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Molecular identification of *Tenualosa toli* (Valenciennes, 1847) and *Tenualosa ilisha* (Hamilton, 1822) from Pakistan coast using Cytochrome-b gene

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Abstract: Species identification is a crucial step in understanding and characterizing biodiversity. Before utilizing molecular identification tools, it's essential to acknowledge the challenges associated with assigning an organism to a biological species. Molecular methods offer significant insights into species diversity by detecting genetic variation at the DNA level, providing valuable data that enhances our understanding of biodiversity. The present study aims to develop a clear and comprehensive guide that accurately identifies Hilsa species in Pakistan coast, providing a valuable resource for individuals seeking to distinguish between them. This study obtained the molecular identification of two fish samples, *Tenualosa toli* (Valenciennes, 1847) and *Tenualosa ilisha* (Hamilton, 1822), from Pakistan in the mitochondrial DNA Cytochrome-b (Cyt-b) gene region. This adds to the existing NCBI sequence database related to the Cyt-b sequence, which is still lower than the Cytochrome Oxidase Subunit I (COI) gene region. This study also proves the accuracy of molecular identification in fish samples with complex morphological characteristics in Clupeid.

1. Introduction

Dorosomatidae is a family of Clupeiform fishes (Gizzard shads and Sardinellas) (Wang *et al.*, 2022). FishBase now recognizes it as a family in its own right; it had been a subfamily of Clupeidae; it contains 35 extant genera (Lavoué *et al.*, 2007), chiefly marine coastal and schooling fishes, some freshwater and anadromous (Canales-Aguirre *et al.*, 2021). The body is usually fusiform, round to strongly compressed (Dizaj *et al.*, 2020). The Clupeoid family is a vital group of fish that comprises many of the world's most valuable food fish species, making it one of the most significant families of commercially important fishes (Birge *et al.*, 2021), which are harvested and processed for human consumption, oil production, or conversion into fish food (Cashion *et al.*, 2017).

Accurate fish species identification is essential for understanding and managing biodiversity (Paris *et al.*, 2018; Ude *et al.*, 2020; Nasri *et al.*, 2021; Kathirvelpandian *et al.*, 2022; Nasri *et al.*, 2024).

However, it's important to acknowledge the challenges of correctly classifying an organism before relying on molecular identification methods. Molecular identification methods reveal species diversity by detecting genetic variations at the DNA level, providing a detailed understanding of species differences (Andriyono *et al.*, 2022). Fish species identification has been based on external physical characteristics. Still, this approach can be limited by the complexity of identifying fish, especially during different stages of their development, where morphological traits may be unclear or variable. DNA-based identification offers a powerful and precise solution, potentially replacing traditional methods with a more accurate identification of species (Teletchea, 2009). Fish species identification has relied on physical characteristics like body shape, colour, scales, fins, and body proportions (Wong and Khoo, 2017). Even in entire specimens, morphological traits may not always be useful for identification and distinction due to intraspecific variances or minor differences across species (Yates *et al.*, 2014). Identification becomes difficult, especially in early life stages (eggs and larvae) are more difficult to identify than adults (Hubal *et al.*, 2014). Due to the challenges in identifying fish species based on physical characteristics, scientists and biologists have developed alternative methods that don't rely on morphological traits. To accurately identify species, researchers must consider the specimen's evolutionary history, taxonomy, and phylogenetic relationships. A thorough understanding of the phylogeny and nomenclature used in previous studies is essential for accurate classification and to avoid confusion (Pereira *et al.*, 2008). These methods are based on the principle that individuals within a species share distinct DNA or protein sequences that set them apart from other species. The spread of these molecular variants across time and space is influenced by factors like reproductive success, migration patterns, and random genetic events, which shape the genetic diversity within and between species (Charlesworth, 2009; Laine *et al.*, 2011).

Currently, these fishes are identified based on morphometric and meristic methods, but the confusion remains in others except one species *Tenualosa ilisha* (Jawad, 2021; Sarker *et al.*, 2021). Ghouri *et al.*, (2020) reported the identification of *Tenualosa ilisha* including fresh and marine water fish species through DNA Barcoding. Despite attempts to use modern molecular biology techniques like RAPD, researchers have struggled to accurately identify these fish species due to the limited resolution of these methods. This confusion has trickled down to fishermen, resulting in misidentification, inadequate handling, and overfishing of hilsa, exacerbating the problem.

