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Validation of *Epinephelus coioides* (Hamilton, 1822) occurrence along north-east coast of India

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Present study is to identify and validation of the species using different methods like morphological and meristic characters including pyloric caeca count and pattern and by DNA bar-coding. Morphological and meristic characters of the species landed in Visakhapatnam and Paradeep are in close proximity to the earlier reports for *E. coioides*, and is distinctly different from *E. tauvina*. Molecular studies using DNA bar-coding also revealed high similarity with *E. coioides* and low similarity with *E. tauvina*. It is therefore concluded that the particular grouper species landed along north-east coast is *E. coioides* and not *E. tauvina*.

[Key Words: DNA-Barcoding, *Epinephelus coioides*, morphological and meristic characters, pyloric caeca.]

Introduction

Groupers belonging to the subfamily Epinephelinae are composed of 16 genera[1] and 203 valid species[2] which are distributed world-wide in the tropical and sub-tropical waters, principally in Indo-Pacific region, the east Atlantic and Mediterranean regions and the inter-tropical American zone[3]. Around 69 species of the subfamily Epinephelinae and 31 species of the genus *Epinephelus* have been reported from the Indian seas and they inhabit mainly coral reefs, rocky areas, sea grass beds and estuaries[4]. Groupers are popular food fish with high market demand in many parts of the world, particularly live seafood markets in several Asian countries such as Hong Kong, China, Taiwan, Singapore and Malaysia [5]. Groupers are protogynous hermaphrodites, mostly inhabiting depths less than 100 m, and juveniles are often found in tide-pools [6]. They are largely piscivorous, but also feed on crustaceans and cephalopods.

Grouper species are usually identified by their colour pattern and morphological and meristic characters[6, 7]. Fresh colour patterns are commonly used for field identification, but are often confusing and cause difficulty in distinguishing many of the species, which often leads to misidentification. Adult groupers often possess indistinct colour patterns and morphological and meristic characters overlap between species, therefore the configuration, form and number of pyloric caeca, which are species specific [6, 8] are used for confirming their identity[9, 10]. Advances in molecular techniques help in resolving the ambiguity in species level identification of different species. Recently, several molecular techniques have been used to confirm species identification in groupers and also to reveal their evolutionary history [11].

Groupers are regularly landed in the commercial marine catches off Visakhapatnam and Paradeep, along the north-east coast of India. No systematic scientific study has been conducted till date on the taxonomic identification of the grouper species landed here. Earlier studies had reported the occurrence of greasy grouper, *E. tauvina* in the waters along the east coast [12, 13, 14] of India, as a result of which researchers attributed the grouper species landed at Visakhapatnam and Paradeep as *E. tauvina* without any definite study. There are reports on frequent misidentification of *E. coioides* as *E. tauvina* because they look similar and have overlapping distributions [6]. Visakhapatnam Regional Centre of Central Marine Fisheries Research Institute had successfully developed

broodstock [15] and had achieved success in induced spawning and larval rearing of grouper in 2013. This success in breeding and seed production has given inspiration to have a re-look at the taxonomy, as correct taxonomical classification is an absolute pre-requisite before seeds are released for culture. Therefore, in the present study, an attempt has been made to identify the grouper species available along north-east coast of India using morphological and meristic characters, pyloric caeca count and pattern as well as by DNA bar-coding.

Materials and Methods

For morphological and meristic study, 30 and 20 freshly caught individuals of the species ranging in size from 11.6 – 69.8 cm standard length (SL) were collected from fishing boats operated off Visakhapatnam and Paradeep respectively, between January and December 2013. Collected fishes were photographed for colour patterns in the landing centres and brought to the laboratory (Fig. 1).

Fig.1: Grouper species landed at Visakhapatnam and Paradeep, along north-east coast of India

Morphological (total length, standard length, head length, eye diameter, interorbital width, upper jaw interorbital width, upper jaw length, snout length, upper jaw snout length, maxilla width, pectoral fin length and pelvic fin length) and meristic characters (lateral line scales, gill rakers, pectoral fin rays count and pelvic fin rays count) were recorded [6, 16] (Fig. 2).

