

Identification of Larval Fishes in Tropical Estuarine Waters Using Morphology and DNA Barcoding Methods (Alamparai, India)

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Abstract

Comprehensive information regarding larval identifications and descriptions of tropical marine and estuarine fishes remains limited, contributing to taxonomic ambiguity and exacerbating the already high diversity of finfish species in these waters. The necessity for precise identification of early developmental stages of fishes cannot be overstated, as it plays a crucial role in various aspects of fishery resource management, including stock assessment, fishery compensation, ecological monitoring, and habitat protection. The primary objective of this study is to assess the efficacy of DNA barcodes in facilitating the identification of larval fish. This evaluation becomes crucial as conventional taxonomic keys, reliant on morphological characteristics, fall short in light of the dearth of discernible larval traits and the swift morphological transformations throughout ontogeny. The collection of larval fishes was carried out using plankton nets within the vicinity of the Alamparai estuary, Tamil Nadu, India, during the period from April 2012 to March 2014. A total of 12,428 finfish larvae were morphologically identified, mostly to the family and genus level. Among them, a total of four individuals were studied for DNA barcoding analysis. Molecular studies employed polymerase chain reaction (PCR) and sequencing of mitochondrial Cytochrome c Oxidase I (*CO I*) gene fragments. Genetic distance analysis of mitochondrial DNA sequences demonstrated unique characteristics distinguishing them from closely related species of all finfish larvae. The highest genetic distance was observed *Trachinotus blochii* (0.100%) followed by *Crossorhombus azureus* (0.097%); *Terapon jarbua* (0.08%) and *Arothron immaculatus* (0.02%). The significance of larval fish taxonomy in fishery management underscores the need for a comprehensive approach that integrates both morphological and molecular identification methods. This strategic combination of morphological and molecular techniques not only addresses the limitations of traditional methods but also enhances the precision and expediency required for robust fishery management practices.

Introduction

Only a few larval identification studies have been described in tropical nations compared to temperate countries (Leis, 2015); this is why studies of fish larvae in tropical countries are not progressing as quickly as they are in temperate regions. The Indo-Pacific regions recent publications on fish larval taxonomy have been active since before the inaugural Indo-Pacific conference (Leis, 2015). Tropical larval fish descriptions for marine and coastal waters are very less; some

notable contributions were made by various researchers. The only guide of Indo-Pacific fish larvae (Leis & Carson-Ewart, 2004) allows identification of early life stages mostly to the family level, more rarely to the genus level.

Studies from tropical regions highlight the taxonomic challenges of larval fish identification due to high species diversity and morphological similarity, particularly at the genus and species levels (Konishi et al., 2012). In Indian coastal and estuarine waters, several morphological surveys have documented larval fishes;

however, identification has largely been restricted to the family level despite extensive sampling (Venkataramanujam, 1975; Prince Jeyaseelan, 1998; Sundaramanickam et al., 2007; Silambarasan, 2018; Ram et al., 2020). These limitations emphasize the need for improved approaches to achieve accurate species-level identification in tropical marine ecosystems.

Morphological identification of fish larvae can be difficult to undertake due to a lack of taxonomic keys and the rapid development of larval to juvenile stages. Furthermore, extensive abilities and taxonomic competence in the field are required. Traditionally, morphological characteristics such as body shape, pigmentation, meristic count and measurements have been used to identify larval fish. However, these characters are insufficient to identify all species, particularly rare and cryptic species that are morphologically identical but genetically diverse, limiting the capabilities of traditional taxonomy such as morphology in the identification process (Ko et al. 2013). Many fish species share the same morphology in their early life history, and their morphometric measures are frequently replicated (Victor et al., 2009). Furthermore, the varying levels of competence and capacities among larval fish taxonomists have made it a critical aspect in the larval fish morphological identification process.

The fundamental limits of traditional morphology-based identification systems, as well as the shrinking pool of taxonomists, have already signaled the need for a new approach to taxon recognition, such as DNA based identification (Hebert et al., 2003). DNA barcoding can be used to validate a taxonomist or laboratory capacity to make or improve larval fish identification. The results of barcoding can be utilized to create more reliable diagnostic keys or an encyclopedia for fish eggs and larvae. The application of DNA barcoding can help solve the “splitter” misidentification problem of larval fishes as well as the “lumper” problem of fish eggs. Morphological identification on batches of eggs with sizes less than 1 mm is impossible. Even if we use the sampling approach to locate some typical dominating species by barcoding, we cannot establish the communities total composition and its spatial and temporal distribution (Ko et al., 2013).

With an increasing abundance of DNA barcodes accessible through publicly available databases like GenBank and the Barcode of Life Data System (BOLD), the scope for identifying additional species of larval fish has expanded significantly through the matching of DNA sequences. This breakthrough has enabled researchers to link larvae to their adult species, providing valuable insights into the questions surrounding their early life stages (Valdez-Moreno et al., 2010). The wealth of DNA sequences associated with adult fishes serves as a crucial tool, facilitating the accurate identification of fishes during their early life history stages. As emphasized by Shao et al. (2002), molecular identification stands as the only method capable of guaranteeing the identification of fish eggs and larvae to

the species level. Consequently, the primary objective of this study was to delineate the taxonomy of larval fishes collected from the Alamparai estuary in Tamil Nadu, India, utilizing both traditional taxonomic keys and DNA barcoding methods, with a concurrent analysis to compare the efficacy of these two distinct approaches.

Materials and Methods

Study Area

The Alamparai estuary (Yedayanthittu) a small brackish water system (12°12' - 12°15' N and 70°56' - 80°00' E) on the western part of the Kaliveli wetland, which plays a significant role in fishery production and constitutes the life line of the local economy. The Alamparai estuary is 16 km long with a width of 157 m at its widest point near the mouth and 70 m in the upper reaches with depths that vary from 2 m in the lower reaches to 3.4 km in the upper reaches. The Alamparai estuary drains a catchment area of 176 km². The estuary extends for approximately 16 km, passing through the villages of Kanthadu, Vandipalayam and Chettikulam from just north of the Marakkanam road bridge to its confluence with the Bay of Bengal near the historic site of Alamparai port, Kanchipuram District, Tamil Nadu (Silambarasan, 2018) (Figure 1).

