

# Taxonomic validation of Areolate grouper, *Epinephelus areolatus* (Perciformes: Serranidae) along the Nizampatnam coast, India

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## ABSTRACT

Groupers (Perciformes: Serranidae: Epinephelinae) are a significant group of marine and estuarine fishes having ecological and commercial importance in tropical and subtropical waters. The genus *Epinephelus* is identified based on the color configuration and morphological characteristics. The homogeneity in the morphological characteristics has created confusion in the species identification of *Epinephelus*. *Epinephelus areolatus* with its delicious taste has high economic value; however, its population showed a remarkable decline in recent times due to overfishing. Due to morphological resemblance, it has often been confused with *Epinephelus chlorostigma* and *Epinephelus bleekeri*, which leads to misidentification. Hence, correct identification of species is needed for the proper management of fish resources. This study aims to identify and validate *E. areolatus* by using morpho-meristic characteristics, including the number and pattern of pyloric caeca, vertebrae count through radiograph imaging, scale and otolith morphology, and DNA barcoding. The morphological and meristic characteristics of *Epinephelus* species observed in Nizampatnam coast are strikingly comparable to those reported earlier as *E. areolatus*. Comparative molecular analysis was also carried along with other morphologically similar species, viz. *E. chlorostigma* and *E. bleekeri*, using mitochondrial cytochrome oxidase I gene sequences. The mean interspecific genetic distance revealed high similarity with *E. bleekeri* ( $0.109 \pm 0.012$ ) and low similarity with *E. chlorostigma* ( $0.079 \pm 0.009$ ). The neighbor-joining tree has shown distinct clades for three species with high bootstrap values. The partial DNA sequence of the mitochondrial cytochrome oxidase I was established for the first time for *E. areolatus* from the Nizampatnam coast in this study.

## 1. INTRODUCTION

Groupers are marine and estuarine fishes representing about 163 species in 16 genera distributed worldwide in the tropical and sub-tropical waters. The sub-family Epinephelinae comprises the large commercially important groupers. Around 54 grouper species of the sub-family Epinephelinae and 38 species of the genus *Epinephelus* have so far been recorded from the Indian waters [1]. Groupers inhabit a diverse range of habitats, mainly coral reefs, rocky areas, seagrass beds, and estuaries [2]. It also exhibits a wide variety of reproductive and growth strategies [3,4]. It was estimated that grouper fish landings in India have been increasing steadily over the years and the output in 2018 was 51,433 tons [5]. The identification

characteristics to distinguish the grouper species are usually based on the color pattern and morphological features [6]. However, taxonomic confusion occurred in identifying individual species of the genus *Epinephelus* due to subtle variations in morphological characteristics [7] and specialties, as well as with a vast number of species [8]. The misidentification of species due to species complexity and synonymies, in addition to the doubts on some generic placements, makes fisheries management and conservation difficult [9,10]. Due to overfishing, some species of groupers are under the threat of extinction. Among 163 species reported worldwide, the International Union for Conservation of Nature (IUCN) Red List of threatened species has categorized 20 species on the verge of extinction and 22 species are considered as nearly threatened [11]. Thus, accurate species identification is important for the analysis of population dynamics, biodiversity assessment, and fishery management.

*Epinephelus* species are frequently landed in commercial marine catches off Nizampatnam, east coast of India. In spite of their

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abundance and commercial value, the data on the population characteristics and their conservation status are meager. Furthermore, systematic scientific study has not been conducted so far, including the taxonomic identification of *Epinephelus* species occurring at Nizampatnam coast. Earlier studies have recorded the prevalence of *Epinephelus chlorostigma* and *Epinephelus bleekeri* along the Visakhapatnam coast of India [12]. However, there were reports on the misidentification of *Epinephelus areolatus* as *E. chlorostigma* or *E. bleekeri* because they look similar with overlapping distributions [6,13,14]. In addition to the color pattern and morphological characteristics, the species-specific characteristics, like pyloric caeca [6,15], scales, and otoliths [16–18], and vertebrae [19–21], are proved to be more useful for the identification of species. Furthermore, the advanced molecular technique, such as DNA barcoding, helps in resolving the ambiguity at species-level identification [22–27]. Hence, this study has been carried to identify and validate *E. areolatus* along Nizampatnam coast, Andhra Pradesh, India, using morpho-meristic characteristics, including pyloric caeca, vertebrae, scales, otoliths, and DNA barcoding. The genetic divergence among the three species of *Epinephelus* was evaluated using K2P model and the phylogenetic relationships were analyzed based on *COI* sequence data.

