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DNA barcoding, phylogenetic study of *Epinephelus spp.* from Andaman coastal region, India

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Present study was carried out for identification of the *Epinephelus spp*. present in Andaman coastal region by bar coding technique and also by other conventional methods to understand the significance of its distribution. DNA sequences of cytochrome C oxidase I gene (COI) of *Epinephelus* spp. were employed to test efficiency of species identification. Study was designed to evaluate mean genetic distances using the Kimura two Parameters (K2P) between the studied *Epinephelus spp* fishes of Andaman coastal region and the same species from world over were analysed. *Epinephelus* spp. shows genetic divergences among *Epinephelus longispinis*, *Epinephelus ongus* and *Epinephelus areolatus,* respectively, 0.0004, 0.0183 and 0.0437. DNA barcoding may be useful in bringing out the evolutionary relatedness between different species. Mitochondrial COI data has an advantage over an individual dataset because of its higher resolving power.

[Keywords: Andaman Sea, DNA barcode, *Epinephelus*, Molecular Taxonomy, Serranidae]

Introduction

DNA barcode is an efficient method for species-level identification using an array of species specific molecular tags derived from 59 region of the mitochondrial Cytochrome c Oxidase I (COI) gene[1]. Phylogenetic systems, in combination with conservation genetics, provide a critical frame work for understanding diversity[2] and predict vulnerability to exploitation of tropical reef fishes[3]. Efficiency of this method hinges on the degree of genetic divergence among species and intra species-level identifications[4]. Hence, DNA bar coding is to identify and to boost the number of unfamiliar taxa in biological conservation and biodiversity surveys, based on sequence diversity[5-6]. Fish Barcode of Life Initiative (FISH-BOL) as a campaign of the International Barcode of Life Project (iBOL) is to build up a standardized database of reference sequences for all fishes and the target is DNA segment of 652 basepairs[1].

Subfamily Epinephelinae of Family Serranidae commonly known as groupers, rock cods, hinds, and sea basses comprises about 159 species of marine fishes in 15 genera[7], *Epinephelus spp*. has often appeared to be closely related. In fact, the separation of the two genera is primarily based on different head structure, caudal fin shape, number of anal fin rays and on the presence of a greatly increased number of gill raker in the species[8].

Separate catch statistics are not reported for most species in Andaman and Nicobar Islands and landings are often summarized as serranidae or groupers[9]. It has been estimated that 90% of the world's harvest of marine food is derived from artisanal fisheries, and groupers are a major component of the artisanal fisheries resource[10]. This lack of species-specific catch data is due to the difficulty of identifying many of the species in the field[11].

The DNA barcoding technique could overcome the difficulty faced in morphological identification and reduces the misidentification of commercially important fishes in all stages (larval to adult). Present study is an attempt made to evaluate existing groupers taxonomy in Andaman Sea. As far as our information is concerned this is the first ever study in these species in this water.

Materials and Methods

Andaman and Nicobar Islands (92° to 94° East and 6° to 14° North), is an archipelago with 572 Islands, stretching over 700 Kilometres (km) from North to South, in the Bay of Bengal. Andaman Islands are

volcanic rock type of land mass surrounded with different sort endemic flora and fauna[12]. These Islands coral reef environment exhibits around 25 species of serranidae family are commonly available in this costal region, in which 14 species are commercially important for exports to various countries[13]. Fish species belonging to family serranidae were collected from local major fish landing sites in Port Blair such as Wandoor and Guptapara (Fig. 1).

The Serranidae fish of different species were collected, phenotypic characterization of fishes were analysed using FAO sheets; morphometric parameters such as body shape, colour and the rays of the dorsal fins were included to find the variations if any[11]. The length and weight were measured. Simultaneously tissue specimens were also collected and stored with 90% ethanol at -20°C for further analysis.

