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ORIGINAL ARTICLE

Exploring Genetic Diversity, Phylogenetics, and Molecular Dating of *Priacanthus tayenus* (Richardson, 1846) from the Odisha Coast, Bay of Bengal, India

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ABSTRACT

The Priacanthidae family plays a significant role in global fisheries, particularly in warm, shallow seas and estuaries. This research aimed to investigate the molecular divergence, genetic characteristics, and evolutionary history of the species within the Priacanthidae family, focusing on Priacanthus tayenus. The study employed DNA barcoding techniques, including the amplification and analysis of the mitochondrial COI gene. The pairwise distance analysis of 14 Priacanthus tayenus nucleotide sequences from different locations, showed an average pairwise distance of 0.04. Haplotype analysis identified eight haplotypes, with two dominant and six low-frequency haplotypes. Phylogenetic investigations using NJ and ML trees demonstrated the species-level relationships within the Priacanthidae family, supporting its monophyly. Molecular dating analysis estimated the divergence times, suggesting that the family Priacanthidae originated approximately 84.66 Mya during the late Cretaceous period. In the same period, the genus Priacanthus separated from other genera within the family. Furthermore, the analysis revealed that the species P. tayenus diverged from its common ancestor around 47.27 Mya, marking an important event in the evolutionary history of the genus Priacanthus. The research emphasizes DNA barcoding's effectiveness in species identification and evolutionary studies. It enhances our understanding of the genetic diversity, divergence patterns, and evolutionary history within the Priacanthidae family, contributing valuable insights for fisheries management and conservation efforts. Furthermore, the investigation validates the use of COI barcode sequences as a reliable tool for species-level identification and phylogenetic analysis in Priacanthidae research.

Keywords: *Priacanthidae, Mitochondrial COI gene, DNA barcoding, Molecular divergence*

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INTRODUCTION

The Earth's oceans encompassing over 70% of its surface water are vital repositories of biodiversity. However, the current state of biodiversity on our planet is under threat due to anthropogenic activities, leading to the alarming prospect of a sixth mass extinction [1],[2]. In this context, understanding and conserving marine biodiversity have become crucial objectives for scientific research. Fish species play a significant role in marine biodiversity, exhibiting a remarkable range of body sizes, shapes, and ecological adaptations [3]. India, with its diverse freshwater and marine habitats, contributes substantially to the global fish diversity, Indian waters house a substantial portion of the world's fish fauna, among these, marine fishes alone comprise 7.4% of the total, with 2,443 species and 927 genera distributed across 230 groups and 40 orders. The rich diversity of marine fish in India directly influences the complexity and uniqueness of its marine ecosystems [4]. The Bay of Bengal, situated along the eastern coast of India, is renowned for its abundant resources and remarkable biological diversity [5]. Odisha, a coastal state in India, boasts a 480 km coastline and is home to approximately 19% of India's marine biodiversity and

1.24% of the global marine biodiversity. Over the past two centuries, numerous fish species have been recorded from the Odisha coast [6],[7]. Among the various families of marine fish, the Priacanthidae family stands out as a distinct group. Commonly known as Bigeyes, these bottom-dwelling marine fishes comprise four genera: *Cookeolus, Heteropriacanthus, Priacanthus,* and *Pristigenys,* encompassing a total of 21 species [8],[9]. Bigeyes, characterized as carnivorous and nocturnal, play a crucial role in the marine food chain, contributing to the overall ecosystem dynamics. *Priacanthus tayenus* [10], is a remarkable species within the Priacanthidae family. It is recognized by its vibrant crimson-red body coloration and distinct blackish red spots on the pelvic fins. This species has a wide distribution in the tropical Indo-Pacific region. *Priacanthus tayenus* primarily inhabits shallow waters, but it can also be found at depths ranging from 150 to 200 meters [11]. It is predominantly distributed in the Northern Indian and western Pacific oceans, with its range extending from the Persian Gulf to the Great Barrier Reef of Australia and north to Taiwan. While it is rare in the East China Sea, *Priacanthus tayenus* is highly abundant in the Andaman Sea and southern South China Sea regions, where it holds significant importance in the commercial trawl fishery [11],[12]. Adult individuals of this species are known to form large schools at certain times and occupy more open bottom areas compared to other priacanthids, which are typically solitary near cover [13]. In the realm of taxonomy and species identification, accurate species composition estimation relies on robust identification methods. Traditional morpho-taxonomy, which relies on physical characteristics, has been complemented by techniques such as cytotaxonomy and immunoassay in the past [14]. However, these methods often face limitations, such as misidentification due to cryptic species, tissue damage during transportation, phenotypic plasticity, and misleading features during larval stages. In recent years, DNA barcoding has emerged as a more reliable and precise tool for species identification in marine environments [15],[16],[17]. This technique, which examines species-level sequence divergence, is widely recognized as the most adaptable method for species delineation and diagnosis [18],[19]. Although concerns exist regarding the potential loss of traditional taxonomy with the adoption of new methodologies [20], it is important to acknowledge the challenges posed by morphological similarities in conventional taxonomy. Integrating traditional morphological and modern molecular tools offers significant advantages [21],[22],[23]. Therefore, this study employed a comprehensive biological strategy using molecular DNA barcoding to overcome morpho-taxonomic obstacles and validate the taxonomic position of species within the examined families. Our goal was to explore the genetic diversity and phylogeny of Priacanthidae species, focusing on the mtCOI gene to determine their divergence time.