## 2. MATERIALS AND METHODS

### 2.1. Sample Collection

Thirty-eight specimens of *E. areolatus* ( $n = 38$ ) were collected from the Nizampatnam fish landing center ( $15^{\circ} 52' 58''$  N and  $80^{\circ} 38' 18''$  E), Guntur district, Andhra Pradesh, India (Fig. 1), during December 2016 to September 2018, except during the fishing ban period (from April to June). Specimens were observed in fresh condition for color pattern and were

photographed (Fig. 2). About 2 g of muscle tissue was collected and preserved in 95% ethanol for molecular analysis and the rest of the specimens were preserved in 5% formalin for morphological study.

### 2.2. Morphological Analysis

Morpho-meristic data of the species were recorded following the standard methods [6 and 28]. Measurements for morphometric characteristics were taken with a digital vernier caliper to the nearest 1 mm. Vertebrae were counted by radiograph imaging through a digital X-ray. Pyloric caeca were counted at their free ends and fresh coloration was noted. Scales were obtained from eight regions of the fish body (Fig. 3) according to the suggested methods [29,30]. Fine forceps were used to remove scales from the left side of the fish and then cleared in a solution of 1% potassium hydroxide. Sagittal otoliths were extracted by using “Up through the gills method” [31]. The right and left otoliths were separately considered. Digital images and dimensions of scales and otoliths were taken using a Nikon stereomicroscope (SMZ18) with an integrated DS-L3 DS camera. Voucher specimens were documented in the museum of the Department of Zoology and Aquaculture, Acharya Nagarjuna University, Guntur, India (Voucher No. DNAB-5/MUS/NOV/2018).

### 2.3. DNA Extraction and Polymerase Chain Reaction (PCR) Amplification

Genomic DNA was extracted from the muscle tissue using DNeasy Blood and Tissue kit (www.qiagen.com). A partial mitochondrial cytochrome oxidase I (*COI*) gene was amplified by PCR by using a set of published universal primers [27], i.e., FishF1 (5'-TCA ACC AAC CAC AAA GAC ATT GGC

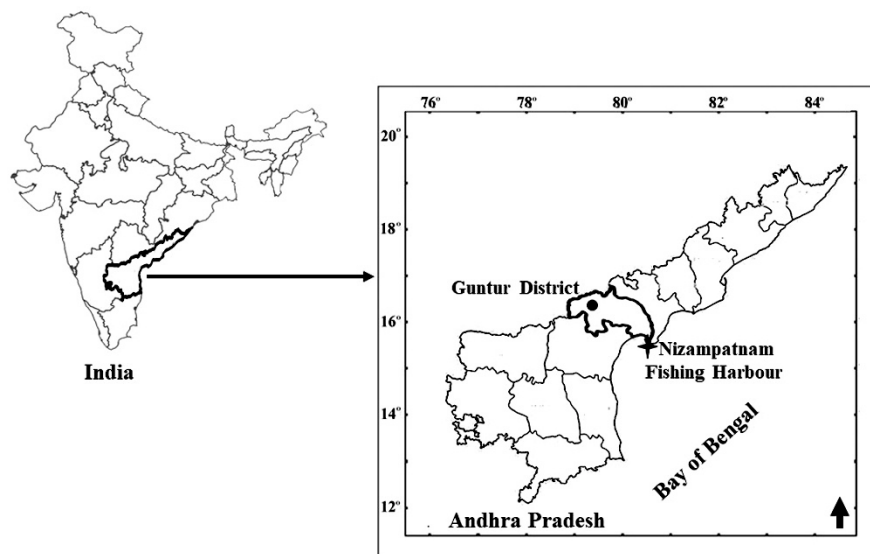


Figure 1: Sampling station at Nizampatnam fishing harbor.



Figure 2: *Epinephelus areolatus*.