Collection and Preservation of Larval Fish Specimens

In the present investigation, finfish larvae were collected monthly from April 2012–March 2014, encompassing all four seasons (Postmonsoon, Summer, Premonsoon and Monsoon) in each year, from the Alamparai estuary. For the sampling process, three stations were selected. Sampling was carried out during the early hours of the day at high tided using a conical plankton net with a 0.5 m mouth diameter (mouth area \approx 0.196 m²), made of bolting silk (No. 10 mesh size; 158 μ m) and fitted with a General Oceanics Inc. flow meter to estimate the volume of water filtered. Larvae were collected from surface and subsurface layers, with two replicate sample per station during each sampling event. Sampling was conducted using vertical hauls with a single net. At stations with a bottom depth of approximately 10 m, the net was hauled from \sim 5 m depth to the surface (0 m), whereas at stations with water depths greater than 20 m, hauls were conducted from \sim 20 m depth to the surface. In addition, at each station, the net was towed for 15 minutes while maintaining an average boat speed of approximately 2 knots (\approx 1 m s⁻¹). After each haul or tow, the net was gently rinsed from the outside, and the flow meter readings were recorded to determine the volume of water filtered. The collected samples were transferred to 500 mL polythene bottles and preserved on site in 95% ethanol, and subsequently transported to the laboratory, where larval fish were sorted out from the remaining plankton for further analysis.

Sorting and Morphological Identification of Larval Fish Specimens

For the taxonomic identification, the fish larvae were sorted out from plankton samples. Initially the fish larvae segregated from preserved planktonic samples. For that, the sample was placed in Petri dishes and analyzed under a stereo zoom microscope (Leica M80 6.4x-48x), at low magnification and the fish larvae were identified, counted, and segregated. Then similar looking fish larvae were stored in a separate container and identified by lowest possible taxonomic level using the standard references of Delsman (1931); Venkataramanujam (1975); Thangaraja (1982); Prince Jayaseelan (1998); Ramaiyan et al., (2005); and Ram et al., (2020); amongst others. Larvae were measured to the nearest 0.1 mm body length (BL) using an eyepiece micrometer for specimens < 10 mm, and vernier calipers for larger specimens. Body length represented notochord length in preflexion and flexion larvae, and standard length in postflexion larvae and early juvenile stages, following the definitions and developmental criteria described by (Neira et al., 1992).

Data Analysis

A non-parametric Kruskal-Wallis's test was used to compare the median values of surface water temperature, salinity and dissolved oxygen among sampling periods. All statistical analysis were performed using the software R version 3.5.1 (R Core Team, 2018).

Taxonomic composition was expressed as percentage composition of each family to the total larval fish abundance. The relative abundance (%) of each family was calculated as:

$$\text{Relative abundance (\%)} = \left(\frac{\text{Number of larvae of a given family}}{\text{Total Number of larvae collected}} \right) \times 100.$$

Larval abundance was standardized as number of individuals per 100 m³ (N/100 m³) using a flow meter readings to estimate the volume of water filtered during each haul. Families contributing less than 2% to the total abundance were considered rare components of the larval assemblage.

DNA Barcoding Using Universal Primers of CO1

The DNA barcoding analysis focused on seven selected larval fishes. Genomic DNA extraction from individual fish larvae was performed using the NucleoSpin® Tissue Kit (Macherey-Nagel) following the manufacturer's guidelines. Amplification of the COI gene was conducted for all samples using the Fish F1 [5'-GGTCAACAAATCATAAAGATATTGG-3'] and Fish R1 [5'-TAAACTTCAGGGTGACCAAAAAATCA-3'] primers (Ward et al., 2005). PCR amplification reactions were executed in a 20 µl reaction volume, comprising 1X Phire PCR buffer (containing 1.5 mM MgCl₂), 0.2 mM each dNTPs (dATP, dGTP, dCTP, and dTTP), 1 µl DNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, and 5pM of forward and reverse primers. The PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) was employed for amplification under the following conditions: initial denaturation at 98°C for 2 min, followed by 30 cycles of 98°C for 30 seconds, 54°C for 40 seconds, and 72°C for 1 minute. The quality of the PCR products was verified through electrophoresis on a 2% agarose gel with a 100bp DNA marker as a size standard, confirming the suitability of the products for sequencing. Subsequently,