Fig. 2: Pattern of gill rakers of the collected specimen of grouper

Measurements were taken with digital vernier calliper to the nearest 0.1 mm. The fishes were then dissected along the ventral side, the pyloric caeca were identified and photographed (Fig. 3). The pyloric caecum is arranged in a whorl around the junction between the stomach and the duodenum, most of it is on the ventral side (Fig. 4). Incisions were made in the alimentary canal near the operculum and in the upper portion of the intestine for isolating the zone harbouring the pyloric caeca. Pyloric caeca were fixed in 10% formalin. The number (counts taken at their free ends), arrangement (one or more groups), form (branched or unbranched) and fresh colouration of pyloric caeca were noted and compared [8]. Morphological and meristic features or standard length [SL] proportions of these features were used to ascertain similarities among fishes using a Bray-Curtis similarity cluster analysis in PRIMER v6 [17].

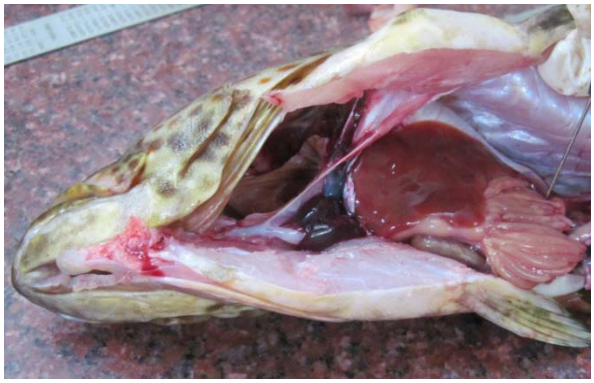

Fig. 3: Dissected ventral side showing the pyloric caeca position

Twelve different individuals (five landed at Visakhapatnam and seven landed at Paradeep) with similar morphology, meristic characters and colour pattern were selected for DNA bar-coding analysis. The genomic DNA was isolated from the muscle tissues using standard phenol-chloroform extraction method [18]. Quality of DNA was ascertained by agarose gel electrophoresis and quantity was estimated using 260/280 nm UV spectrophometer (Genesys 10 UV, Thermofisher, USA) method. For DNA bar-coding, approximately 650 bp of small DNA fragment from the Cytochrome oxidase I (COI) gene in mitochondrial DNA was amplified using universal primers (forward: 5'-TCAACCAACCACAAAGACATTGGCAC-3'; reverse: 5'-TAGACTTCTGGGTGGCCAAAGAATCA -3') (Bioserve Biotechnologies Pvt. Ltd, India). Polymerase Chain Reaction (PCR) was performed using genomic DNA with the universal primers.

Standard PCR reaction at 94 °C for 4 min; 30 cycles at 94 °C for 1 min, 56 °C for 1 min and 72 °C for 2 min and final extension of single cycle at 72 °C for 10 min were followed using thermocycler (Bioer Little Genius). Amplified PCR product was visualized on 1.5% agarose gel, and subsequently the single amplified products was purified by PCR purification kit (Qiagen, Germany) and then used for DNA sequencing. Nucleotide sequencing was carried out by Bioserve Biotechnologies Pvt. Ltd., India by ABI

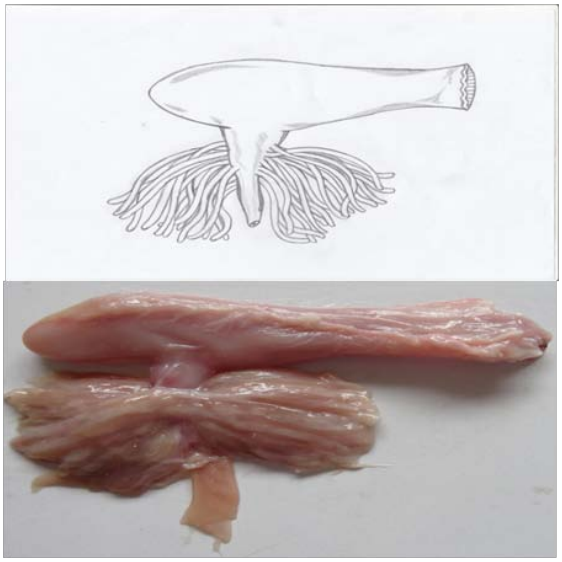

Fig. 4: Pattern of pyloric caeca (thin tubular strands arranged in groups)