The fish species *Tenualosa toli* and *Tenualosa ilisha* are vital to Pakistan's fishing industry, yet their population dynamics and genetic diversity along the country's coasts remain unknown. This study aims to bridge this knowledge gap by employing DNA barcoding (COI) for molecular characterization. This will enable accurate identification, classification, and understanding of population structure, genetic diversity, and evolutionary relationships. The findings will inform effective management and conservation strategies for these species on the Pakistan coast.

2. Methodology

2.1 Sample collection

Total (N=20) fish samples of *Tenualosa toli* and *Tenualosa ilisha* were collected from two locations, Kharochan (67◦34’42’ N, 24◦04’31E) and Jhangisir (67◦37’34’ N, 24◦11’18E), between March 2021 to 2022 (Figure 1). The samples (Figure 2) were carefully cleaned with distilled water, stored in labelled polythene bags, and transported to the laboratory for further analysis. One sample from each species was identified by molecular identification. The fish were stored at -20°C until DNA extraction. The length and weight of the samples were recorded, ranging from 30.4-32.2 cm and 290-360 g for *Tenualosa toli*, and 18.00-23.00 cm and 218-266 g for *Tenualosa ilisha*. Morphological

identification was conducted using morphometric and meristic characteristics, with species-level identification confirmed using the Field Identification Guide to the Living Marine Resources of Pakistan (FAO, 2016).

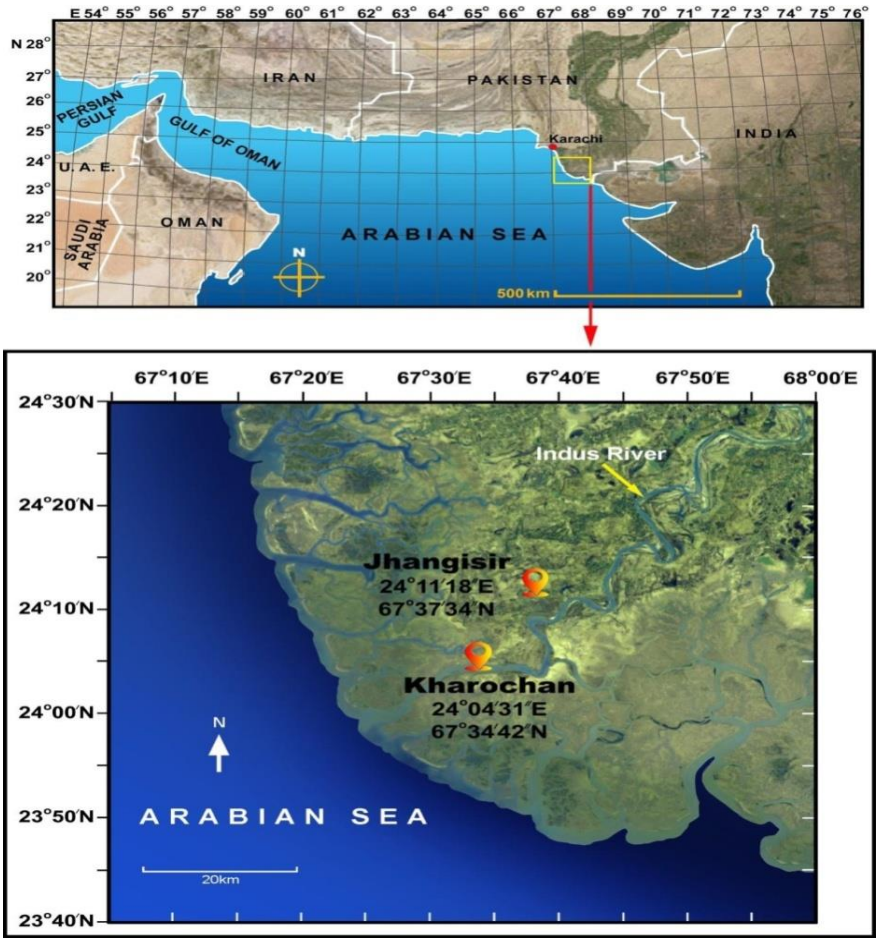

Figure 1. Map of the sampling area, Kharochan (67◦34'42' N, 24◦04'31E) and Jhangisir (67◦37'34' N, 24◦11'18E).