MATERIAL AND METHODS

Sample Collection and Storage

Fish specimens were collected from the Boxipalli fish landing centre of Gopalpur-on-Sea, Odisha, India (Figure 1). Upon collection, the specimens were immediately transported to the laboratory under freezing conditions. Morpho-taxonomic categorization was conducted, followed by the extraction of fin and muscle tissues in aseptic conditions. These tissues were then stored at -20 °C to facilitate further investigations.

Morphometry

A meticulous approach was employed to group the specimens based on their taxonomic features during the initial analysis, as well as subsequent re-descriptions and taxonomic revisions. The morphological variables were carefully assessed, and their accuracy was validated by comparing them to established taxonomic references, such as "Commercial Sea Fishes of India" [24], and "Fishes of The World" [9]. The classification of the specimens at the species level was achieved using approved taxonomic keys provided in Eschmeyer's Catalogue of Fishes [8].

DNA Extraction, Amplification, and Sequencing

Genomic DNA was extracted from muscle tissue using an improved version of the salting-out method as proposed by Sambrook and Russell [25]. The extracted DNA underwent analysis through 1.5 percent agarose gel electrophoresis and was subsequently stored at -20°C for future use [26]. This isolated DNA served as a template for PCR amplification of the mtCOI gene. Fish cocktail primers, namely FR1d_t1, FishR2_t1, FishF2_t1, and VF2_t1, targeting the 5' region (as listed in Table 1) were utilized for COI amplification [27]. For the amplification, a reaction mixture of 25 microliters was prepared, consisting of 10 µM of each primer, 1.0 U of DreamTaq DNA polymerase (Thermo Scientific, USA), a 10 mM dNTPs mix, and 1X PCR Assay buffer containing 20 mM MgCl2. The PCR conditions included an initial denaturation step at 95°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 1 minute. A final extension step was performed for 10 minutes at 72°C. The amplified products were then subjected to 2 percent agarose gel electrophoresis.

Later, successfully amplified products of the DNA samples were sent to Bioserve Biotechnologies Pvt. Ltd., Hyderabad, India, for commercial sequencing using the di-deoxy chain termination technique.

Sequence Data Analysis

Electropherograms were processed using the Codoncode aligner software to perform base-calling. High-quality sequence repeats with a quality value (QV) greater than 20 were selected for further analysis. The BioEdit program [29], was employed to remove any noisy sequences from both ends. Barcode sequences were accurately generated, aligned, and consensus sequences were created following the protocols outlined by Hall [29]. To identify the presence of insertions, stop codons, or deletions, the Expasy software was utilized. The BLASTN feature on the NCBI (National Center for Biotechnology Information) web service was employed to confirm if the sequences corresponded to the intended locus. Fragments that exhibited perfect alignment without any gaps, insertions, or deletions were selected. Finally, the obtained sequences were submitted to NCBI, where they were assigned unique accession numbers.

Genetic Diversity

The study utilized mtCOI sequences of *Priacanthus tayenus* obtained from various geographic areas, which were sourced from the NCBI database. The sequences were aligned using ClustalW in MEGA X software, and the resulting alignment was exported in mega format for further analysis. Nucleotide composition, overall transition/transversion bias, and average pairwise distance of the sequences were determined using MEGA X. DNA polymorphism analysis of the sequences was conducted using DnaSP6. To investigate the genetic relationships between haplotypes of *Priacanthus tayenus*, Median Joining Networks based on Bandelt et al. [30], were constructed using PopART software. The significance of the dataset was assessed using Tajima's D test [31], as well as Fu and Li's D and F tests [32].

Phylogenetic analysis

In the phylogenetic study of the Priacanthidae family, a total of 35 mtCOI sequences were analyzed. Among these sequences, 34 corresponded to four different genera within the Priacanthidae family, while one sequence was used as an outgroup. After alignment, K2P distances were calculated between all sequences of the Priacanthidae family. Phylogenetic analyses were conducted using MEGA X software [33]. Neighbor-joining (NJ) and maximum-likelihood (ML) trees were generated for the analysis. The Kimura-2-parameter substitution model [34] was selected for the NJ tree, and the substitution model for the ML tree was determined based on the best-fit DNA/Protein model study.