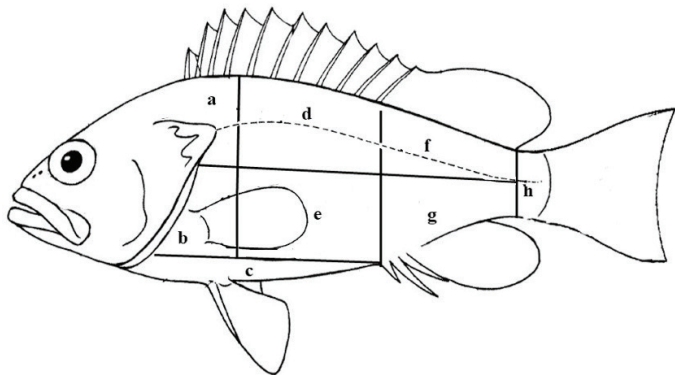


Figure 3: Image showing eight regions (a–h) from where scales were collected.

AC-3') and FishR1 (5'-TAG ACT TCT GGG TGG CCA AAG AAT CA-3') in Eppendorf Master Cycler (Nexus gradient, 230 V) (Eppendorf, Hamburg, Germany). A total of 20 µl reaction mixture was prepared with 5 µl of PCR Smart Master Mix 2X (www.thermofisher.com), 1 µl of forward primer (10 nm/µl), 1 µl of reverse primer (10 nm/µl), approximately 1 µl of DNA template (0.5–1 µg), and 12 µl dH<sub>2</sub>O. The thermal profile of the PCR reaction was as follows: an initial denaturation at 94°C for 3 minutes, denaturation at 94°C for 45 seconds, annealing temperature of 50°C for 45 seconds, and elongation temperature of 72°C for 1 minute for 30 cycles, and concluded with a final

elongation step at 72°C for 7 minutes, followed by a hold at 4°C. The amplicons were verified on 1.5% agarose gel stained with ethidium bromide (EtBr) (10 mg/ml). Amplicons were sent to MacroGen Inc., Seoul, South Korea (www.macrogen.com), for purification and single direction sequencing following the BigDye terminator method [32].

2.4. Sequence Analysis

The *COI* partial gene sequence obtained for *E. areolatus* was subjected to nucleotide Basic Local Alignment Search Tool (BLAST) search to know the sequence identity and was further verified for the presence of internal stop codons by using the translate tool in ExPASy ProtParam tool and submitted to NCBI-GenBank to obtain an accession number. The sequence data of *E. areolatus* were compared with the morphologically similar *E. chlorostigma* and *E. bleekeri* by complementing with sequences acquired from the public domain database, NCBI-GenBank (Table 1). The submitted and retrieved sequences were subjected to alignment using ClustalW analysis tool [33] for comparison. Inter- and intra-species genetic divergences in various hierarchical levels were analyzed using Kimura 2 Parameter method [34], with 1,000 bootstrap replicates. The pairwise deletion option was selected to treat the gaps. The neighbor-joining (NJ) tree was generated among the sequences with a suitable out group (*Cephalopholis sonnerati*, JQ431575) by K2P method [35], using 5,000 pseudo replicates [36].

Table 1: List of species used in phylogenetic analysis with NCBI-GenBank accession numbers.

S.No.	Species	Accession number	No. of sequences
1	<i>E. areolatus</i>	MK184954, KJ607969, KM226238, KM226237, KM226236, KM226235, JX674969, JX674968, JX674967	9
2	<i>E. chlorostigma</i>	EU392204, EU392203, EU392202, EF609515, EF609514, KT835686, KT835685, KM226245, KM226244, JX674981, JX674980, JX674979, KF434771	13
3	<i>E. bleekeri</i>	MF978166, KM226243, KM226242, KM226241, KM226240, KM226239, JX674973, JX674972, JX674971, JX674970	10

### 3. RESULTS

The following characteristics were noted: body was moderately elongated; skin was thick and leathery; inter orbital space convex; caniniform teeth in both jaws; preopercle was finely serrated with small spines at its lower angle; scales were ctenoid; pectoral fins were longer than the pelvic and anal fins; slightly convex caudal fin in juveniles, slightly emarginated or truncated in adults; depth of the body was less than head length (HL) and contained 2.5–2.8 times standard length (SL) (Table 2); HL contained 2.8–3.0 times in SL; HL was almost similar to the prepelvic distance; pectoral-fin length contained 1.5–1.6 times in HL; pelvic-fin length contained 2.1–2.4 times in HL. The body, head, and fins had numerous yellowish brown or yellow spots; the largest spots were about the size of the pupil; the dorsal fin

was gray with small brown spots; soft dorsal distal edge with a white margin; pectoral fin was paler with dark brown spots; pelvic fin was light with dark brown spots; caudal gray with dark brown spots, rear edges with a white margin; anal base was light gray with no spots.