Genomic DNA was extracted from dorsal muscles using the standard Barcode of Life protocol (Ivanova *et al.*, 2006). The process involved dissecting dorsal muscle tissue and crushing it into a fine powder using a mortar and pestle, suspending the powder in a digestion buffer (100 mM NaCl, 10 mM TRIS-Cl, 25 mM EDTA, SDS, pH 8-4) with 0.1 mg/ml proteinase K (Sigma), incubating the mixture at 37°C for a variable period, purifying the nucleic acid through an organic extraction step, involving, adding an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) to the proteinase K digests, mixing thoroughly and centrifuging to separate the aqueous and organic layers. This process yielded high-quality genomic DNA for further analysis.

2.2 DNA Extraction and PCR Condition

All gathered specimens underwent meticulous preservation in 90% ethanol to ensure their structural integrity for subsequent laboratory analyses. In the preliminary stages of genomic DNA extraction, 0.5-gram tissue samples from each specimen were aseptically dissected and then rinsed to eliminate ethanol residue, utilizing distilled water. Following this cleansing procedure, the tissue samples were transferred into microtubes containing 6X lysis buffer and subjected to mechanical disruption using the Tissue Lyser II device (Qiagen). Genomic DNA extraction was executed using the Accuprep® Genomic DNA Extraction Kit (Bioneer), adhering to the manufacturer's stipulated protocol. The

concentration of the extracted DNA was quantified using a nano Drop spectrophotometer (Thermofisher Scientific D1000) and was either promptly utilized in PCR applications or stored at -70°C for prospective analyses.

The amplification of genomic DNA from the samples employed a universal primer set that had previously demonstrated success in fish identification. This primer set comprised forward, 5′-AAC CAC CGT TGT TAT TCA ACT ACA A-3′ and reverse 5′-CCGACTTCCGGATTACAAGACCG-3′)(Bautista et al., 2007).The target DNA amplified was the Cyt-b region, approximately 900 bp in length. The PCR mixture had a volume of 20 µL, comprising 11.2 µL ultra-pure water, 1 µL each of forward and reverse primers (0.5 µM), 0.2 µL Ex Taq DNA polymerase (TaKaRa, Japan), 2 µL 10X ExTag Buffer, 2 µL dNTPs (1 µM, TaKaRa, Japan), and 2 µL genomic DNA as a template. The PCR conditions were as follows: an initial denaturation at 95°C for 5 min, followed by denaturation at 95°C for 30 s in 40 cycles, annealing at 50°C for 30 s, extension at 72°C for 45 s, and a final extension at 72°C for 5 min. After obtaining the desired bands on the electrophoresis gel (1.5% agarose), the PCR products were purified using the AccuPrep® Gel purification kit (Bioneer, Korea).

2.3 Data Analysis and Phylogenetic Reconstruction

All sequences were aligned using MegaX software (Kumar *et al.*, 2018), which encompassed sequences acquired from the GenBank database for comparative analysis. The raw DNA sequence data underwent trimming by Chromas software, which is open-source and downloaded (http://technelysium.com.au/wp/chromas/). Trimming involved excising the sections of the DNA sequence with suboptimal signals (quality value <20) to ensure the integrity of the DNA sequence. This trimming procedure was applied to both the forward and reverse sequences. In the case of the reverse sequence, prior to alignment with the forward sequence, a reversal process was conducted using an open-source web bioinformatics tool (https://www.bioinformatics.org/sms/rev_comp.html).