BigDYE terminator method. The sequence from the sequencer was obtained in a chromatogram, which was analyzed using CHROMASLITE 201 software. The obtained sequences from all the individuals were analyzed for both forward and reverse sequences using Gene Runner software and then multiple and pair-wise alignment was done using CLUSTALW tool. The aligned sequences were subjected to nucleotide BLAST search and BOLD System v3 to know the sequence identity and further analysis of the sequences. Sequences for *E. tauvina* (GenBank accession number KC500708) and *E. coioides* (GenBank accession number JN208591) were acquired from the public domain database GenBank for comparison. Intraspecific sequence divergence and a neighbor-joining phylogenetic tree were concluded using MEGA6[19] with the Kimura two-parameter model[20] using 1000 bootstrap replicates.

Results and Discussion

Grouper species landed along north-east coast exhibit characteristic orange to reddish-brown colouration on their body, with no white spots or blotches. The observed morphological and meristic characters are presented in Table 1. The observed colour pattern, morphological and meristic characters and also the comparison with the results of earlier workers [6, 8, 16], revealed that the grouper specimens landed at Visakhapatnam and Paradeep are similar to *E. coioides*. Further, specimens from Visakhapatnam and Paradeep demonstrated a high degree of heterogeneity, supportive of the specimens being the same species (Fig. 5). In *E. tauvina*, small faint white spots and blotches are observed on the body, but in the present specimens, there are no white spots

Table 1. Morphological and meristic characters of grouper species landed along north-east coast and a comparison to earlier authors

Characters	Visakhapatnam (VSKP)	Paradeep (PRDP)	Roy, 2004 for *E. coioides*	Roy and Gopalakrishnan, 2011 for *E. coioides*	Heemstra and Randall, 1993 for *E. coioides*
Total length (mm)	141.9 – 830.8	250 - 650	-	-	-
Standard length (mm)	116.0 – 697.5	216.8 - 563.7	-	-	-
Head Length (mm)	46.1 – 320.3	80.1 - 220.1	-	-	-
Head length to Standard length	2.1 – 2.8 times Standard length	2.6 - 2.7 times Standard length	-	-	2.3 – 2.6 times Standard length
Eye diameter (mm)	8.3 – 30.2	15.2 - 17.5	-	-	-
Interorbital Width (mm)	6.4 – 49.7	16.1 - 40.1	-	-	-
Interorbital width to Head length	3.8 – 7.1 times Head length	5.0 - 5.5 times Head length	-	-	5.0 – 6.2 times Head length
Upper Jaw Interorbital Width (mm)	10.4 – 94.1	24.2 - 60.1	-	-	-
Upper Jaw Length (mm)	19.7 – 124.2 (14.5 – 19.1 % of Standard length)	36 - 95 (16.6 - 16.9 % of Standard length)	-	-	17 – 20 % of Standard length

Snout Length (mm)	8.8 – 99.8	15.4 – 40.0	-	-	-
Upper Jaw Snout Length (mm)	6.6 – 77.3	12.0 - 40.1	-	-	-
Maxilla Width (mm)	4.8 – 27.1(3.1 – 5.9 % of Standard length)	8.1 - 19.1 (3.4 – 3.7 % of Standard length)	-	-	4.2 – 5.5 % of Standard length
Pectoral Fin Length (mm)	24.8 – 139.2	43.7 - 113.601	-	-	-
Pectoral fin length to Head length	1.84 – 2.3 times Head length	1.8 - 1.9 times Head length	1.6 – 2.2 times Head length	1.6 – 2.2 times Head length	-
Pelvic Fin Length (mm)	20.6 – 106.4	36.3 - 94.4	-	-	-
Pelvic fin length to Head length	2.18 – 3.01 times Head length	2.2 - 2.3 times Head length	1.9 – 2.7 times Head length	1.9 – 2.7 times Head length	-
Lateral Line Scales	61 - 63	61 - 63	58 - 65	58 - 65	-
Gill Rakers	8 – 10 on upper limb; 14 – 17 on lower limb; total 23 - 25	8 - 10 on upper limb; 14 -17 on lower limb; total 23 - 25	8 – 10 on upper limb; 14 – 17 on lower limb; total 23 - 26	8 – 10 on upper limb; 14 – 17 on lower limb; total 23 - 26	8 – 10 on upper limb; 14 – 17 on lower limb; total 23 - 26
Pectoral Fin Rays count	18	18	18-20	-	-
Pelvic Fin Rays count	1 - 5	1 - 5	-	-	-
Pyloric caeca count	51-58	52 - 56	-	50 - 60	50 - 60
Pyloric caeca arrangement	Groups	Groups	-	Groups	-
Pyloric caeca form	Branched	Branched	-	Branched	-
Pyloric caeca colour	Flesh coloured	Flesh coloured	-	Flesh coloured	-