The pyloric caecum was present as a whorl in the junction between the stomach and duodenum, mostly on the ventral side, with the flesh colored 12–17 caeca. Vertebrae are 24 (abdominal-10 and caudal-14) (Fig. 4); first two abdominal vertebrae with supraneural spines are directed upward. Scales vary in size in different regions of the body (Fig. 3); the order of scale size is e>h>d>c>g>f>b>a (Table 3 and Fig. 5); 4–8 radii; longer radii are present in scales from regions f to h. Otoliths are slightly

**Table 2:** Morphometric and meristic characteristics of *E. areolatus* ( $n = 38$ ).

Reference		Present study	Sachithanandam <i>et al.</i> [26]	Rao <i>et al.</i> [37]	Heemstra and Randall [6]
<b>Morphometric (mm)</b>					
Total length		140–310	330	–	130–350
SL		116–260	300	–	140–310
Body depth		46–92	103		50–94
Body depth (times in SL)		2.51–2.8	2.9	2.52–2.65	2.8–3.3
Head length		46–107	–	–	–
Eye diameter		8–22	–	–	–
Preorbital		12–31	–	–	–
Predorsal distance		44–106	–	–	–
Prepectoral distance		40–94	–	–	–
Prepelvic distance		47–110	–	–	–
Preanal distance		81–189	–	–	–
Dorsal base		61–142	–	–	–
Anal base		14–34	–	–	–
Dorsal spine height		13–30	–	–	–
Soft dorsal height		7–23	–	–	–
Pectoral length		30–65	–	–	–
Pelvic length		22–50	–	–	–
Anal length		26–66	–	–	–
Anal spine height		8–25	–	–	–
Maxillary width		16–52	–	–	–
<b>Meristic</b>					
Gill rakers	Upper arm	8–10	–	–	8–10
	Lower arm	14–16	–	–	14–16
Dorsal fin	Spines	XI	XI	XI	XI
	Rays	14–16	17	15–16	15–17
Anal fin	Spines	III	III	III	III
	Rays	8	11	8	8
Pectoral fin rays		17–19	15	17–18	17–19
Lateral line scales		49–53	49–51	49–51	49–53
Lateral-scale series		96–116	–	–	97–116
Pyloric caeca count		12–17	–	–	11–17
Vertebrae		24	–	–	–



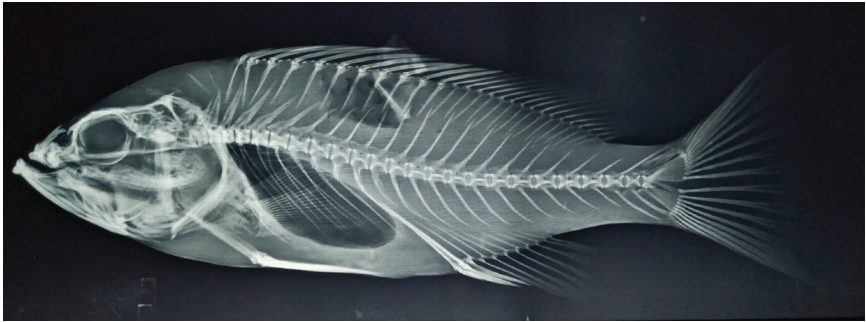


Figure 4: Radiograph of *E. areolatus* showing vertebrae.

Table 3: Scale and otolith dimensions of *E. areolatus*.

Type	Region <sup>a</sup>	Length (μm)	Width (μm)
Scale	A	2,160.67	1,274.98
	B	3,318.03	1,534.72
	C	4,176.65	2,407.54
	D	4,344.00	2,538.46
	E	5,252.14	3,078.66
	F	3,951.08	2,211.16
	G	4,031.12	2,269.34
	H	5,092.30	2,737.98
Otolith	Right	9,194.97	4,417.21
	Left	9,396.32	4,580.81

<sup>a</sup>Regions on the body (a–h) are shown in Figure 3.