Molecular dating

The divergence time of species within the Priacanthidae family was estimated using the RelTime technique. To perform the estimation, mtCOI gene sequences of the Priacanthidae family, including the species studied and an outgroup sequence, were obtained from the NCBI database. These sequences were used to construct a phylogenetic tree, which was saved in newick format for calibration of the time tree. The best-fit model was also determined for the analysis. In the RelTime technique, only minimum and/or maximum measurement parameters are required. For this study, the species *Pristigenys niphonia* and *Cookeolus japonicus* (16 Mya) were used for the minimum fossil evidence time calibration boundary and the species *Priacanthus harmour* and *Priacanthus meeki* (46.6 Mya) [35], were selected for maximum fossil evidence time calibration boundary. To address the challenge of testing for equal rates of evolution between in-group and out-group sequences, the out-group clade was excluded during evaluation.

RESULTS

Priacanthus tayenus [10], (Figure 2) a species belonging to the Priacanthidae family, Actinopterygii class, and Acanthuriformes order, was collected from the Gopalpur-on-Sea located on the coast of Odisha, India. The identification of these specimens was successfully accomplished using traditional morpho-taxonomic identification keys.

Morphometric characters

The specimen was identified to be Purple-spotted bigeye fish, *Priacanthus tayenus*. It primarily inhabits rocky reefs, with occasional presence in more open areas. Body, head and iris of eye were pink to reddish or silvery white with pink tinges. The body, head and caudal fin base were covered with scales. Anterior profile was a little asymmetrical, enormously protruding lower jaw with a little above the level of midline of the body. Detailed morphometric and meristic characteristics of *Priacanthus tayenus* are presented in Table 2.

Sequence data analysis

No insertions, deletions, or stop codons were identified in any of the sequences, indicating that each sequence represents a functional mitochondrial COI sequence. The sequence of the *Priacanthus tayenus* specimen, consisting of 502 base pairs (bp) of good quality sequence, was subsequently uploaded to the NCBI database, and the accession number ON248034 was assigned to it.

Genetic diversity

The nucleotides compositions of *Priacanthus tayenus* were found to be 30.08% (T), 30.28% (C), 21.51% (A) and 18.18% (G) respectively, but average nucleotide composition of all the 14 sequences were found to be 29.55% (T), 29.42% (C), 21.92% (A) and 19.11% (G) respectively. Nucleotide composition of COI gene sequences of *Priacanthus tayenus* showed that AT content (52%) was higher than GC content (48%). Nucleotide frequencies were found to be 21.92% (A), 29.63% (T/U), 29.46% (C), 19.00% (G). The transition/transversion rate ratios were estimated to be $k_1 = 5.337$ (purines) and $k_2 = 5.853$ (pyrimidines). The overall transition/transversion bias R was 3.032. In codon usage bias analysis, in the first codon position of *Priacanthus tayenus*, G content (31.10%) was more comparision to A (25.72%), T (18.94%) and C (24.37%). In the second codon position, T (42.01%) content was more comparision to A (14.80%), G (13.89%) and C (29.37%). In the third codon position, G (12.45%) content was less compared to A (25.38%), T (27.88%) and C (34.37%) (Figure 3). The average intraspecies K2P distance between 14 sequences of *Priacanthus tayenus* was found to be 0.04. The median joining network of *Priacanthus tayenus* provided an overall complex pattern of star-like elements (Figure 4). There were 55 polymorphic sites including 42 (76.36%) parsimony informative sites and 13 (23.63%) singleton variable sites. A total of 58 mutations were discovered within the dataset. The 14 sequenced individuals produced a total of 8 haplotypes, with 6 unique haplotypes (69.23%) and 2 shared haplotypes (30.77%). Hap_1 (Gopalpur-on-sea, India; Banda Aceh, Indonesia); Hap_3 (Panay Island, Philippines; Tuban, Bali, Indonesia; Hong Kong, China; South China Sea; Batangas, Philippines; Vietnam) were shared haplotypes and the rest others were unique haplotypes. The nucleotide diversity (π) and haplotype diversity (*Hd*) of *Priacanthus tayenus* populations were estimated to be 0.03743 and 0.8242 respectively. Higher *Hd* and lower π indicated the modest level of genetic variability in populations under study. COI sequence of the collected specimen from Gopalpur-on-sea represented shared haplotype with Banda Aceh, Indonesia.

