fish family and for investigating their evolutionary relationships. In future, these DNA barcodes will help detect cryptic species and contribute to the study of new species within the family. Precise species identification is an essential first step toward the future conservation and management of fishery resources in Pakistani waters.

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1.0 Introduction

Ecosystem stability and sustainability are at serious risk due to the rapid biodiversity loss driven by human activities such as overexploitation, pollution and climate change (Anjum et al., 2025). Biodiversity, which plays a fundamental role in maintaining ecosystem productivity and nutrient recycling, has undergone a sharp decline due to the direct impacts of human activities on marine life, resulting in socio-economic consequences (Elahi et al., 2015). Conservation planning and resource management can only be made possible through effective species identification. Precise identification of fish species is crucial not only for taxonomic studies but also for effective conservation of fisheries, ecological studies, and management (Kochzius et al., 2010). Traditional methods are commonly based on morphological identification, which is often constrained

by phenotypic plasticity among closely related species and by the difficulty of identifying the early development stages of these species. Molecular biological analysis is a proven and successful method for species identification (Ziaei et al., 2025).

DNA barcoding has been widely adopted for species identification and taxonomy (Liu et al., 2017). An approximately 650 base pairs fragment of the mitochondrial cytochrome c oxidase subunit I (COI) is used for DNA barcoding in animals (Ko et al., 2013). Studies have demonstrated the effectiveness of COI barcoding in freshwater and marine fishes, with reliable results of species identification (Zemlak et al., 2009). The Terapontidae fish family is an important coastal fish assemblage in Pakistan that contributes to local fisheries. Despite its economic and ecological importance, no molecular identification studies or reference DNA barcode data are currently available for Terapontidae species from Pakistan. Furthermore, no COI-based DNA barcoding studies have been reported for Terapontidae fishes inhabiting Pakistani waters (Samreen & Manzoor, 2024).

This study aims to generate and analyze COI barcode sequences for selected Terapontidae species from Pakistani waters. The study further envisages assessing the divergence patterns of generated sequences. The findings are expected to effectively contribute baseline molecular data for species identification, and thereby supporting biodiversity conservation and the development of DNA barcode reference libraries for marine fishes in Pakistan.

2.0 Material and Methods

2.1 Specimen collection and morphological identification

Thirty (30) samples each of *T. jarbua* and *T. puta* were collected from the coastal waters (24°47'38.9"N 66°53'48.2"E) of Karachi, Sindh, Pakistan. Specimens were labelled with names, date, and coordinates. Standard taxonomic keys were used for morphological identification (Bianchi, G., 1985).

2.2 DNA extraction and PCR amplification

Genomic DNA was extracted from fin tissue of each sample using the standard CTAB method (Godinez-Vidal, et al., 2025). The extracted DNA was stored at -20 °C for further analysis. A ~658 bp fragment of mitochondrial COI gene was amplified using universal fish primers (Asiah et al., 2020) (Table 1). Standard cycling conditions were adopted for PCR amplification (Lorenz, 2012). PCR products were visualized on 0.5% agarose gel stained with ethidium bromide, and successful amplicons were sequenced bidirectionally using Sanger sequencing.

2.3 Sequence analysis and species identification

MEGA X software was used to edit and align the raw sequences. Species identification was performed through BLAST similarity searches against the NCBI nucleotide database. Sequences showing >98% similarity were considered reliably identified. Sequences of *T. theraps* and *P. quadrilineatus* were retrieved from GenBank as reference sequences for comparative studies. *Sphyraena barracuda* was used as the outgroup in the phylogenetic analysis.

Table. 1. Primers used for PCR amplification

Primer	Sequence (5'-3')
FishF1	TCAACCAACCACAAAGACATTGGCAC
FishR1	TAGACTTCTGGGTGGCCAAAGAATCA
FishF2	TCGACTAATCATAAAGATATCGGCACA
FishR2	ACTTCAGGGTGACCGAAGAATCAGAA

2.4 Phylogenetic analysis and genetic divergence analysis

Kimura 2-Parameter (K2P) model in MEGA X was used to calculate the genetic distances. Presence of barcode gap was assessed by comparing the intra- and interspecific divergence values. Evolutionary relationships were visualized by constructing a phylogenetic tree based on K2P distances using Maximum Likelihood method (Nishimaki & Sato, 2019) Barcode Index Numbers (BINs) were assigned using the Barcode of Life Data Systems (BOLD), and operational taxonomic units were evaluated using the Refined Single Linkage (REFL) algorithm (Stein & Gailing, 2025).