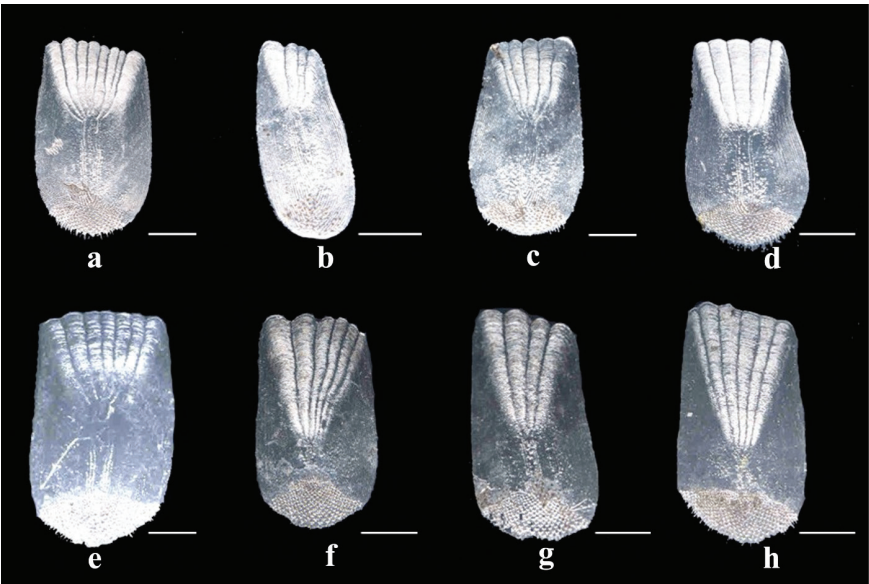
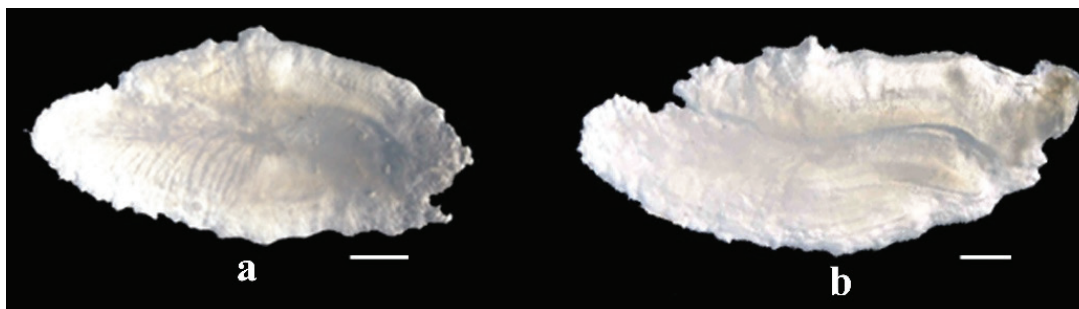


Figure 5: Scales from different body regions of *E. areolatus* (a–h) (Scale: a: 500 μm, b–h: 1,000 μm).



**Figure 6:** Otolith images of *E. areolatus* (a-Right; b-Left) (Scale: 1,000  $\mu$ m).

**Table 4:** Intra-species divergence of *Epinephelus* species using *COI* gene based on K2P model.

Species	Mean distance $\pm$ SE
<i>E. areolatus</i>	0.012 $\pm$ 0.003
<i>E. chlorostigma</i>	0.032 $\pm$ 0.004
<i>E. bleekeri</i>	0.001 $\pm$ 0.001

**Table 5:** Pairwise inter-genetic distances of *Epinephelus* species using *COI* gene based on K2P model.

Species 1	Species 2	Mean distance $\pm$ SE
<i>E. areolatus</i>	<i>E. bleekeri</i>	0.109 $\pm$ 0.012
<i>E. areolatus</i>	<i>E. chlorostigma</i>	0.079 $\pm$ 0.009
<i>E. bleekeri</i>	<i>E. chlorostigma</i>	0.104 $\pm$ 0.012

oval; right and left otoliths varied in shape and size (Fig. 6 and Table 3). The morphometric and meristic characteristics of the present specimen along with those of earlier works [6,26,37] on this species are shown in Table 2.