Phylogenetic analysis

Besides, 32 sequences of the family *Priacanthidae* along with *Apogon aurolineatus* as an outgroup were considered for phylogenetic tree construction. Average interspecies K2P distance of the genus *Priacanthus* was found to be 0.15 and the intergenus K2P distance of family *Priacanthidae* was found to be 0.24. HKY+G+I was identified as the best fit model for construction of ML tree with BIC value of 9667.051. In both ML (Figure 6) and NJ (Figure 5) phylogenetic trees, the sequences from same species (newly obtained in current study and retrieved from NCBI) clustered together in a monophyletic clade showing homology and more or less conspecific distance between them. Both the phylogenetic trees showed almost similar topology, which inferred that sequence of the species *Priacanthus tayenus* from Gopalpur-on-sea was closer to the sequences from Indonesia followed by Saudi Arabia comparision to other sequences of same species. The genus *Heteropriacanthus* was found to be the ancestor of rest of the genus of the family *Priacanthidae*.

Molecular dating

The family *Priacanthidae* had originated about 84.66 Mya during the late Cretaceous period and at the same time the genus *Priacanthus* separated from the other genera of the family. However, the species of the genus *Priacanthus* started separating during the early Eocene sub-epoch, which was around 47.27 Mya and the species *Priacanthus tayenus* was the first species of the genus *Priacanthus* to be diverged from their common ancestor at the same time around 47.27 Mya (Figure 6).

Table 1. Primer Specifications Utilized in This Investigation to Produce COI Barcode Sequences

Primer	Primer sequence (5′ to 3′)	mtDNA target	Amplicon size (bp)
Primer cocktail for fish DNA barcoding (ratio 1:1:1:1)		Cytochrome C oxidase 1 (COI) gene	652
VF2_t1	TGTAAAACGACGGCCAGTCAACCAACCACAAAG ACATTGGCAC		
FishF2_t1	TGTAAAACGACGGCCAGTCGACTAATCATAAAG ATATCGGCAC		
FishR2_t1	CAGGAAACAGCTATGACACTTCAGGGTGACCGA AGAATCAGAA		
FR1d_t1	CAGGAAACAGCTATGACACCTCAGGGTGTCCGA ARAAYCARAA		
M13F*	TGTAAAACGACGGCCAGT		
M13R*	CAGGAAACAGCTATGAC		

Note - *Sequencing primers for M13-tailed PCR products [27][28].

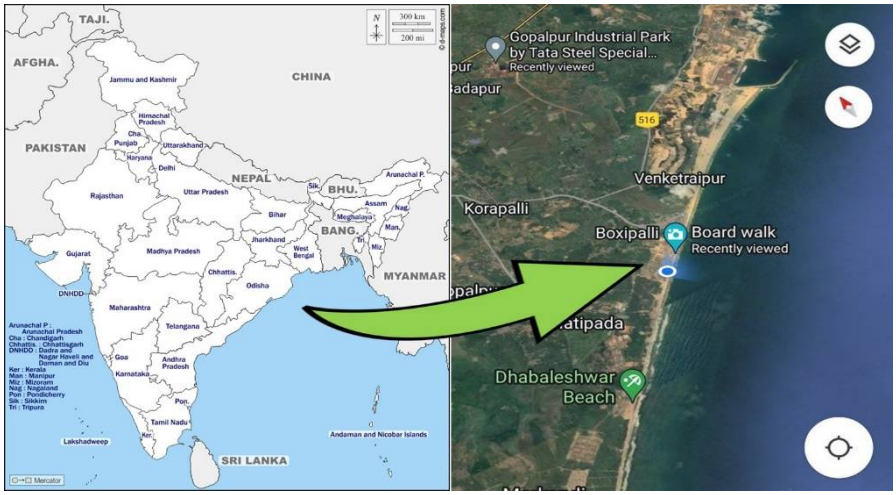

Figure 1. The Collecting Site's Map (84.90°E, 19.25°N)

Figure 2. *Priacanthus tayenus* [10]

Table 2. Morphometric and meristic data of *Priacanthus tayenus*.

Morphometric Parameters	Measurements
Total length (TL)	15.9 cm
Standard length (SL)	13.6 cm
% TL (Total length)	
Fork length	99.7
Pre-anal length	46.6
Pre-dorsal length	27.5
Pre-pelvic length	22.7
Pre-Pectoral length	27.5
Body depth	27.8
Head length (HL)	27.8
% HL (Head length)	
Eye diameter	46.5
Pre-orbital length	23.5
Meristic counts, Number	
Dorsal fin (Spine+ Soft Rays)	X+12
Pectoral fin (Soft Rays)	18
Anal fin (Spine+ Soft Rays)	III+13
Pelvic fin (Spine+ Soft Rays)	I+5