3.0 Results and Discussion

3.1 Specimen morphological identification

Morphological characteristics of *T. jarbua* and *T. puta* are shown in Fig. 1. *Terapon jarbua* has a silvery body with three to four dark stripes along its body. Its caudal fin contains stripes, and the lobes have darkened tips (Fig.1b). *Terapon puta* is silvery-grey with four stripes along the body; the upper stripes are black, whereas the lower ones are pale yellow. Its caudal fin has dark and yellow stripes (Fig.1d).

3.2 Sequences analysis, BLAST identification and genetic divergence

The COI sequences obtained from *T. jarbua* and *T. puta* showed high-quality reads; no insertion, deletion or stop codons were observed. BLAST analysis revealed high sequence similarity, with percent identities ranging from 97.25% to 100% and query coverages exceeding 98% (Camacho et al., 2009). K2P genetic distance analysis revealed low intraspecific divergence (0.007) and comparatively higher interspecific divergence (0.687). This demonstrated a clear barcode gap between the two species. The *T. jarbua* and *T. puta* sequences were submitted to the NCBI GenBank database with accession numbers PZ476806 and PZ476809, respectively.

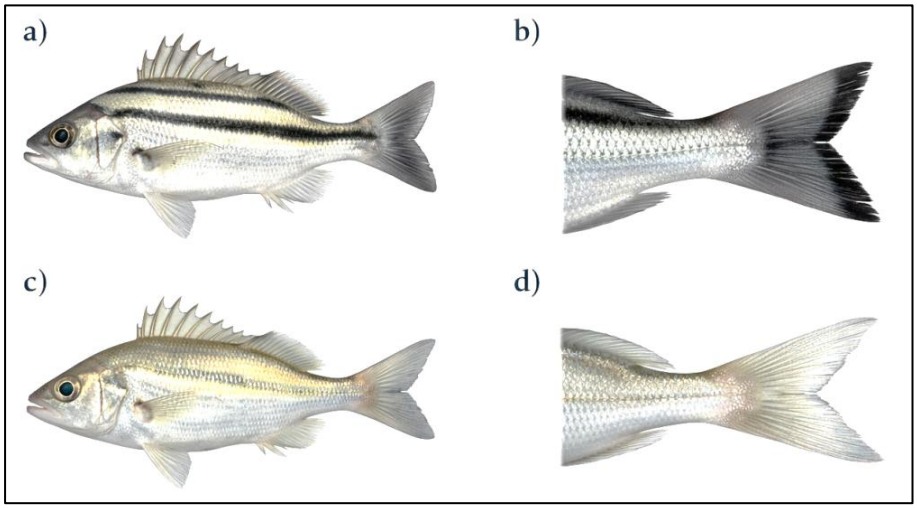

Fig. 1. a) *Terapon jarbua;* b) Caudal fin of *T. jarbua;* c) *Terapon puta;* d) Caudal fin of *T. puta*

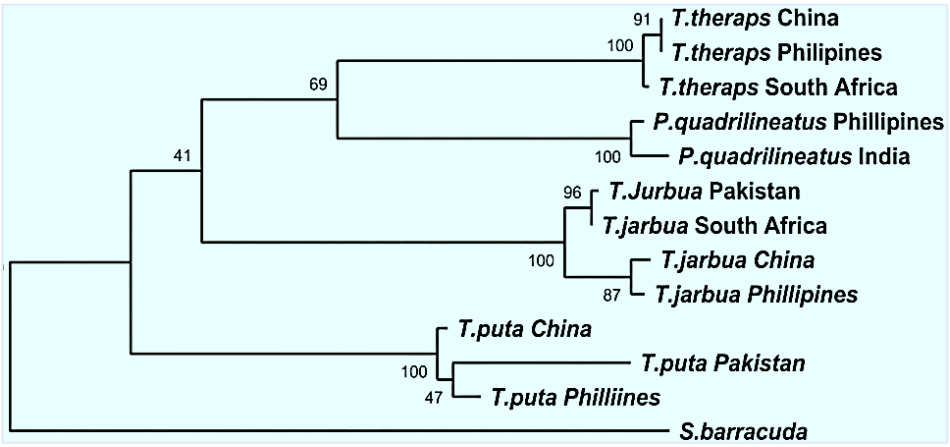

Fig. 2. Maximum Likelihood phylogram generated using the K2P model. *Sphyraena barracuda* is used as an outgroup species. According to the phylogenetic tree, *T. jarbua*, has a close phylogenetic relationship with *T. quadrilineatus* and *T. theraps*, while *T. puta* has a distant relationship.