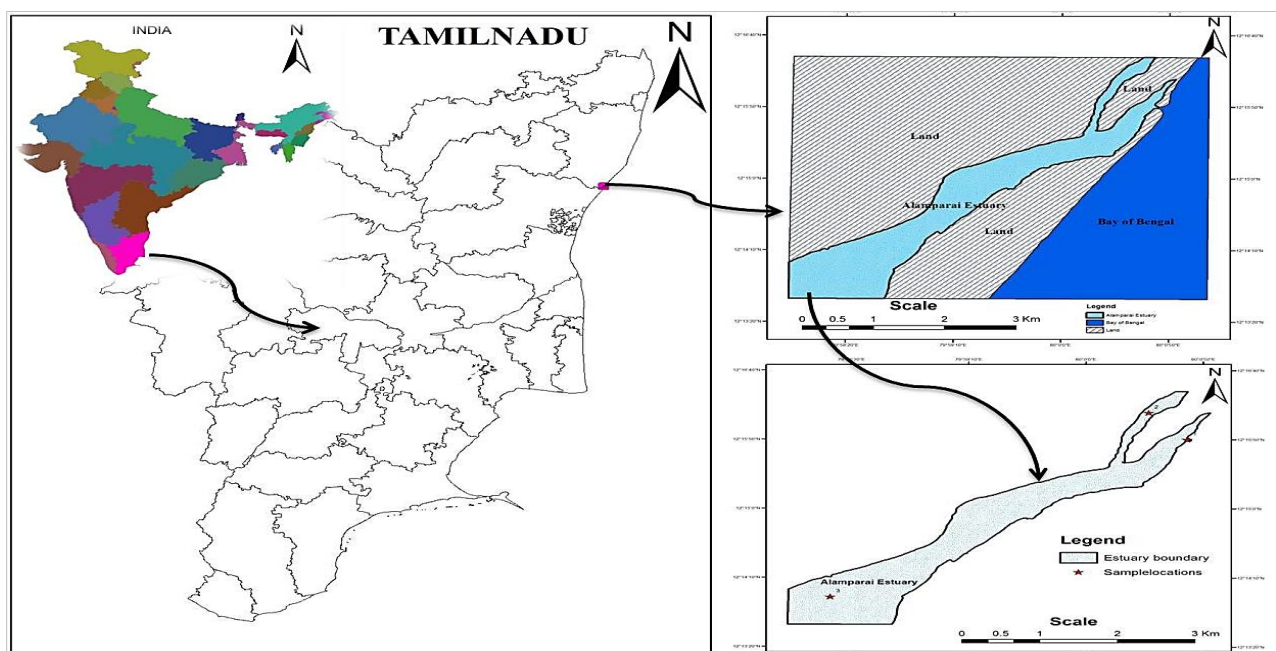


Figure 1. Map showing the collection sites of Alamparai estuary.

purified PCR products were prepared for cycle sequencing using the Big Dye® Terminator 3.1 sequence kit (Applied Biosystems, Foster City, California, USA) following the manufacturer's instructions. The sequencing reaction was carried out in the GeneAmp PCR System 9700 (Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA), adhering to the manufacturer's protocol.

DNA Sequence Analysis

The assessment of sequence quality was conducted using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and necessary editing of the acquired sequences were performed using Geneious Pro v5.1 (Drummond et al., 2010). To facilitate further analysis, all larval sequences underwent conversion to FASTA format and were subsequently submitted to the NCBI website's nucleotide BLAST tool (<http://www.ncbi.nlm.nih.gov/>). The top five sequence matches, displaying the highest similarity to database references (mat ident), were scrutinized for species consistency. Additionally, all sequences underwent submission to the BOLD identification system (<http://www.boldsystems.org/>) for comparison with the extensive repository of species-level barcode records of animals, comprising 148,815 sequences as of November 9, 2014. The outcomes of these submissions were then analyzed based on percent similarity scores, providing a comprehensive evaluation of the concordance between the obtained larval sequences and existing reference databases.

Neighbour-Joining Tree Analysis

Taxonomic classifications were established through a meticulous phylogenetic analysis of mitochondrial DNA (mtDNA) gene sequences. The Bioedit software (Version 7.2.5) (Hall, 2004) was employed for the analysis of the sequences. The final alignment, comprising four sequences, underwent comparison with sequences deposited in the GenBank database at the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST).

To visually represent the divergence patterns between species, Neighbor-Joining (NJ) trees were constructed based on Kimura two-parameter (K2P) distances (Saitou et al., 1987). This graphical representation was generated using MEGA 6.0 software, with 1000 bootstrap replications performed to enhance statistical robustness (Kumar et al. 2004). The Kimura two-parameter (K2P) genetic distances were applied to define species, genus, and family levels, aligning with the methodology outlined by Ward et al. (2005).

Results

Physico-chemical Parameters

A distinct seasonal pattern was observed in the Alamparai estuarine mangrove environment when comparing surface water temperature, salinity and dissolved oxygen (Figure 2). Surface water temperature varied between 24.0°C to 36.0°C, while the lowest values recorded during the monsoon season and the highest during the summer season. Salinity exhibited pronounced seasonal variation throughout the study period, ranging from 18.0 to 36.0 PSU. The lowest salinity (18.0 PSU) was observed during the monsoon season, followed by a gradual increase during post-monsoon period, reaching maximum values (36.0 PSU) during the summer season. Dissolved oxygen concentration showed a considerable fluctuation, varying from 3.2 to 7.07 mg l⁻¹. The lowest value DO content (3.2 mg l⁻¹) were recorded during the summer season, whereas the highest values (7.07 mg l⁻¹) occurred during the monsoon season. The observed seasonal differences in surface water temperature ($\chi^2 = 18.011$, df = 3, p = 0.0004375), salinity ($\chi^2 = 17.262$, df = 3, p = 0.0006243), and dissolved oxygen ($\chi^2 = 19.484$, df = 3, p = 0.0002171) were statistically significant, as confirmed by the Kruskal–Walli's rank sum test.

Taxonomic Composition of Larval Fishes

In Alamparai estuary, a total of 12,428 finfish larvae belonging to 28 families, 37 genus and 47 species were collected during the study period. The larval fish assemblages were not so diverse and were numerically dominated by 10 families (75.1%) of the total catch. Clupeidae is the most abundant family comprising (14.8%) followed by Engraulidae (14.6%), Teraponidae (11.1%), Ambasiidae (10.3%), Mugilidae (9.6%), Leiognathidae (4.5%), Lutjanidae (3.1%), Cichilidae (2.8%) (Mean 64.2 ± 16.7 N/100m³), Sillaginidae (2.2%) and Gerridae (2.1%). Other families that were not well represented and contributing less than 2% were Carangidae (2.0%), Centropomidae (1.9%) Hemiramphidae (1.5%), Chanidae (1.5%), Apogonidae (1.4%), Tetraodontidae (1.3%), Atherinidae (1.3%), Elopidae (1.1%), Gobidae (1.1%), Scatophagidae (1.0%), Platycephalidae (0.9%), Bothidae (0.9%), Scombridae (0.9%), Belonidae (0.8%), Syngnathidae (0.8%), Cyprinodontidae (0.5%) and Haemulidae (0.4%). Damaged specimens comprised 3.3% of total larval collection.

Morphological Identification of Larval Fishes

Morphological examination of larval fishes collected from the Alamparai Estuary revealed distinct diagnostic characters that enabled reliable taxonomic identification. Key morphological traits analyzed included body shape, gut configuration and relative

length, presence and visibility of the gas bladder, head and snout morphology, mouth position, eye size and orientation, head spination, pigmentation patterns, fin ray counts, and vertebral numbers, which collectively enabled reliable taxonomic assignment and are summarized in (Tables 1 and 2 & Figure 3). These characters were consistent with established larval fish identification keys and exhibited clear interspecific variation among *Trachinotus blochii*, *Terapon jarbua*,

Crossorhombus azureus, and *Arothron immaculatus*. Morphological identification was completed prior to molecular analysis, and concordance between morphological and genetic results confirmed that all sequenced individuals belonged to the morphologically assigned families. Owing to limited sample size and the qualitative nature of diagnostic larval characters, statistical analyses of morphological variation were not performed.