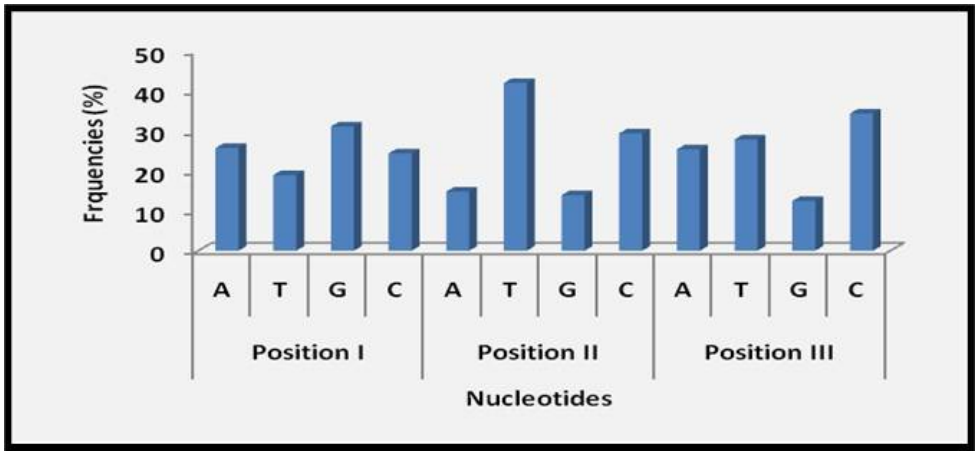

Figure 3. Distribution of nucleotides within codons in the barcodes of the fish species *Priacanthus tayenus*

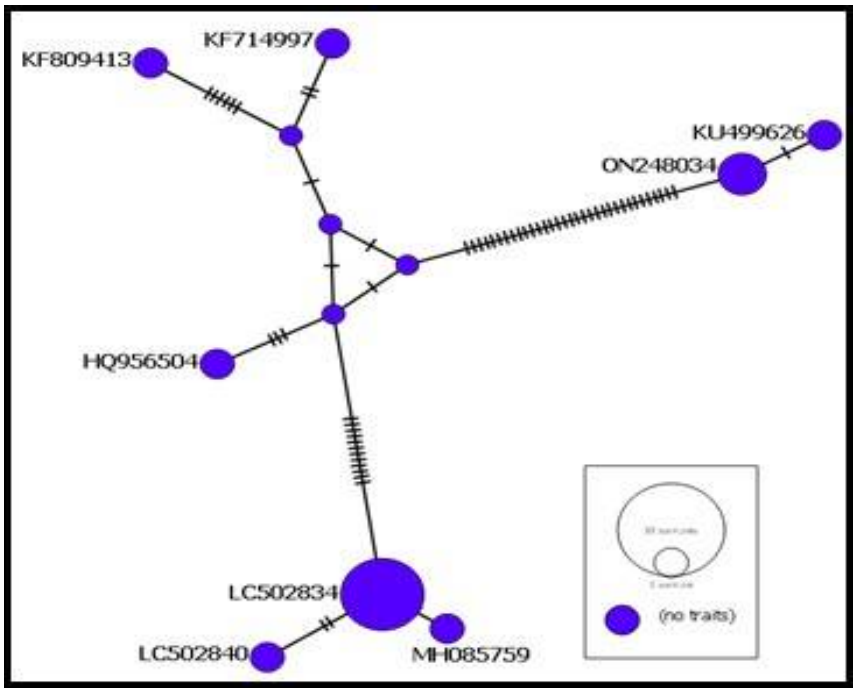

Figure 4. Median-joining haplotype networks of COI gene of *Priacanthus tayenus.* Size of the circles designates the frequencies of individual appear in sample. Mutations are represented by lines and the rate of mutation is shown by dashes along the lines.