DNA extraction and PCR reaction

Total DNA was extracted from 0.25 g of tissue by using lysis buffer and followed by standard proteinase-K/phenol-chloroform-isoamyl alcohol-ethanol precipitation method[14]. Concentration of DNA was estimated using a 260/280 nm UV spectrophotometer method. Subsequently the DNA was diluted to final concentration of 100ng/µl for further use. The 650-655 bp section of the mitochondrial (mt) DNA genome from the COI gene was amplified using already published universal primer[4] synthesised by Sigma Aldrich Chemicals India Pvt. Ltd. Forward primer: FishF1-5'TCAACCAACCACAAAGACATTGGCAC3' and Reverse primer: FishR1-5'TAGACTTCTGGGTGGC CAAAGAATCA3'. Polymerase chain reaction (PCR) was carried out in 50 µl consisting of approximately 100 ng/µl of DNA,10X PCR buffer with $MgCl_2$ 1.5 mM, 2.5 mM dNTPs, 3U *Taq* polymerase (Bangalore Genei) and Forward and Reveres Primers at 10 µM/µl concentration. PCR was carried out in Applied Bio Systems AB-2720 Thermal Cycler. Initial denaturation was performed at 94°C for 5min, followed by denaturation at 94°C at 30 sec., annealing at 56°C for 60 sec., and extension at 72°C for 60 sec., for 40 cycles followed by final extension at 72°C for 7 min. PCR products were resolved in 1% agarose containing 0.5 µg/mL of ethidium bromide and viewed under UV Transilluminator and documented.

Nucleotide sequencing was performed using the Sanger method[15] modified by Chen and Seebug[16]. Sequencing was performed using BigDye Terminator