(A) (B)

Figure 2. Speciment of *Tenualosa toli* (A) and *Tenualosa ilisha* (B)

The forward and reversed sequences were then merged to generate an extended sequence, enhancing accuracy in identifying the Cyt-b region. After the combination of the two sequences by ClustalW (https://www.ebi.ac.uk/Tools/msa/clustalo/), alignment was further conducted with the online databases at NCBI through BLASTN, available in an open-source format (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This step aimed to ascertain the species serving as the identity for the DNA sequences in this study. Once all DNA sequences were assigned species identity, the ensuing step involved constructing a phylogenetic tree using the bioinformatic software MEGAX. The pairwise evolutionary distance among the species was determined utilizing the Kimura 2-Parameter method. The construction of a Neighbour-Joining (NJ) tree, coupled with a 1000 bootstrap

analysis, was executed using MegaX (Kumar *et al.*, 2018). This algorithm stands as a fitting and standardized analytical procedure for DNA barcoding sequences, previously applied in studies by Collins and Cruickshank (Collins and Cruickshank, 2013).

3. Results and Discussion

3.1 Molecular Identification

In this study, we morphologically identified two types of fish that are one of the important commodities of coastal communities in Pakistan. These fish are called hilsa although there are slightly different morphological characteristics. Molecular identification results proved that the two fish species were identified as *Tenualosa toli* and *Tenualosa ilisha*. Online alignment with the sequence in the GenBank database confirmed that the two sequences were valid with an accuracy of 100% on the reference sequence in NCBI (Table 1).

Phylogenetic reconstruction of two sequences (t04 and t09) found that they cluster in the same clade of the genus *Tenualosa* (Flower, 1934) and are more closely related to its sister genus *Hilsa* (Regan, 1917). The phylogenetic tree also shows that the genus Sardinella is slightly more distant from the genus *Tenualosa* (Hunnam,2021; Samante *et al.*, 2009). In this phylogenetic tree reconstruction, the sequence of Lutjanus vitta (DQ900677) was added which was accessed through the NCBI web (Figure 3).

Table 1. BLAST of two sequences of *Tenualosa* from Pakistan

No.	Sample ID	Species name	% Identity	Reference from NCBI Database
1	Hil-t04	*Tenualosat oli*	100	KX859103
2	Hil-t09	*Tenualosa ilisha*	100	KX859108

Phylogenetic tree reconstruction

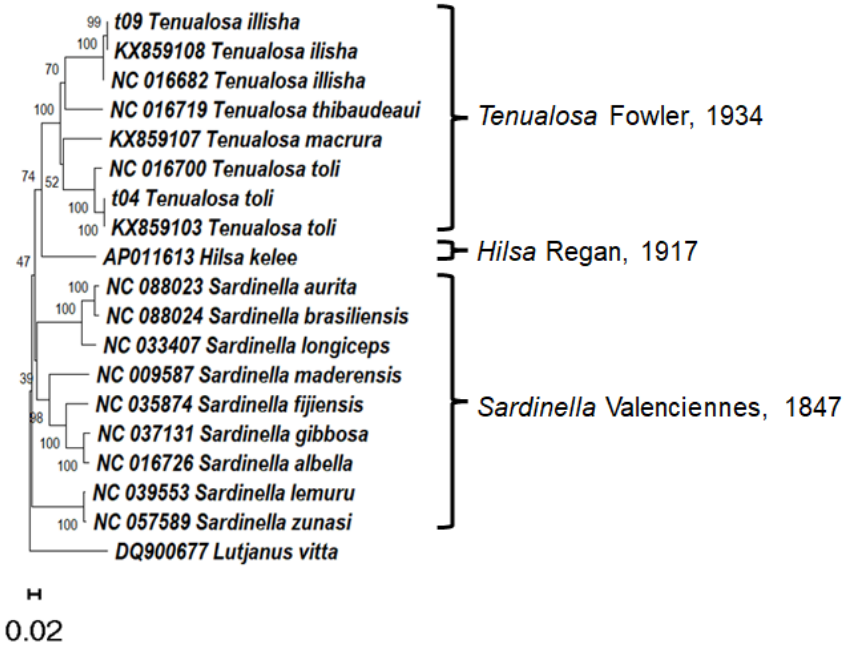

Figure 3. Phylogenetic tree of *Tenualosa ilisha* and *Tenualosa toli* from Pakistan.