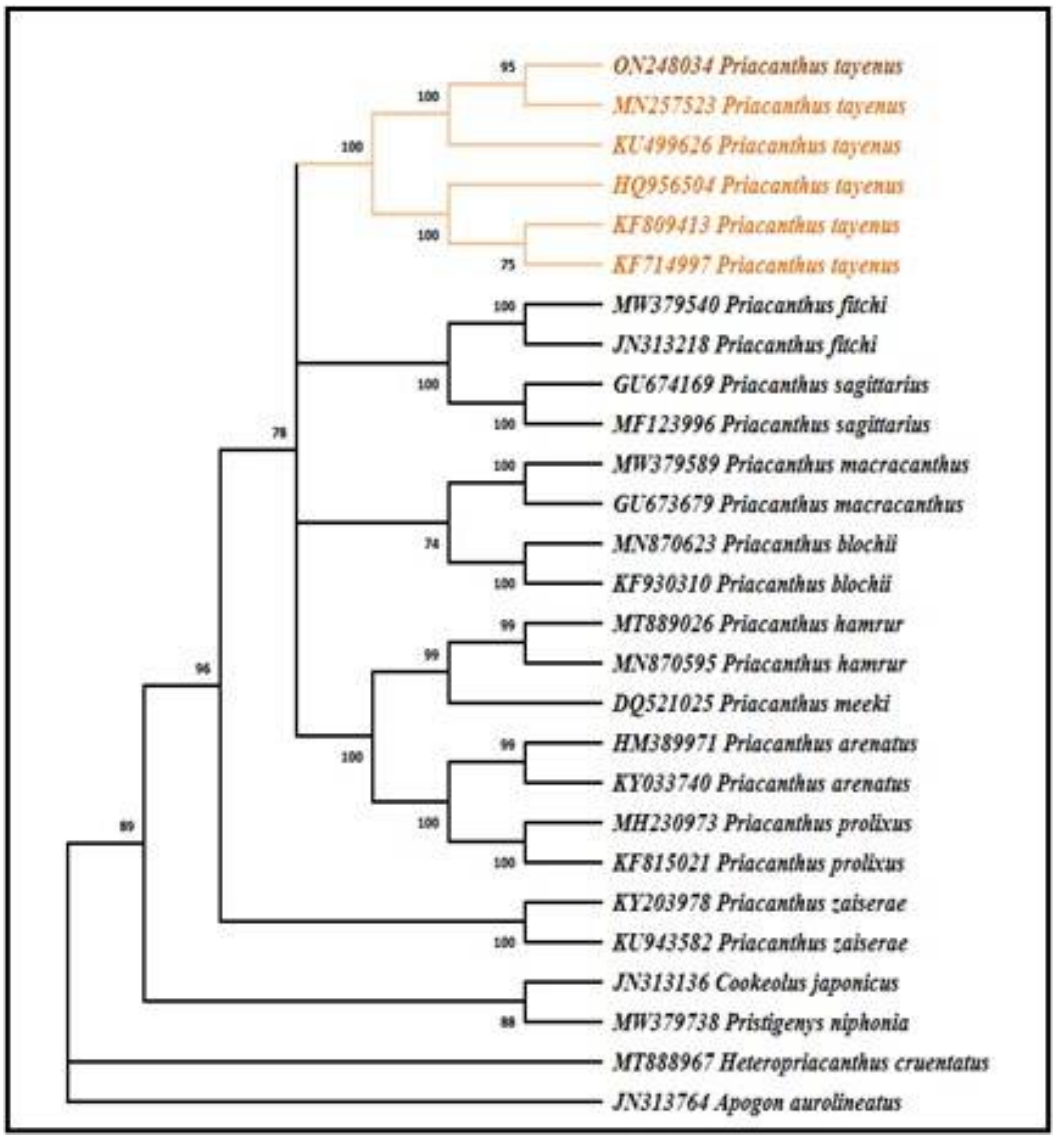

Figure 5. Neighbor-joining phylogenetic tree of the family Priacanthidae [36]. Evolutionary history of taxa under study was assumed to be represented by bootstrap consensus tree obtained from 1000 replicates [37]. Branches resulting in partitions less than 50% bootstrap replicates were collapsed. Evolutionary distances were measured in base substitutions per site using Kimura 2-parameter method [34]. Accession No.: ON248034 was generated in the present study.