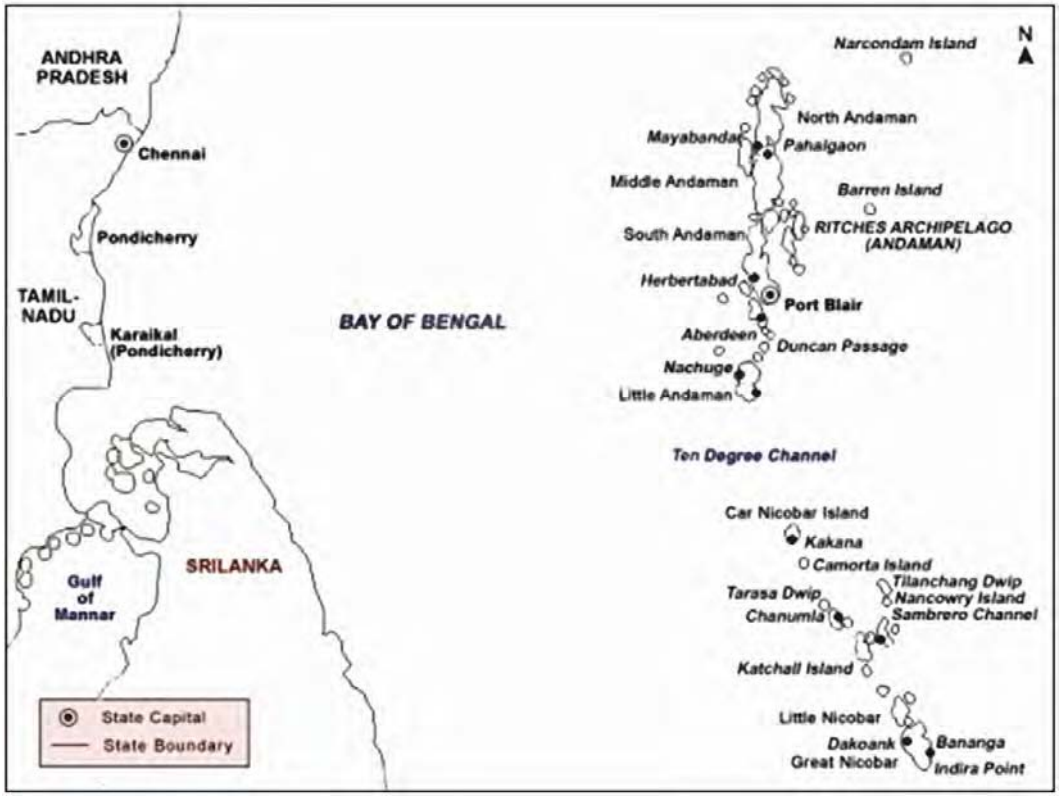

Fig.1—Study Area

Cycle Sequencing kit, following manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The sequencing was done both in the forward and reverse directions.

DNA sequences were analysed both forward and reverse for every individual fish were assembled using the SeqMan II version 5.03 (DNASTAR). Sequence analysis was done along with reference sequences of various species belong to the family Serranidae retrieved from NCBI GenBank. Nucleic acid sequences were the multiple and pairwise alignment done using CLUSTALW tool and phylogenetic molecular evolutionary analyses conducted using MEGA version 4 (Molecular Evolutionary Genetics Analysis). A neighbour-joining (NJ) tree was recovered with MEGA. Bootstrap values for NJ tree were estimated using searches with 1000 pseudo replicates[17-19]. Aligned sequences were also subjected for nucleotide BLAST search to know the identity and further strengthen our results.

Results and Discussion

DNA sequences extracted from the above said three species were submitted to GenBank (PubMed) and their Accession Numbers are shown in Table 1. Out of 650– 655 bp basic taxonomic sequence length, it was able to get 516 bp for *E.longispinis*, 522 bp for *E. ongus* and 318 bp for *E.areolatus*. Consequently phylogenetic analysis was completed for *E.longispinis* and *E.ongus* together but *E.areolatus* as alone.

In the phylogenetic analysis the phenotypically characterized gene sequence of Andaman *E.areolatus* was closely related to conspecific *E.areolatus,* the group wise mean genetic distance also very small with worldwide *E.aerolatus* ($K_2P = 0.0437$) than other species. Further the pairwise genetic distance analysis between *E.areolatus* species of Andaman and South Africa (HQ945841:0.000633) was found comparatively smaller than the other congenetic species from various part of the world as follows Western-Australian (FJ237762:0.04939), Italy (DQ107869:0.05278), South-China Sea (GU324187:0.05278), Australian species (FJ237761:0.04939), the Australian species (FJ237763:0.04939) and South China species (DQ107866: 0.04596).

Table 1—Andaman coastal region Grouper fishes Species their COI Sequence GenBank accession numbers

Sl. No.	Name	Common Name	GenBank ID Accession Number
1.	*Epinephelus longispinis*	Longispinis grouper	JF414596
2.	*Epinephelus areolatus*	Areolatus grouper	JF414593
3.	*Epinephelus ongus*	White-streaked grouper	JF414595

The sequence of *E.ongus* from Andaman Sea was grouped with the reference sequences of same species from worldwide. The pairwise genetic distance were more or less similar with the congenetic species in global such as Philippines (DQ107858:0.01779), Cuba water species (FJ583397:0.01932), Australian species (DQ10785:0.01779), Cuba water species (FJ583399:0.01930) and Cuba water species (FJ583398:0.01779). The group wise mean congenetic distance was ($K_2P = 0.018398$) also fall between the other two species.

In the phylogenetic analysis *E.longispinis* grouped with the reference sequences of same species from various part of the world (GU805000, EF609522 and EF609521), there were no significant pairwise conspecific distance between Andaman *E.longispinis* gene sequence and the gene sequences from different part of the world (0.0, 0.00146 and 0.0). The group wise mean congenetic distance were also exhibit very small ($K_2P = 0.0004$) as compare to all the other species.