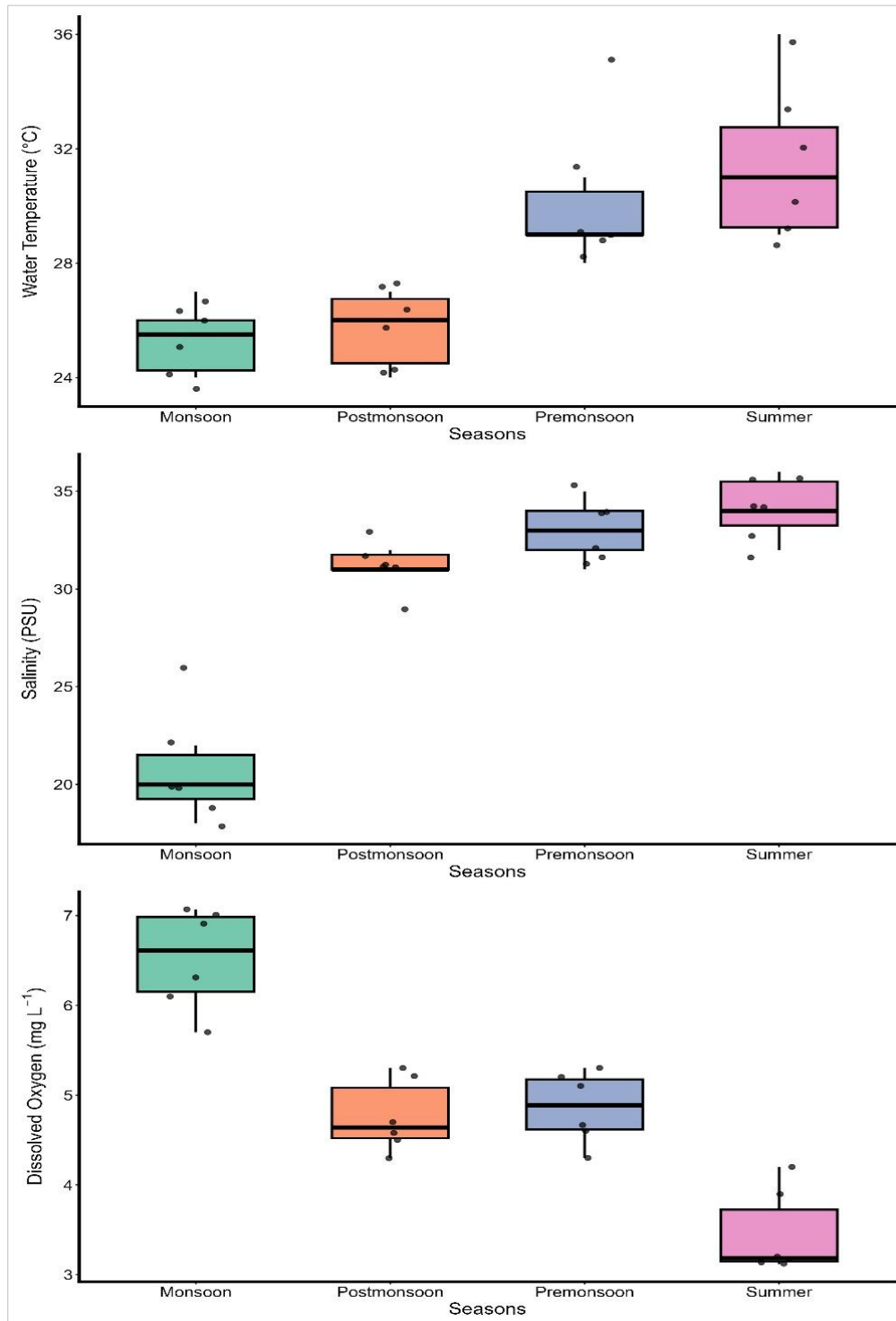


Figure 2. Seasonal variation in surface water temperature (°C), salinity (PSU), and dissolved oxygen (mg L⁻¹) in the Alamparai estuarine mangrove environment during the study period (April 2012–March 2014). Box plots represents interquartile ranges, horizontal lines indicate medians, and points denote individual observations.

Larval Fish Samples through DNA Barcodes

A total of 12,428 finfish larvae were collected, only 7 individuals that ranged from 5.6 to 30.0 mm total length (TL) and comprised various families were chosen for molecular identification. The *COI* gene was successfully amplified in 4 individuals using the barcoding primers. The 3 samples that failed to amplify were from the families Elopidae, Gobidae and mugilidae each (n=1).

DNA extraction from four larval fish samples (*Trachinotus blochii*, *Terapon jarbua*, *Crossorhombus azureus* and *Arothron immaculatus*) were successfully amplified, sequenced and analyzed DNA sequences extracted from the above said four species were submitted to GenBank and their accession numbers are presented in Table 3, sequencing of the *COI* gene produced 655 nucleotide base pairs per taxon. Simplicity and ambiguity were observed among all the sequences, and no insertions, deletions or stop codons were

observed in any of the sequences. The average percentage of base composition of the *COI* gene fragment in four fish species was as follows: *Trachinotus blochii*, A: 24.3, T: 30, G: 17.9, C: 27.6; *Terapon jarbua* A: 22.4, T: 29.1, G: 18, C: 30.2; *Crossorhombus azureus* A: 22.4, T: 29.6, G: 20.3, C: 27.5 and *Arothron immaculatus* A: 22.6, T: 25.2, G: 19.4, C: 32.6. The highest AT content (24.3-30%) was observed in *Trachinotus blochii* followed by *Arothron immaculatus* (22.6-25.2%), *Crossorhombus azureus* (22.4-29.6 %) and *Terapon jarbua* (22.4-29.1%) (Figure 4).