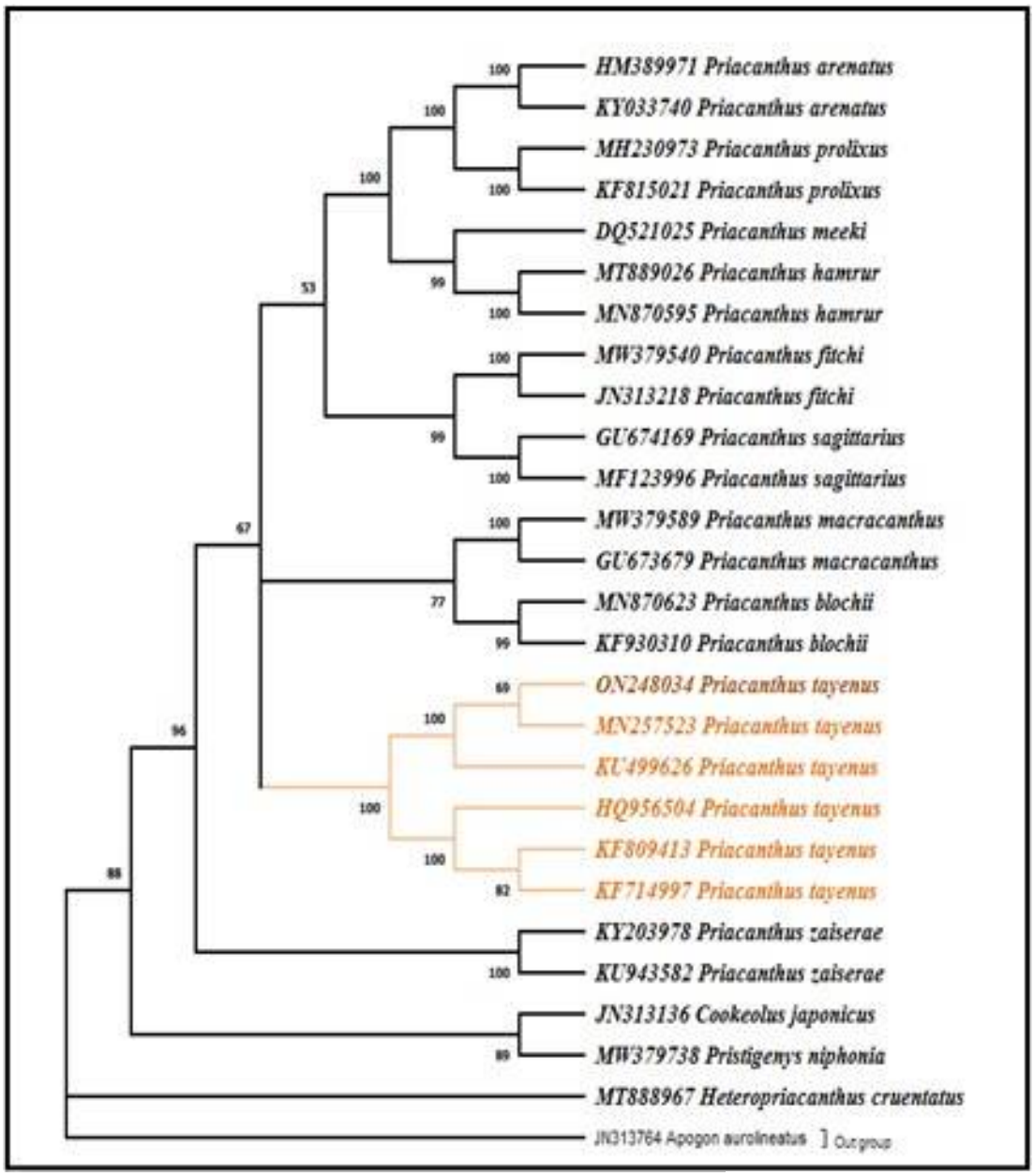

Figure 6. Maximum likelihood phylogenetic tree of Priacanthidae using Hasegawa-Kishino-Yano model [38]. The percentage of trees in which the associated taxa clustered together was shown next to the branches. Branches that belong to partitions have been replicated in fewer than 50% of bootstrap replicates were collapsed. Tree with highest log likelihood (-4318.01) was shown. The discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.0424)). Rate variation model allowed for some sites to be evolutionarily invariable ([+I], 57.00% sites). Accession No.: ON248034 was generated in the study. Accession No.: ON248034 was generated in the present study.