The blast search analyses of sequences were also carried out for further strengthening of these sequenced data. The query coverage 100% and maximum indenty were ≥99% recorded. The phenotypical identification of the present studied species of *E.longispinis* showed 99% identity with existed GenBank *E.longispinis* (Access No: GU 805000). Similarly, *E.ongus* matched 99% with identified. Gene Bank species (Access No: FJ 107872). The *E.areolatus* showed 99% similarity with same species sequence in GenBank (Access No: HQ 945841) (Figs 2 & 3).

One of the important resources target by the coastal fisheries in tropical and subtropical areas is Groupers[11]. However, the *Epinephelus* species are often incorrectly identified in the field because of their closely related to the morphological features. The taxonomic variation through the molecular level may provide a better understanding of the species with reference to their commercial exploitation as well as for their sustainable fisheries. In this regard, Andaman and Nicobar waters which are one of the least studied on the species of *Epinephelus spp.* has been considered to understand their molecular taxonomy with reference to three of the major species existed in this waters.

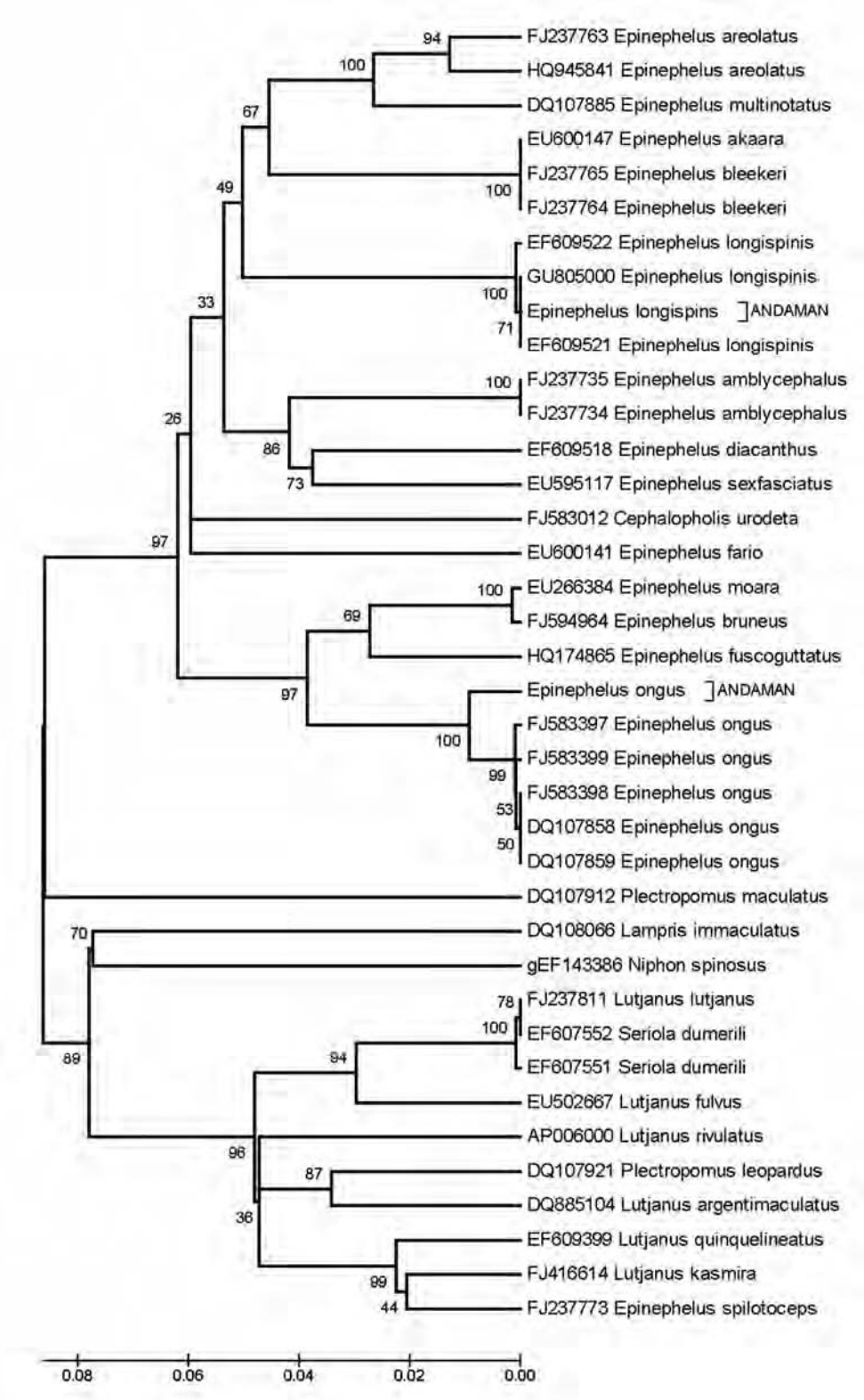

Fig. 2—Neighbour-joining tree based on the mtDNA COI nucleotide sequences of Epinephelinae species analyzed in the present work and of GenBank species. Numbers at nodes are bootstrap values based on 1000 replicates. The scale bar represents an interval of Tamura-Nei genetic distance for *Epinephelus ongus* and *Epinephelus longispinis*