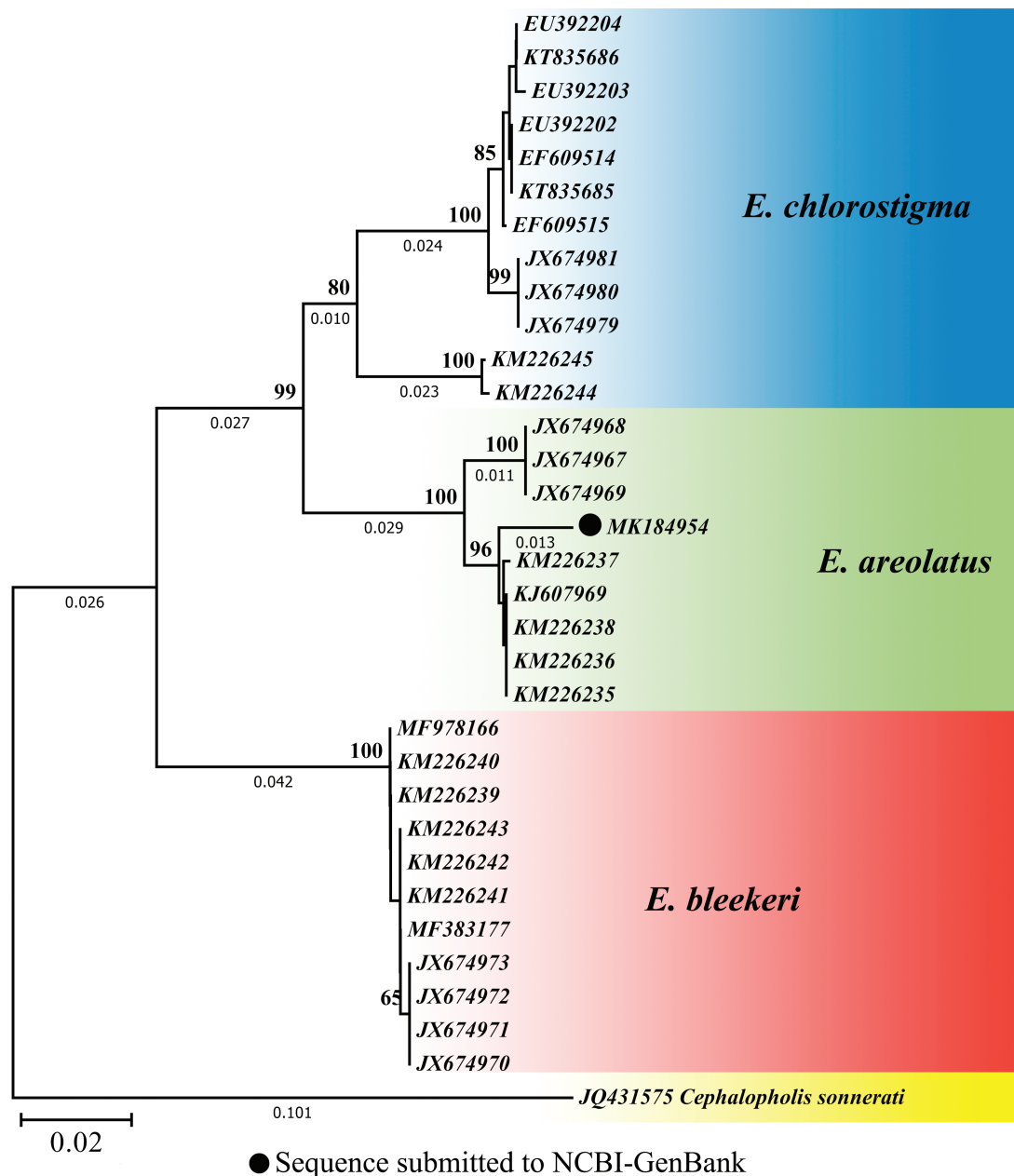
An overall 677 base pair (bp) sequence of mitochondrial *COI* for *E. areolatus* was obtained after alignment. The sequence was deposited in NCBI-GenBank with the accession number MK184954. However, the sequences for *E. chlorostigma* and *E. bleekeri* were retrieved from the NCBI-GenBank. The multiple sequence alignment led to 649 bp per taxon after eliminating the primer sequence. Sequences were without any ambiguities. No indels (insertions and deletions) and internal stop codons were noted when they were verified in the translate tool using ExPASy ProtParam tool. The pairwise inter- and intra-specific genetic distances among the three species of *Epinephelus* based on K2P method are presented in Tables 4 and 5. The NJ tree revealed distinct clades for three species with high bootstrap values (Fig. 7).