Neighbour-Joining Analysis of CO I Barcode Sequences

The dendrogram analysis revealed a distinct clustering pattern among the four species, grouping them into two primary clades, as illustrated in Figure 5. The first major clade further divides into three sub-clades, encompassing *T. blochii*, *T. jarbua*, and *C. azureus* species. In contrast, the second clade is

Table 1. Comparison of morphological characters of larval fishes collected from the Alamparai estuary

Main characters	<i>Trachinotus blochii</i>	<i>Terapon jarbua</i>	<i>Crossorhombus azureus</i>	<i>Arothron immaculatus</i>
Body shape	Moderately deep bodied	Elongate to moderately compressed	Strong and laterally compressed	Broadly rounded
Gut	Round shaped and coiled; extends beyond the middle of the body	Triangular and compact; reaches about 55% body length (BL)	Coiled; reaches to 15-33% BL in the late larval stages.	Triangular and compact.
Gas bladder	often concealed by heavy pigmentation	Very small; difficult to discern	Initially inconspicuous; disappears before right eye migration	Invisible
Head	Large	Large and round shaped	Small to moderate and round	Large and blunt
Snout	Slightly pointed or blunt	Short and slightly pointed	Steep to slightly rounded; anterior projection of the dorsal-fin base slightly reduced	Short
Mouth	Oblique; extends beyond mid eye	Oblique; reaches the anterior margin of the pupil	Small and oblique; usually does not reach the anterior margin of the eye	Jaws with fused teeth.
Eyes	Round; moderate to large	Moderate and round	Small to moderate and round; right eye migrates to the left side of the body through an opening between the dorsal-fin base and cranium.	Very large and rounded.
Head spination	Weak spination	Spines present on the opercle and cleithrum.	Spination absent	Spination absent
Pigment	Slight pigmentation	Melanophores increase laterally on the body; faint vertical broad pigment bands on trunk and tail.	Pigment develops along dorsal and ventral margins; head, dorsal, anal and pelvic fins, and on fleshy membranes of elongate dorsal-fin rays,	Pigmentation becomes reduced after the post-flexion stages

Table 2. Comparison of meristic counts in larval fishes collected from the Alamparai estuary

Species	Dorsal fin	Anal fin	First pectoral fin	Second Pectoral fin	Caudal fin	Vertebrae
<i>Trachinotus blochi</i>	VI+I, 18	II+I, 23	19	I, 5	8+9	10+14=24
<i>Terapon jarbua</i>	XI, 10	III, 9	14	I, 3	9+8	10+15=25
<i>Crossorhombus azureus</i>	79 (soft rays)	63 (soft rays)	9	6	17	10+24=34
<i>Arothron immaculatus</i>	0+9	0+10	17	--	9	--

comprised solely of *Arothron immaculatus* species. Examining the branch lengths within the *T. blochii* clade, a noteworthy similarity emerged between the branch lengths leading to related species. This similarity extended to the *T. jarbua* and *C. azureus* clade, suggesting a shared characteristic among *T. blochii*, *T. jarbua*, and *C. azureus*, possibly indicative of unique heterogeneity within these species.

The phylogenetic construction, employing the NJ method, consistently depicted the two clear branches observed in the dendrogram analysis. Notably, there were no alterations in this branching pattern, affirming the stability of the identified clades. Furthermore, all species exhibited robust node stability, supported by high bootstrap values (1000), reinforcing the reliability of the branch structures in the phylogenetic tree.