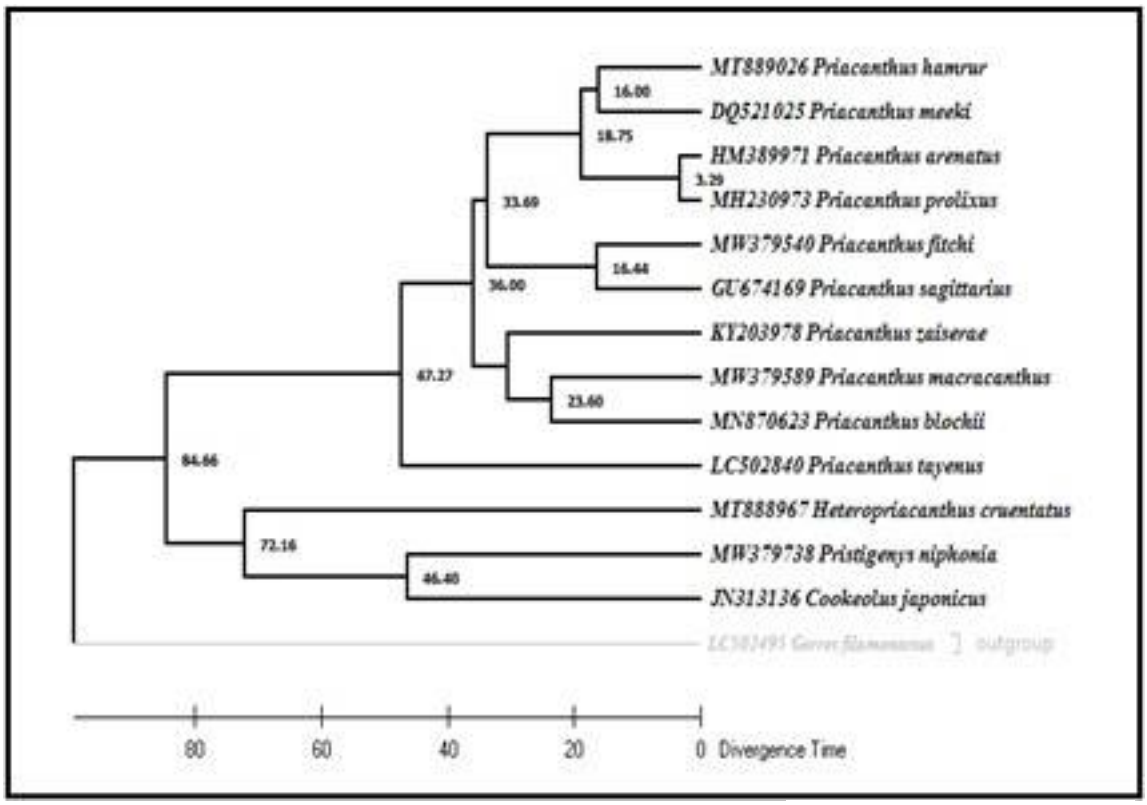

Figure 7. Time-calibrated phylogenetic tree of family *Priacanthidae*. Time tree inferred by applying RelTime method [39],[40], to user-supplied phylogenetic tree whose branch lengths were calculated using the Maximum likelihood (ML) method and Hasegawa-Kishino-Yano substitution model [38]. The estimated log likelihood value of the tree is -3771.39. The discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+*G*, parameter = 0.6784)]. The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 31.49% sites). The analysis involved 14 nucleotide sequences. Numbers on the branches represent the time (in Mya) of different nodes. Accession No.: ON248034 was generated in the present study.

DISCUSSION

In previous classifications, Priacanthids were categorized as Perciformes according to "Commercial Sea Fishes of India" [9],[24]. However, they have recently been reclassified as Acanthuriformes according to "Eschmeyer's Catalogue of Fishes" [8]. This study, successfully sequenced and amplified the barcode sequence COI gene of *Priacanthus tayenus*, a species belonging to the Priacanthidae family. Additionally, we did not detect any base deletions or insertions, indicating that all the amplified sequences originated from functional mtCOI sequences [26]. Analysis of the base composition of the COI gene sequence in *Priacanthus tayenus* from Gopalpur-on-sea revealed an overall higher AT content compared to GC content. The codon usage bias analysis of all the studied *Priacanthus tayenus* species showed that there was a clear anti-T bias in the first codon position, and T bias and anti-G bias in the second and third codon positions respectively which has been observed in other studies as well [41][42]. The investigation of COI genes in this study also revealed a higher frequency of transitions compared to transversions, indicating varying levels of base-mutation selection pressure during species evolution, which may contribute to base usage bias. Haplotype analysis showed the presence of eight haplotypes in *Priacanthus tayenus*, among which two were dominant. The NJ and ML analyses of the Priacanthidae family generated phylogenetic trees with similar branch lengths and robust bootstrap values. These trees depicted the genetic divergence among the studied species and supported the monophyly of the Priacanthidae family, as evidenced by the distinct clustering of Priacanthidae species from the outgroup. Based on molecular dating phylogeny, it was determined that the family Priacanthidae originated approximately 84.66 million years ago during the late Cretaceous period. At the same time, the genus *Priacanthus* separated from other genera within the family. Subsequently, around 47.27 million years ago during the early Eocene sub-epoch, the species of the genus *Priacanthus* started to diverge, with *Priacanthus tayenus* being the first species to diverge from their common ancestor. Tajima's D values of the species *Priacanthus tayenus* were weak positive,

which was indicative of neutral evolution [31], [43]. These inferences were also supported by the Fu and Li's D and F test values [32]. However, these values were not reached the level of significance ($P < 0.05$).

CONCLUSIONS

Species within the Priacanthidae family have a significant role in global fisheries [44]. Our investigation successfully demonstrated the utility of COI divergences in distinguishing various Priacanthid fishes. The results of this study highlight the effectiveness of the mtDNA barcode technique in species classification and understanding their evolutionary origins. While our analysis aimed to emphasize the most important findings from COI-based studies, it is worth noting that COI sequences are highly reliable in distinguishing closely related species, with shorter distances observed within species compared to between species. By analyzing the mtCOI gene, we have enhanced our understanding of the evolutionary history of the Priacanthidae family. Our base composition analysis revealed a higher AT content compared to GC content in the species, along with a clear anti-T bias at the first codon position. Additionally, our evaluation of COI genes indicated a higher frequency of transitions than transversions. The NJ and ML trees of the Priacanthidae family demonstrated strong monophyletic clustering of species, affirming the reliability and efficiency of DNA barcoding in species identification and tracking their evolutionary history. Molecular dating phylogeny indicated that the family Priacanthidae originated 84.66 million years ago in the late Cretaceous period. Genus *Priacanthus* separated from other genera at that time. During the early Eocene sub-epoch, approximately 47.27 million years ago, species within the genus *Priacanthus* began to diverge, with *Priacanthus tayenus* being the first species to diverge from their common ancestor.

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ETHICS APPROVAL

The authors confirmed that they adhered to all relevant international, national, and institutional guidelines for sampling, handling, and experimental use of fishes in this study. They obtained the necessary approvals required for conducting the research.

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