Six specimens each of *Tenualosa toli* and *Tenualosa ilisha* were collected from two locations - Kharochan (67°34'42" N, 24°04'31" E) and Jhangisir (67°37'34" N, 24°11'18" E) - between July 2020 and March 2021. These specimens were used for molecular characterization of the two species using the Cyt-b gene. PCR amplification of the extracted DNA was carried out using Cyt-b gene-specific primers, yielding a 910 bp amplicon for *Tenualosa toli* and a 905 bp amplicon for *Tenualosa ilisha*. The resulting PCR products were then separated and visualized on a 2% agarose gel stained with Ethidium Bromide.

The current study found that *Tenualosa toli* and *Tenualosa ilisha* possess distinct DNA barcodes that can be clearly differentiated from each other. This finding is consistent with previous research conducted in Bangladesh, which used the Cyt-b gene to molecularly characterize three hilsa shad species *Tenualosa ilisha*, *Tenualosa toli* and *Hilsa kelee* and reported unique barcodes for each species (Sultana et al. 2022). The research reveals the existence of three distinct species of hilsa: *Tenualosa ilisha* and *Hilsa kelee*. The Cyt-b gene is identified as a reliable genetic marker for accurately identifying and confirming the identity of these species.

DNA barcoding is a taxonomic tool that utilizes molecular techniques to identify and distinguish between different species (Antil *et al.,* 2023). This approach typically involves analysing a short, standardized genetic sequence from the mitochondrial cytochrome c oxidase subunit I (COI) gene, which serves as a unique barcode for each species (Hubert and Hanner, 2015). The Cyt-b gene in identifying fish species in Pakistan's freshwater and marine environments, including *Tenualosa ilisha* (Ghouri *et al.,* 2020). Their study revealed the nucleotide composition of the Cyt-b gene, with adenine (A) occurring 216 times, thymine (T) 262 times, guanine (G) 163 times, and cytosine (C) 284 times. The average nucleotide composition was estimated to be 48.3% GC (guanine-cytosine) and 51.7% AT (adenine-thymine). The current study's findings revealed a nucleotide composition of A (259), T (201), G (128), and C (222) for the Cyt-b locus. This barcode analysis enabled the identification of mysterious fish specimens, uncovering unexpected diversity among them, consistent with previous research (Colihueque *et al.,* 2021). Phylogenetic analysis using the neighbour-joining method showed a clear clustering of *Tenualosa ilisha* and *Tenualosa toli*, with a 100% bootstrap value indicating a very close relationship between these two species (Figure 3).

The phylogenetic tree indicates a close evolutionary relationship between *Tenualosa ilisha* and *Tenualosa toli*, suggesting that they should share similar physical characteristics and overall morphology. This similarity likely contributes to the difficulties in distinguishing between the two species based on morphological features alone, as previously noted by Rahman *et al.* (2018).

Three species of hilsa *Tenualosa ilisha, Tenualosa toli*, and *Hilsa kelee* reported from Pakistan (FAO, 2016). Hilsa is a highly valued and significant resource in Pakistan's fisheries sector. However, its popularity has led to exploitation, largely due to misidentification. A major challenge lies in fishermen's struggle to accurately distinguish between the three *Hilsa* spp., leading to unsustainable fishing practices and potential depletion of this vital resource. Research on the morphological and molecular characteristics of *Tenualosa ilisha* and *Tenualosa toli* has been limited, with a few studies conducted in Bangladesh. Earlier studies proposed the existence of three hilsa types based on biometric measurements (Pillay and Rosa, 1963). Later, RAPD (Random Amplified Polymorphic DNA) techniques were employed to identify and distinguish three distinct forms of hilsa, supporting the idea of multiple species within the genus (Dahle *et al.,* 1997). Contrary to previous findings, a comprehensive morphological study revealed distinct characteristics that unequivocally distinguish three separate species of hilsa. Furthermore, DNA barcoding and evolutionary distance analyses also support the existence of three distinct species, providing strong evidence for the classification of Hilsa

into three separate species. The phylogenetic tree revealed a clear divergence of the species into two distinct groups within a single genus and a third species in a more distantly related genus (Rahman *et al.*, 2018). While the study is limited by a small sample size, the robust bootstrap values provide strong support for the inferred relationships, suggesting a reliable reconstruction of the species' evolutionary history. Future studies should aim to increase the sample size and include specimens from closely related genera within the same family to further validate the findings and provide a more comprehensive understanding of the population dynamics and phylogenetic relationships (Wohl *et al.*, 2021).