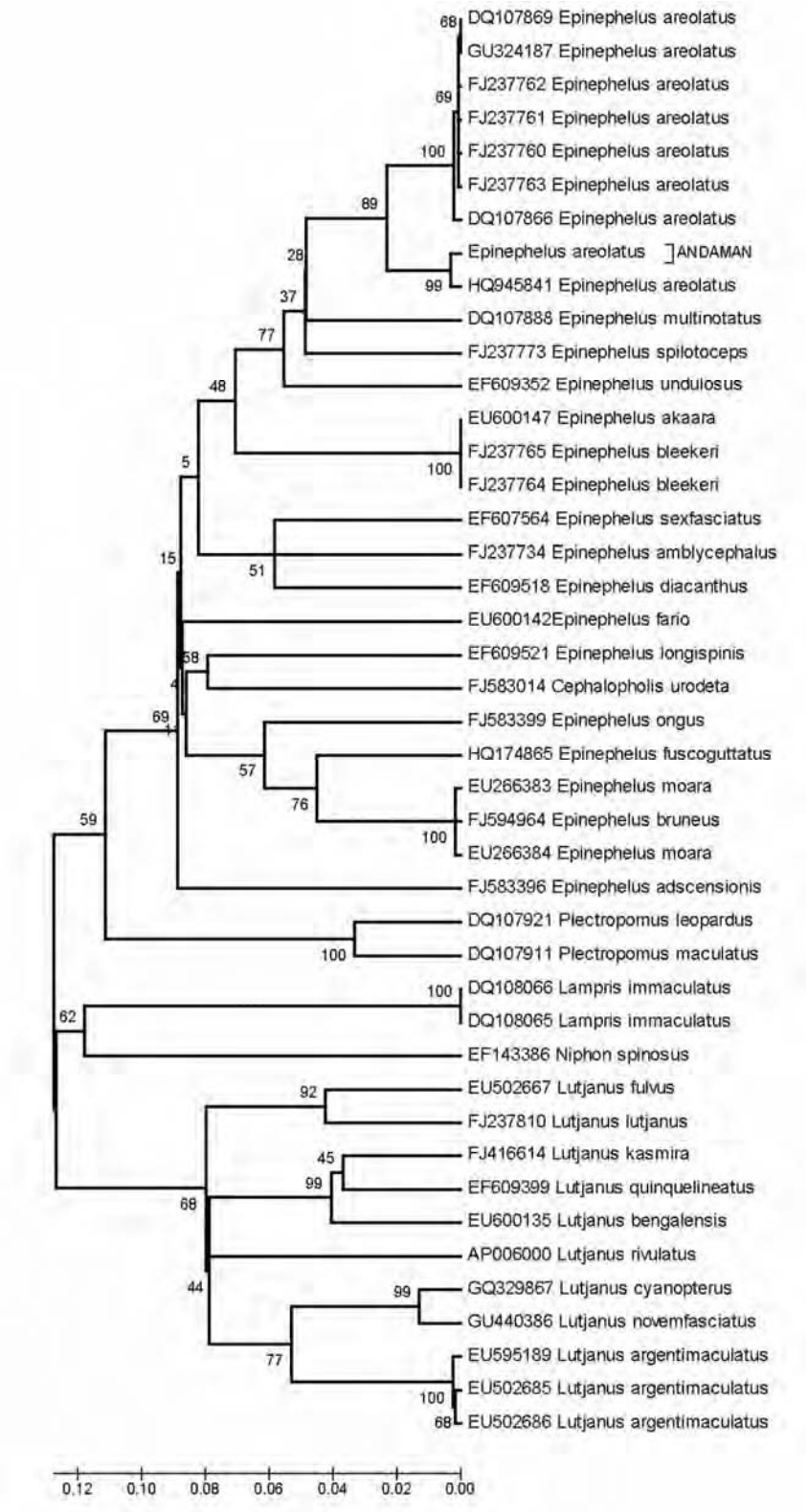

Fig. 3—Neighbour-joining tree based on the mtDNA COI nucleotide sequences of Epinephelinae species analyzed in the present work and of GenBank species. Numbers at nodes are bootstrap values based on 1000 replicates. The scale bar represents an interval of Tamura-Nei genetic distance for *Epinephelus areolatus.*

Identification of *Epinephelus spp.* was carried out based on the morphometric characters and compared with Indo-Pacific region *Epinephelus spp.* which had been reported by Heemstara & Randal, Rajan, Allen et al., and Rao, et al. [11,13, 20-21] (Tables 2, 3 & 4).

The present study of Andaman species showed variation noticeably when compared with Indo-Pacific waters. The species *E.ongus* distinctly differ from their wavy stripes which are absent in this waters. Other changes of the species identified in the waters of Andaman exhibit 15 numbers of dorsal rays. Similarly, *E.longispinis* specifically differs from the major characteristic of a dark brownish pattern on the body and 17 numbers of dorsal rays. The species of *E.areolatus* also differ from lighter tone on the body surface in this water and 17 numbers of dorsal rays. This has been identified and considered as a major morphological identification key of Andaman Nicobar Islands waters.

COI gene is a reliable species tag and DNA barcoding can deliver species-level identifications[22-28]. The sequence data of *E.longispinis*, *E.areolatus* and *E.ongus* were further analysed to understand the species divergence with worldwide sequences of same species suggested that *E.longispinis* of Andaman Sea shows almost similar character of South African as well as Arabian Sea species. This suggested that the stock of this species has not been undergone much of the variation with reference to the South Africa and west coast of Indian environment. Further, the morphometry (Table 2) also supports that the variation between the *E.longispinis* available in Andaman Sea as well as surrounding waters of India and other parts of the world are not varied. So, this supports that the molecular taxonomy results also implied the same by the way of low conspecific distance ($K_2P = 0.0004$).

The *E.ongus* showed distinct divergence from the Philippines, Cuba and Australian water species. Similarly, in the case of morphometry the Andaman *E.ongus* (Table 3) with FAO specified description as well as other parts of the reef fishes existed in the world suggests that this species shows a characteristic variation with reference to the dorsal

Table 2—Morphometric comparison of the *E. longispinis* with the existing literature