and blotches. The upper jaw length and maxilla width as proportion to standard length in the present specimens, differs from that reported for *E. tauvina* by Heemstra and Randall [6]. Lower limb gill rakers, lateral line scales and pyloric caeca counts for *E. tauvina* are 17 – 20, 63 – 74 and 16 – 18, respectively [6, 16], but in the present specimens, their numbers are 14 – 17, 61 – 63 and 51 – 58 (Table 1). Therefore, it is evident that the species landed along north-east coast is not *E. tauvina*. There was no variation observed in number of pyloric caeca between juvenile and adult specimens in this study.

The DNA extracted from twelve different samples was successfully amplified with the COI universal primer. The resulting PCR products were sequenced to obtain full length DNA barcodes averaging 650 bp in length. Insertions, deletions or stop codons were not observed in any of the COI sequences. It showed that all the amplified sequences are functional mitochondrial COI sequences of the fish. The identified gene sequences were submitted to NCBI GENBANK. NCBI BLAST search and DNA BOLD analysis of the obtained sequences revealed that all the selected samples showed very high similarity with the species *E. coioides*. Hence, molecular results are consistent with the morphological and meristic results. There was no intraspecific sequence divergence between Visakhapatnam and Paradeep samples, which indicates that both the samples represent the same species. Interspecific sequence divergence between the present species and *E. coioides* obtained from GenBank (JN208591) was nil and between the present species and *E. tauvina* obtained from GenBank (KC500708) was 23.8 %. The divergence of the present species from *E. tauvina* was well above genetic divergence levels used to distinguish between sister fish species [21]. The Cyt *b* neighbor-joining tree produced a single, highly supported clade containing present specimens and *E. coioides*, whereas *E. tauvina* was distantly located (Fig. 6). This depicts genetic similarity of the present specimens with *E. coioides* and dissimilarity with *E. tauvina*.

Conclusion

Accurate taxonomic identification is essential for the proper management of any fishery resource [22]. Grouper species landed at Visakhapatnam and Paradeep is morphologically and genetically identical, hence is a single species. Using morphological and meristic characters, pyloric caeca count and pattern and by

DNA bar-coding, it is conclusive that the grouper species landed along north-east coast is *E. coioides* and not *E. tauvina*. Similarly, study made by earlier researchers such as Roy and Gopalakrishnan [8] could not find a single specimen to confirm the occurrence of *E. tauvina* from the Indian waters even though, earlier authors [12, 13, 14] have reported on its occurrence. The occurrence of *E. tauvina* in Indian waters needs further investigation and it could be quite possible that previous reports of this species in Indian waters were based on misidentification of *E. coioides*, as these two species share more or less similar morphological characters and therefore are often confused in the literature [8].