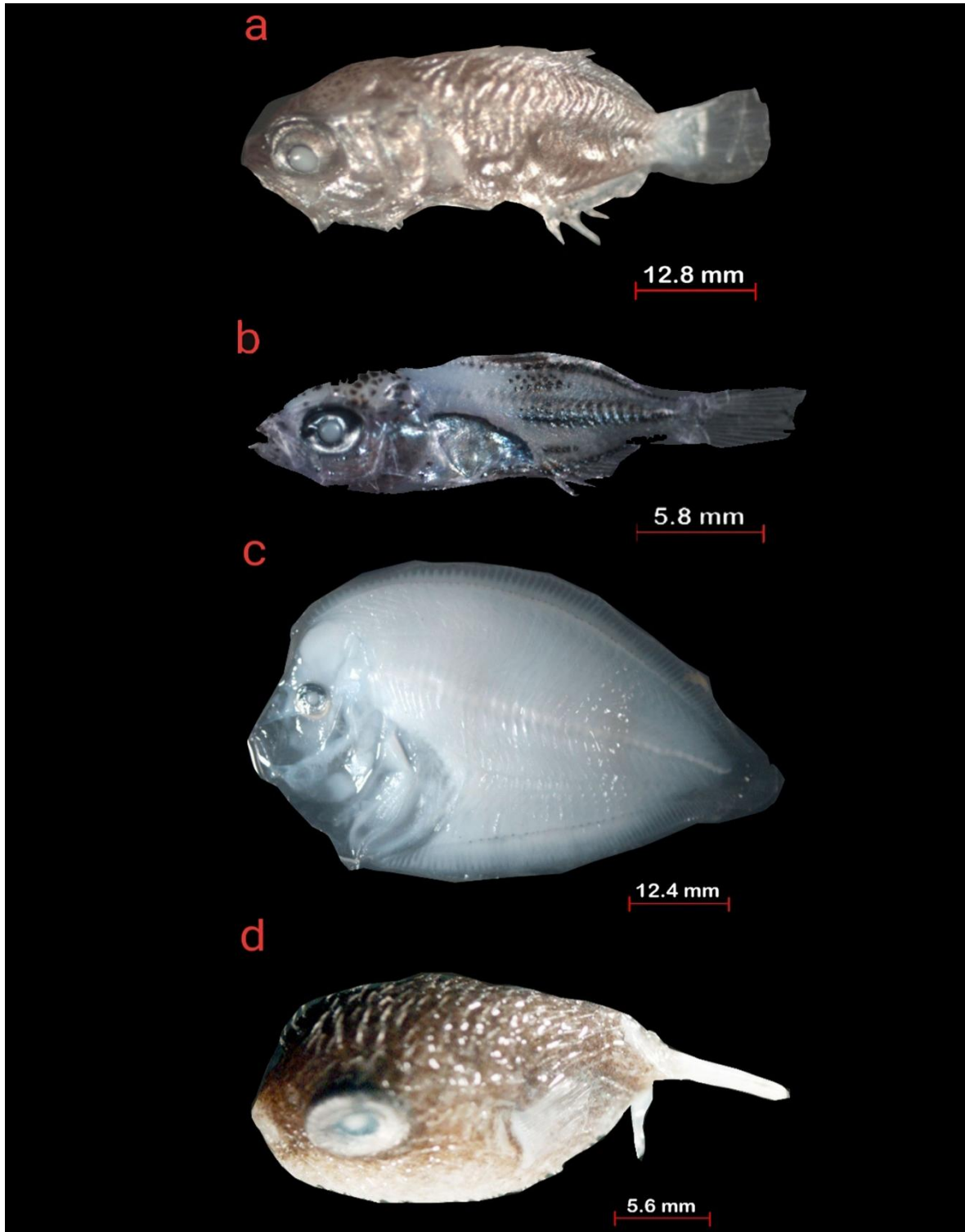


Figure 3. Samples identified correctly through morphological and DNA barcoded larval fishes. Their total length (TL) and % sequence identity (ID) are given: (a) *Trachinotus blochii* 12.8 mm TL, 100% (ID); (b) *Terapon jarbua* 5.8 mm TL, 100% (ID); (c) *Crossorhombus azureus* 12.4 mm TL, 100% (ID) and *Arothron immaculatus* 5.6 mm TL, 100% (ID).

BLAST search revealed that the segment control of mtCOI, has 99% sequence similarity with *T. blochii*, *T. jarbua* and *A. immaculatus* and 89% similarity with *C. azureus*. The nucleotide and protein sequences of *T. blochii*, *T. jarbua*, *C. azureus* and *A. immaculatus* were primarily compared and analyzed with relevant databases using BLASTn. Maximum identity was observed in different strains of *T. blochii*. Maximum identity of *T. jarbua* was with the nucleotide sequence of *T. jarbua* Te A2. Maximum identity of *C. azureus* was observed with the nucleotide sequence of *C. azureus* (DCCO1058) and *C. azureus* (734QYP) (Table 4). Maximum identity of *A. immaculatus* was observed with the nucleotide sequences of *A. immaculatus* CIFE-MUM-AL.

The computed pairwise genetic distances among the four species vary within distinct ranges: 0.008% to 0.100% for *T. blochii*, 0.002% to 0.008% for *T. jarbua*, 0.097% to 0.191% for *C. azureus*, and 0.02% to 0.107% for *A. immaculatus* (Table 4, 5, 6, and 7). Notably, the lowest genetic distance signifies a closely related status, suggesting genetic similarity, while the highest genetic distance implies a highly diverged status, indicating significant genetic differences among the species.

Discussion

India, being a mega biodiversity country with a primary focus on conservation studies, underscores the importance of understanding the early life history (ELH)

of marine fishes for effective marine resource management. Despite this imperative goal, species identification during the early stages poses significant challenges due to the difficulty in relying solely on morphological characteristics. The potential for misidentifications introduces substantial biases in biodiversity surveys and community structure assessments (Nammalwar et al., 2009).

Compounding the complexity, most fishes undergo metamorphosis during ontogeny, leading to dynamic changes in morphometric characteristics such as pigmentations and the number of myomeres. This ontogenetic development further complicates the species identification of finfish eggs and larvae, creating a challenge characterized by overlapping meristic characteristics (Graves et al., 1989; Chow et al., 2003).

Over a decade ago, DNA barcoding technology emerged with the primary objective of providing a reliable method for species-level identification (Chac & Tinh, 2023). This method relies on the average genetic distance among individuals within a species, ensuring it does not exceed the average genetic distance between sister species (Pradhan et al., 2015). Since the introduction of DNA barcoding technology worldwide, numerous studies have utilized DNA barcodes, contributing to a deeper understanding of species identification (Hebert et al. 2003; Ward et al. 2005; Nelson et al. 2007; Johnkress et al. 2014).

The utilization of DNA barcoding for species identification across all life-history stages proves

Table 3. Barcoded larval fishes from Alamparai estuary and their NCBI accession numbers

Order	Family	Name of the species	N	Accession Numbers
Carangiformes	Carangidae	<i>Trachinotus blochii</i>	01	KX0189885
Centrarchiformes	Teraponidae	<i>Terapon jarbua</i>	01	KX018987
Pleuronectiformes	Bothidae	<i>Crossorhombus azureus</i>	01	KX018986
Tetraodontiformes	Tetraodontidae	<i>Arothron immaculatus</i>	01	KX018988

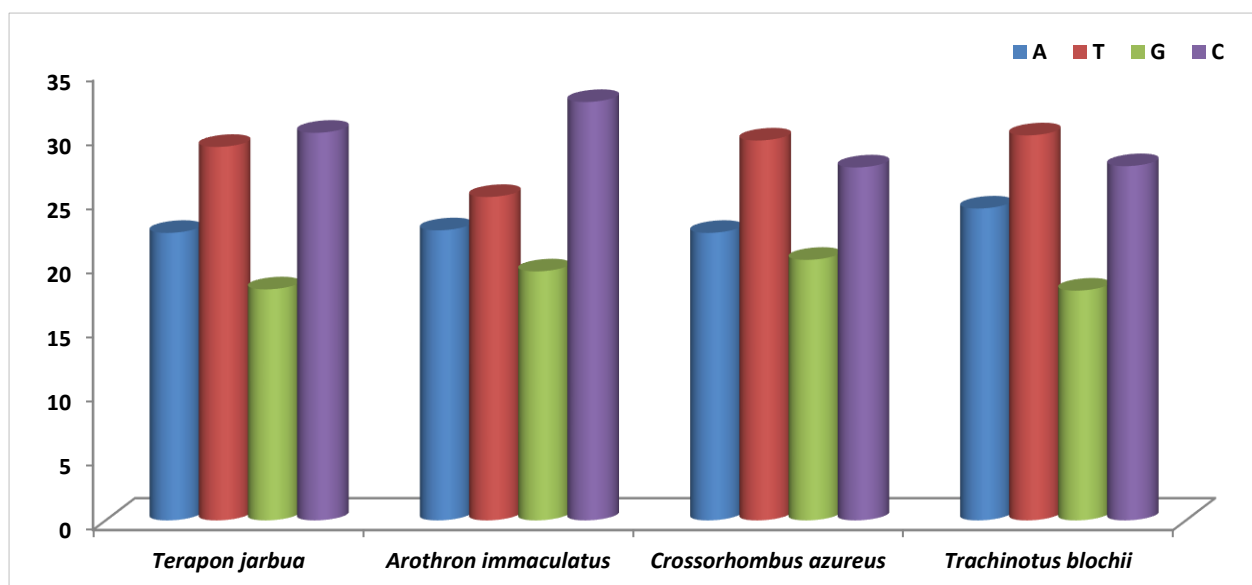


Figure 4. Nucleotide percentage composition of CO I gene in the four fish species.