#### 4. DISCUSSION

Identification of *Epinephelus* species appears to be challenging because of their closely related morphological characteristics [38–40]. The species *E. areolatus* has usually been confused with *E. chlorostigma* and *E. bleekeri*, and these three species are considered as a single species in the study area. He *et al.* [13], Heemstra and Randall [6], and Sanaye [14] also reported that these three species are often misidentified as single species

due to homogeneity in their morphological characteristics. The morpho-meristic analysis showed some valid indices for species identification. *E. areolatus* can be distinguished by the rounded margin of the anal fin vs. angular or pointed in *E. chlorostigma* and *E. bleekeri*; 14–16 dorsal fin rays vs. 16–18 in *E. chlorostigma* and *E. bleekeri* [6]; and 12–17 pyloric caeca vs. 26–52 in *E. chlorostigma* [6] and 26–36 in *E. bleekeri* [41]. Moreover, the color configuration of these three species has also been considered for species identification. *E. areolatus* can be identified by its whitish to gray color with numerous yellowish brown or yellow spots, and caudal fin with a white margin. *E. bleekeri* has a grayish brown color with numerous orange to orangish red spots, caudal fin with two different colors, upper side yellow, and lower side dusky, whereas *E. chlorostigma* has a pale green gray color body with small circular yellow or yellowish brown to dark brown spots, caudal fin with a white rear margin [6].

Identification of groupers based on morphological characteristics may be inconclusive [7,22,24,26,41] and even a single systematic external morphological characteristic has not yet been found to differentiate these commercially important groupers [6]. Hence, in this study, to overcome the taxonomical confusion raised by the overlapping characteristics of closely allied *Epinephelus* species, an attempt has been made by using DNA barcoding as a promising molecular approach to discriminate the species. The barcode established for *E. areolatus* (MK184954) in this study is the first sequence submitted to the NCBI-GenBank from Nizampatnam coastal waters. The gene sequence for *E. areolatus* is lacking Indels (insertions and deletions) signifying that the sequence does not comprise nuclear mitochondrial DNA (NUMT). The sequence of *E. areolatus* matches 98.79% with the sequences of *E. areolatus* (KU499782 and KU499599) accessible in the NCBI-GenBank. The mean intraspecific genetic divergence analysis revealed higher values in *E. chlorostigma* (0.032  $\pm$  0.004), followed by *E. areolatus* (0.012  $\pm$  0.003) and *E. bleekeri* (0.001  $\pm$  0.001) (Table 4). The mean interspecific genetic distance showed high similarity with *E. bleekeri* (0.109  $\pm$  0.012) and low similarity with *E. chlorostigma* (0.079  $\pm$  0.009) (Table 5). The phylogenetic analysis from NJ tree clearly shows the genetic divergence of the three species with high bootstrap values (Fig. 7). Hence, it is evident from this study that *E. areolatus*, *E. bleekeri*, and *E. chlorostigma* are distinctly valid species and are clearly separable from other congeners.



**Figure 7:** Neighbor-joining tree of *COI* gene sequences for *Epinephelus* species.

## 5. CONCLUSION

Perfect taxonomic identification of species is crucial for proper management of any fishery resource. In this study, the morphological data of *E. areolatus* were cataloged to differentiate with other congeneric species, more precisely with reference to the shape of the anal fin, dorsal fin rays, and pyloric caeca. The *COI* gene sequence and phylogenetic analysis revealed genetic divergence and relatedness of the three species. Hence, both morphological data and gene sequencing are crucial for the validation of species and will continue to improve our understanding in the identification of grouper fishes. The occurrence and genetic diversity of *Epinephelus* in Indian waters need further investigation for

more insights in view of their nutritional and commercial importance.

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## CONFLICT OF INTEREST

Authors declared that there is no conflicts of interest.



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