Morphometric Characters	*E. longispinis* (Present study)	*E.longispinis* (*Rajan* 2002)	*E.longispinis* (*Rao* 2000)	*E. longispinis* (*FAO* 1991)	*E. longispinis* (*Allen et al.,* 2010)
Body depth	2.9 times in SL (moderately elongate)	Body moderately elongate	----	2.8 – 3.3 times in SL	----
Standard length	21 cm	----	----	13 – 35 cm	20 inch
Total length	25.5cm	15- 45cm		12 – 37 cm	To 50cm
Dorsal fin	XI	XI	XI	XI	---
Dorsal rays	17	12-19	16	16 -17	----
Anal spins	III	III	III	III	---
Anal rays	8	7-10	8	8	----
Caudal fin	convex	Slight rounded	Rounded	convex	----
Pectoral fins	17	----	18	17 – 19	----
Lateral line scales	50	----	----	49 – 53	----
Colours	Head and body Pale brown. Two blotches. Caudal fins with a white margin.	Head and body Pale; brown. Reddish brown spots,	----	Head and body Pale; greyish brown. 2 blotches. Caudal fins with a white margin. Red brownish spots in Head and front half of the boby.	Greyish brown with paler blotches; brown spots on head and diagonally-elongate spots on body, Pair of Large dark blotches on dorsal fins.
Geographical distribution	Indian Ocean	Indian ocean	Indian ocean	Indian Ocean	Indo –Asian Pacific: E.African to Lesser sunda Is. In Indonesia
Habitat	Coral reefs or rocky areas	Coral reefs or rocky areas	Coral reefs or rocky areas	Coral reefs or rocky areas	Coastal region & reefs
Depth	1-7 m	----	----	----	1-70 m

- SL- Standard Length
- cm – Centimetre
- m- Meter

Table 3—Morphometric comparison of the *E.ongus* with the existing literature

Morphometric Characters	*E.ongus* (Present study)	*E.ongus* (*Rajan* 2002)	*E. ongus* (*Rao* 2000)	*E. ongus* (*FAO* 1991)	*E. ongus* (*Allen et al.,* 2010)
Body depth	2.9 times in SL	----	----	2.7-3.2 times in SL	----
Inter orbital	Flat	Flat to slight convex	----	Flat	----
Standard length	20 cm	10-25cm	22.8-21cm	11- 25cm	12 – 33 cm
Total length	24cm	30cm		11 – 20 cm	12 – 35cm
Dorsal fin	XI	XI	XI	XI	----
Dorsal rays	15(third &fourth spine longest)	12-19	15	14 – 16	----
Anal spins	III	III	III	III	----
Anal rays	8	7-10	8	8	----
Caudal fin	Rounded	Rounded	Rounded	Rounded	----
Pectoral fins	17	----	16	15 – 17	----
Lateral line scales	50	----	----	48 – 53	----
Colours	Body – Brown, with white spots, wavy white spots. Head brown. Dark edged white spots in round.	Body brown with numerous small spots on body. posterior edge of caudal fin narrow white margin with blackish sub marginal hand	Body brown with numerous small spots on body. irregular horizontal rows and stripes; fin black	Wavy white lines; irregular pale blotches (eye sized or longer); head brown, posterior margin margin blackish with white edge.paried fins greyish colours.	Brown with large white blotches; numerous small pale spots on head, body &fins, the spots join to wavy stripes on larger individuals. Solitary and cryptic.
Geographical distribution	Indo-Pacific	Western Indian ocean to Western Pacific	Indian ocean to Western Pacific	Indo – Pacific region, east coast of Africa. It does not occur in Arabian Sea, India, Sri Lanka or Asia.	Indo-West Pacific (E.Africa to Fiji- S.W. Japan to Australia).
Habitat	Coral reefs or rocky areas	Coral reefs or rocky areas	Coral reefs or rocky areas	Coral reefs or rocky areas	Near caves and ledgers of coastal and lagoon reefs
Depth	5-27 m	------	------	5- 25 m	5 – 25 m

- SL- Standard Length
- cm – Centimetre
- m- Meter

rays as well as the surface patterns. This is also very clearly established by the way of the moderate conspecific distance when comparing other species ($K_2P = 0.018398$).