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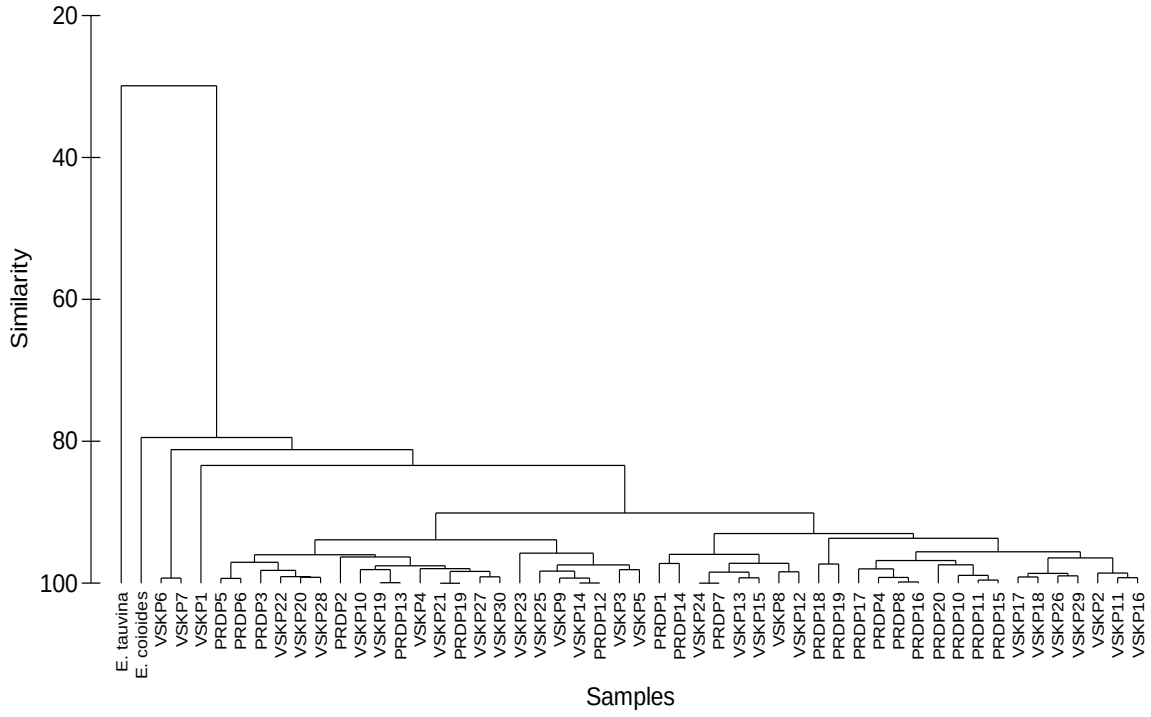

Fig. 5: Dendrogram of morphological characters and meristic values using Bray-Curtis similarity matrix (scale bar represents percent similarity among specimens); VSKP: specimens collected from Visakhapatnam (n=30) and PRDP: specimens collected from Paradeep (n=20) for the comparison of morphological characters and meristic values of E. coioides 8 and E. tauvina 6

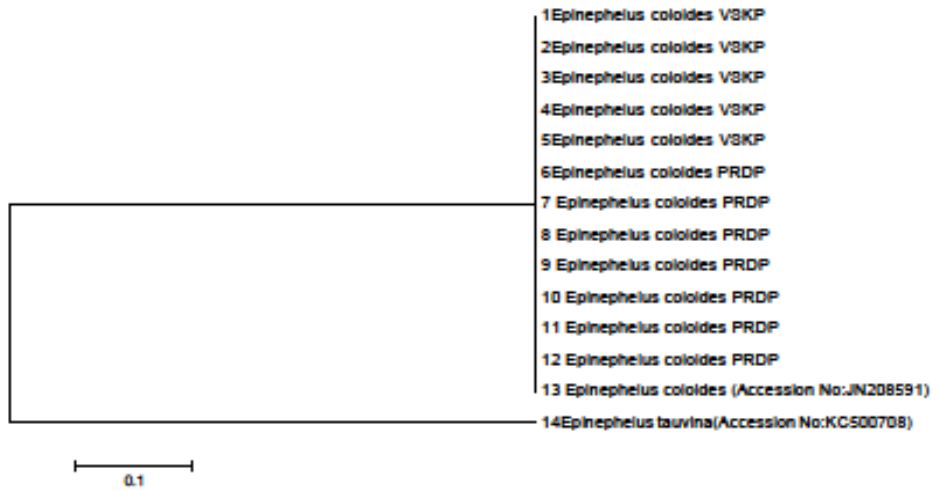

Fig. 6: Neighbor-joining phylogenetic tree of Cyt *b* sequence data with 1000 bootstrap probability (Cyt *b* Kimura 2-parameter)

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