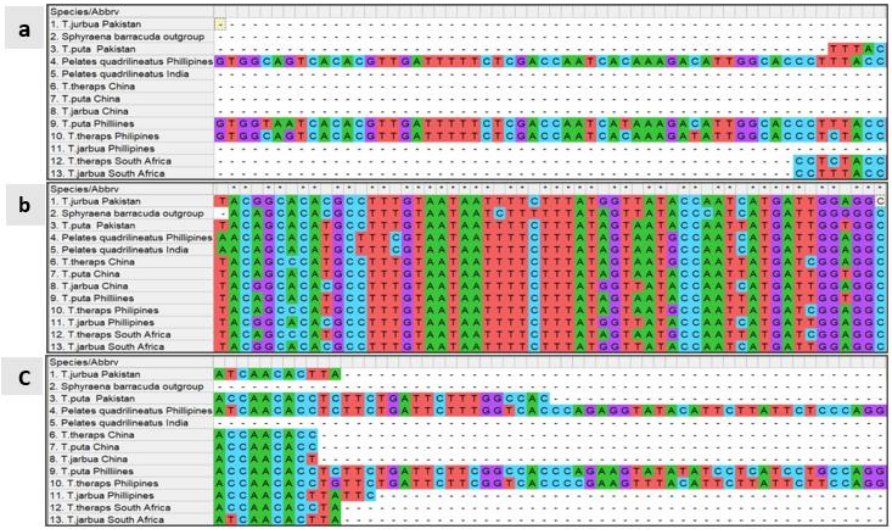

Fig.3. MUSCLE Alignment. a) extreme left; b) middle; c) extreme right regions of the sequence

3.3 Phylogenetic relationships & DNA barcode

The phylogram generated by the Maximum Likelihood (ML) method based on the K2P model as shown in Fig. 2. *Terapon theraps* was grouped in clade 1 and *P. quadrilinieatus* in clade 2. Similarly, *T. jarbua* and *T.*

puta were grouped in clades 3 and 4, respectively. This clustering of sequences into distinct clades demonstrated the reliability of the COI marker in distinguishing species. Moreover, multiple sequence alignment of sequences from different species of the Terapontidae fish family is shown in **Fig. 3**. Phylogenetic analysis grouped sequences into distinct clades, with different genera forming separate clusters. The clear separation of these clades further supports the effectiveness of the COI marker for discriminating species within Terapontidae. These findings reveal that the COI marker is a reliable genetic marker for species identification and discrimination within the family Terapontidae.

4.0 Discussion

This study successfully demonstrates the utility of COI-based DNA barcoding for species identification of Terapontidae fishes from Pakistani coastal waters**.** The observed pattern of low intraspecific and high interspecific divergence is consistent with findings from previous fish barcoding studies and confirms the presence of a distinct barcode gap (Zhang et al., 2023). Phylogenetic analysis further supported and validated species boundaries by clustering species into separate groups. The absence of stop codons in all analyzed sequences further confirms that the obtained sequences represent functional mitochondrial COI genes rather than nuclear pseudogenes. According to the phylogenetic tree, *T. jarbua* has a close phylogenetic relationship with *T. quadrilineatus* and *T. theraps*, while *T. puta* has a distant relationship. Despite the global adoption of DNA barcoding, its application in Pakistan remains limited (Ghouri et al., 2020). The molecular data generated in this study provide an important addition to regional reference genetic databases and will support future applications such as fisheries stock assessment, identification of early life stages, detection of cryptic species, and monitoring of biodiversity changes.

5.0 Conclusion

This study represents the preliminary first report providing unambiguous identification of marine Terapontidae fishes from Pakistan using COI barcoding. The results confirm that DNA barcoding can be used for the accurate identification of fish species. In future, DNA barcoding can be effectively used in taxonomic studies in combination with comprehensive morphological analysis.

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Author contributions:
Atta-ur-Rehman Khan: writing original draft, methodology and data analysis
Faizan Muneer: conceptualization, project management, drafting, reviewing and writing the draft. Both authors reviewed and approved the final manuscript.

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