The sequence of *E.areolatus* collected in Andaman coastal region showed less divergence with South Africa. However, the Western Australian species and Italy species exhibit much variation than the Andaman species. Further, a slight modification was noticed in South China Sea species than the Australian species, but the Andaman Species varied much with South China species. The comparatively higher conspecific distance ($K_2P = 0.0437$) of *E.areolatus* with other parts of the world. This suggests that, three studied species of the Andaman waters is specially varied in their light (*E.areolatus*) and dark colour (*E.longispinis* and *E.ongus*) respectively (Table 4).

When comparing the all the three species *E.longispinis* did not show much variation among the species. *E.areolatus* show maximum divergence among the species of the world. *E.ongus* fall in between the above two species but shows some divergence on their stock. This inference further supported by Ward et al.,[4] study on the COI based barcoding for the *Epinephelus* species which suggested that it has its own validity for the species differentiation as well as comparison of the species originated from the different parts of the world.

Further, it also suggested that the variation of the species conspecific and congenetic distances are due to the migration of the species and its new environmental significance, mutual geographical isolation as well as polymorphism based on their evolution or adaptation as reported by Duo et al.,[29].

Table 4—Morphometric comparison of the *E. areolatus* with the existing literature

Morphometric Characters	*E.areolatus* (Present study)	*E.areolatus* (Rajan,2002)	*E.areolatus* (Rao,2000)	*E.areolatus* (FAO,1991)	*E.areolatus* (Allen et al., 2010)
Body depth	2.9 times in SL	Moderately elongate	2.65 times in SL	2.8 – 3.3 times SL	----
Standard length	30cm	13- 30cm	----	14 – 31 Cm	16 inch
Total length	33cm	40cm	-------	13- 35 cm	To 40cm
Dorsal fin	XI	XI	XI	XI	---
Dorsal rays	17	16	15-16	15 – 17	----
Anal spins	III	Rounded	III	III	---
Anal rays	11	11	8	8	----
Caudal fin	Slight convex	Emarginated	Emarginated	Slight convex	----
Pectoral fins	15	Pectoral fin usually reaching anus	17-18	17 – 19	----
Lateral line scales	49 -51	------	------	49 -53	----
Colours	Head, Body and fins pale; Brown, brownish yellow, pectoral fin pale with small dark spots on the rays. Caudal fins with a white margin.	Polygonal brown spots in body & fin	Polygonal yellow brown spots in body & fin	Head, Body and fins pale; covered with numerous close set Brown, brownish yellow, greenish yellowish spots. Pectoral fin pale with small dark spots on the rays. Caudal fins with a white margin.	Gray to whitish with numerous large close – set brown spots, that become smaller and more numerous maturity; narrow white straight margin on tail
Geographical distribution	Indo-Pacific region	Indo-Pacific region	Indo-Pacific region	Indo-Pacific region	Indo-West Pacific; Red Sea and E. Africa to Fiji – S.W. Japan to N.W. Australia.
Habitat	Sea-grass bed, rocky area, dead coral reefs.	Sea-grass bed, rocky area, dead coral reefs.	Sea-grass bed, rocky area, dead coral reefs.	------	Fine sediment bottoms
Depth	6- 200 m	shallow	shallow	------	To 200 m

- SL- Standard Length
- cm – Centimetre
- m- Meter

The present study suggested that morphometrically the species *E.longispinis*, *E.areolatus* and *E.ongus* are different which are also confirmed by the molecular taxonomic results. Mitochondrial COI gene, as an ideal region for "species barcode", its high efficiency in species identification has been reported in Australia marine fishes[4], so, this tool may be used for the rapid analysis for the commercial purposes especially confirmation for the particular species. This tool would prove to be a pathway in sustainable conservation of fishery resource and better understanding of fishery ecology in Andaman coastal region. This is the first ever study in these species in this water. The mitochondrial COI data has an advantage over an individual dataset because of its higher resolving power.

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