beneficial in gaining a deeper understanding of ecological mechanisms (Katoh 2001; Pegg et al., 2006; Rocha-Olivares & Chávez-González 2008; Victor et al. 2009), without diminishing the role of taxonomic experts, as argued by Ebach & de Carvalho (2010). Nevertheless, both DNA barcoding and traditional taxonomy exhibit their respective limitations, including issues such as the potential misinterpretation of the barcode gap (Collins & Cruickshank 2012) and the establishment of consistent thresholds, which vary among taxonomic groups (Krishnamurthy & Francis 2012).

In certain taxonomic groups, the scarcity of expert taxonomists poses a challenge, and even well-described species often lack comprehensive or reliable information regarding systematic classification (Romano & Palumbi 1996; Balakrishnan 2005; Neigel et al., 2007; Swartz et al., 2008). Recognizing the efficiency of species identification, some ichthyologists advocate for the

incorporation of a DNA barcode in the formal description of species (Victor 2007; Hulley et al., 2018; Rathnasuriya et al., 2021).

Both DNA barcoding and traditional taxonomy contribute significantly to scientific knowledge, and the application of an integrative taxonomy approach (Krishnamurthy & Francis 2012), combining molecular and traditional taxonomy methods, emerges as a promising strategy for enhancing taxonomic research findings. This integrative approach proves particularly valuable in larval fish identification and the acknowledgment of new species (Baldwin & Johnson 2014).

Ajmal Khan et al., (2010) barcoded 42 fish species belonging to 32 genera and 23 families while, Lakra et al., (2011) documented 115 marine finfish species belonging to 37 families and 79 genera from Indian waters, demonstrating the effectiveness of DNA barcoding for fish identification. In the present study,

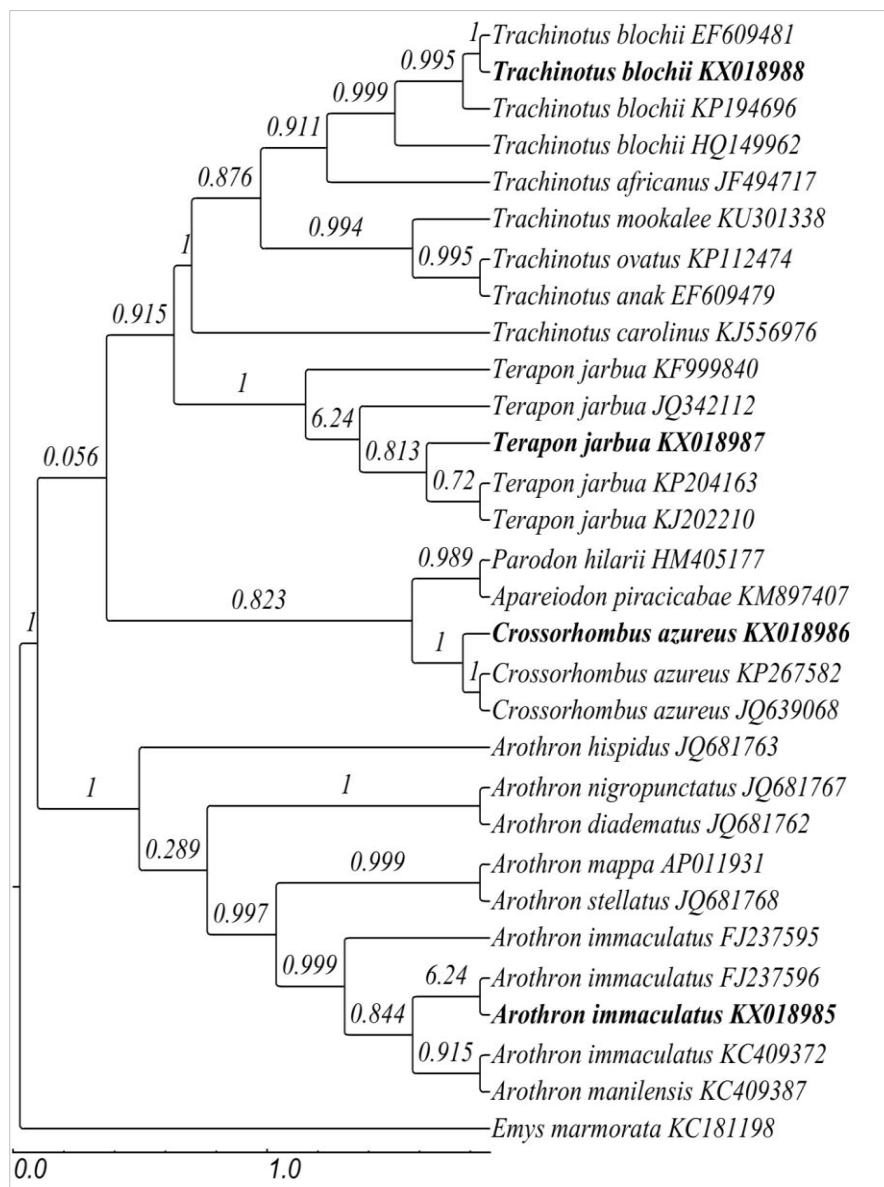


Figure 5. Phylogenetic analyses of *Trachinotus blochii*, *Terapon jarbua*, *Crossorhombus azureus* and *Arothron immaculatus* by Neighbor-Joining method using MEGA v 5.0.

molecular phylogenetic cluster analysis revealed a similar topology, consistent with previous records (Ajmal Khan et al., 2010; Lakra et al., 2011; Thirumaraiselvi et al., 2015). The average nucleotide base composition of the COI gene fragment observed in the present investigation also agrees with previous studies (Lakra et al. 2011; Thirumaraiselvi et al. 2015). Specifically, the average nucleotide composition of *T. jarbua* was A: 22.4, T: 29.1, G: 18, C: 30.2, while *T. blochii* exhibited A: 24.3, T: 30, G: 17.9, C: 27.6., which closely matches the findings of Ajmal Khan et al., (2010) and Thirumaraiselvi et al., (2015).