The phylogenetic tree indicates a close evolutionary relationship among the species, which is likely responsible for the similarities in their physical characteristics and overall structure, leading to challenges in identifying them based on morphology alone. In a related study, the molecular characterization and expression profile of oestrogen receptor subtypes in female hilsa (*Tenualosa ilisha*), providing further insights into the species molecular biology. This study marks the first investigation of oestrogen receptors in a seasonally spawning female fish throughout its entire reproductive cycle The expression of oestrogen receptor mRNAs in the liver and ovary of female Hilsa, provided novel insights into the molecular mechanisms governing its reproductive biology (Roy *et al.*, 2024). Environmental factors significantly influence gonadal development, which continues until the final breeding stage (Ahammad *et al.*, 2021). In a separate study, previous researcher made a significant contribution by publishing the first draft genome assembly and identifying single nucleotide polymorphisms (SNPs) in the hilsa shad (*Tenualosa ilisha*) from the Bay of Bengal, laying the foundation for further genetic research on this species. The draft genome assembly of the Hilsa shad yielded a total length of 710.28 Mb, with an N50 scaffold length of 64,157 bp and a GC content of approximately 43% (Mollah *et al.*, 2019). For comparison, the whole genome assembly of the Atlantic herring, a prominent Clupeid fish, was 808 Mb with an N50 scaffold length of 1.84 Mb and a GC content of 44% (Martinez Barrio *et al.*, 2016). Notably, repetitive elements comprised 31% of the Atlantic herring assembly, based on short reads with insert sizes ranging from 170 bp to 20 kb. There are controversies regarding the number of hilsa stocks in Bangladesh waters. Studies involving morphological and genetic analyses using allozyme, Random Amplification of Polymorphic DNA (RAPD) (Ahmed *et al.*, 2004) and Mt DNA-restriction fragment length polymorphism (RFLP) (Mazumder and Alam 2009) markers proved to be insufficient in resolving the stock disputes of this species (Dahle *et al.*, 1997).

The aim of the present study was to develop a clear and comprehensive guide that enables accurate identification of hilsa species in Pakistan, providing a valuable resource for individuals seeking to distinguish between them. We reported here the first report on the molecular characterization of *Tenualosa toli* from Pakistan using the Cyt-b gene. The assembled genome can be used as a reference for genetic studies of *Tenualosa ilisha*, *Tenualosa toli* and related species. It also provides a valuable resource for resolving stock disputes and phylogenetic or adaptation investigation of the Dorosomatidae family and species. The precise identification and classification of hilsa species are essential for sustainable fisheries management, ensuring food authenticity, understanding species dynamics, and uncovering hidden biological diversity. To mitigate the decline in Hilsa populations and safeguard their genetic diversity in Pakistan, proactive conservation and management measures are imperative. Furthermore, the DNA barcodes provide strong supporting evidence for our findings and offer a valuable tool for informing management and conservation efforts, enabling more effective protection and sustainability of Hilsa populations.

Conclusion

The results of this study obtained the results of the molecular identification of two fish samples, *Tenualosa toli* (Valenciennes, 1847) and *Tenualosa ilisha* (Hamilton, 1822), from Pakistan in the mitochondrial DNA cytochrome b gene region. This adds to the existing NCBI sequence database related to the Cyt-b sequence which is still lower than the COI gene region. This study also proves the accuracy of molecular identification in fish samples with complex morphological characteristics in Clupeids.

Disclosure statement: *Conflict of Interest:* The authors declare that there are no conflicts of interest.
Compliance with Ethical Standards: This article does not contain any studies involving human subjects.

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