Among the four species identified, *Trachinotus blochii* is a marine dependent species; however, its larval

and juvenile stages are known to migrate into estuarine environments in tropical regions (Nazar et al, 2017). The consistent occurrence of *T. blochii* larvae during the present study may be attributed to its extended spawning season under warm tropical conditions, together with tidal incursion and coastal circulation patterns that facilitate larval transport from adjacent marine waters into estuaries. Furthermore, fluctuation of environmental variables, particularly surface water temperature plays a crucial role in prolonged reproductive periods and expanding larval distribution ranges. These combined factors likely explain the presence of *T. blochii* larvae throughout the sampling period in the estuarine waters.

Table 4. Genetic distance of *Trachinotus blochii* (KX018988) sequence from its related species

Species	1	2	3	4	5	6	7	8
<i>Trachinotus carolinus</i> KJ556976								
<i>Trachinotus ovatus</i> KP112474	0.091							
<i>Trachinotus anak</i> EF609479	0.084	0.008						
<i>Trachinotus mookalee</i> KU301338	0.085	0.044	0.038					
<i>Trachinotus africanus</i> JF494717	0.096	0.085	0.079	0.078				
<i>Trachinotus blochii</i> HQ149962	0.097	0.091	0.089	0.095	0.065			
<i>Trachinotus blochii</i> KP194696	0.100	0.097	0.093	0.093	0.061	0.016		
<i>Trachinotus blochii</i> EF609481	0.100	0.097	0.093	0.093	0.061	0.016	0.000	
<i>Trachinotus blochii</i> KX018988	0.100	0.097	0.093	0.093	0.061	0.016	0.000	0.000

Table 5. Genetic distance of *Terapon jarbua* (KX018987) sequence from its related species

Species	1	2	3	4
<i>Terapon jarbua</i> JQ342112				
<i>Terapon jarbua</i> KP204163	0.007			
<i>Terapon jarbua</i> KJ202210	0.007	0.000		
<i>Terapon jarbua</i> KF999840	0.007	0.003	0.003	
<i>Terapon jarbua</i> KX018987	0.008	0.002	0.002	0.005

Table 6. Genetic distance of *Crossorhombus azureus* (KX018986) sequence from its related species

Species	1	2	3	4
<i>Crossorhombus azureus</i> KP267582				
<i>Crossorhombus azureus</i> JQ639068	0.006			
<i>Parodon hilarii</i> HM405177	0.188	0.191		
<i>Apareiodon piracicabae</i> KM897407	0.185	0.188	0.115	
<i>Crossorhombus azureus</i> KX018986	0.097	0.099	0.170	0.174

Table 7. Genetic distance of *Arothron immaculatus* (KX018985) sequence from its related species

Species	1	2	3	4	5	6	7	8	9
<i>Arothron immaculatus</i> FJ237596									
<i>Arothron immaculatus</i> FJ237595	0.004								
<i>Arothron immaculatus</i> KC409372	0.017	0.019							
<i>Arothron mappa</i> AP011931	0.053	0.048	0.064						
<i>Arothron stellatus</i> JQ681768	0.055	0.051	0.067	0.002					
<i>Arothron manilensis</i> KC409387	0.009	0.011	0.007	0.057	0.059				
<i>Arothron hispidus</i> JQ681763	0.086	0.086	0.102	0.081	0.083	0.091			
<i>Arothron nigropunctatus</i> JQ681767	0.093	0.088	0.107	0.081	0.083	0.098	0.099		
<i>Arothron diadematus</i> JQ681762	0.093	0.093	0.107	0.085	0.088	0.098	0.099	0.015	
<i>Arothron immaculatus</i> KX018985	0.002	0.005	0.015	0.050	0.053	0.007	0.083	0.090	0.090

Neighbor-Joining Analysis of CO I Barcode Sequences

The Kimura two-parameter (K2P) distance model was used because it is widely accepted in fish DNA barcoding and provides a consistent framework for species-level comparisons with publicly available reference sequences. K2P genetic distances indicated lower intraspecific variation than interspecific divergence. The highest genetic distance was observed in *Crossorhombus azureus* (0.107) and *Arothron immaculatus* (0.107), followed by *Trachinotus blochii* (0.100), while the lowest was recorded in *Terapon jarbua* (0.008). This pattern is consistent with previous studies showing that genetic divergence increases from within species to between genera or families (Blaxter & Floyd 2003; Borda et al., 2004; Hebert et al., 2003), confirming the suitability of the COI barcode region for species identification.

Conclusion

In conclusion, the present findings unequivocally underscore the limitations associated with the accuracy of traditional morphological identification of fish larvae, revealing notable disparities among different taxonomists and researchers. In contrast, DNA barcoding emerges as a valuable molecular tool for the classification and identification of larval fish, offering a means to validate the precision of traditional identification methods. Moreover, it is recommended that taxonomic studies of larval fish adopt an integrative approach, combining both traditional taxonomy (based on morphology) and molecular taxonomy (utilizing DNA barcodes). Traditional morphological characters, while more conservative, are optimal for identifying specimens up to the genus level, surpassing their efficacy at the species level. This dual approach helps mitigate the risk of incorrect species identification, which ranges from 66% to 97% under exclusive reliance on traditional morphology.

Ethical Statement

Not applicable.

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Author Contribution

K.S. – Conceptualization, Project Administration and Supervision, Funding Acquisition, Formal Analysis, Investigation; **P.S.** – Methodology, Data Curation, Visualization; **A.S.** – Resources, Software, Writing original draft; **A.P.** – Supervision, Writing review and editing.

Conflict of Interest

The authors state that they have no known conflicts that could have influenced the work presented in this paper.

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