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Research Article

First report of *Plectropomus pessuliferus* from Indian Peninsula with species delimitation analysis based on COI gene

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The molecular phylogeny and species delimitation analysis based on Cytochrome C Oxidase I (COI) gene of *Plectropomus pessuliferus*, shows proximity with nine gene sequences of the same species retrieved from the NCBI and BOLD database. However, some gene sequences of *P. pessuliferus* retrieved show a close affinity with some sequences of two closely related species, *viz*. *P. leopardus* and *P. maculatus*, and their hybrids. The overlapping species delimitation results for *P. pessuliferus* and *P. leopardus*, along with fewer nucleotide differences and their appearance in the same clade in the Maximum Likelihood (ML) tree, support the earlier hypothesis of probable natural hybridisation and ongoing introgression between species of the genus *Plectropomus* in the marine environment. Additionally, many similarities in the morphological assessment further corroborate this claim. The finding of coral reef trout *P. pessuliferus* constitutes its first record from the potential fishing zones of the Indian peninsula.

[**Keywords**: COI gene, East coast of India, Hybridization, Introgression, New record, Taxonomy]

Introduction

Hybridisation of marine fishes is not uncommon and may be contributing to the evolution of marine fishes. However, its role in evolutionary significance is debatable[1]. There have been reports of naturally occurring hybrids in the genus *Plectropomus* Oken, 1817, especially between the species *P. leopardus* (Lacepède, 1802) and *P. maculatus* (Bloch, 1790)[1,2]. Further, it is also pointed out that the mitochondrial markers like Cytochrome C Oxidase I (COI) and Cytochrome b of the mitochondrial genome are not sufficient to distinguish between pure breeds and hybrids of the above two species. Comparative studies based on the molecular analysis of COI gene sequences of *Plectropomus pessuliferus* (Flower, 1940) and the two previously mentioned species support the idea of potential hybridisation and introgression among related species within the genus Plectropomus, which includes several commercially important groupers[2].

From the biodiversity point of view, the present work reports a new addition of one coral reef grouper species, the roving coral grouper, *P. pessuliferus* (Flower, 1940), for the first time from the coast of peninsular India. This species was first described from Padang, Sumatra, Indonesia, but is sparsely distributed in the Indo–Pacific region[3]. The normal distributional range is from East Africa (Zanzibar), St. Brandon's Shoals, Maldives, Sri Lanka and Sumatra and Fiji[4], and Tonga[5] in the east. In the present study, molecular characterisation of the species *P. pessuliferus* based on its COI gene, comparative morphological data, molecular phylogeny and species delimitation analysis of three related species *viz*., *P. leopardus*, *P. maculatus*, and *P. pessuliferus*, which share overlapping morphological features based on COI gene sequences retrieved from the NCBI and BOLD databases, are provided.

Materials and Methods

A single specimen was collected during a local field survey from Gopalpur fish landing centre (19°15′36.08″ N, 84°54′47.90″ E) on 9th November, 2020. The specimen was photographed and measured after bringing to the laboratory on a 1 mm gradation measuring scale and digital calipers. Lateral line

scales were counted by using a Leica EZ4 microscope. The fresh pectoral fin tissues (frozen) of about 100 mg were stored in a refrigerator at –20 °C for molecular study. Later, the whole specimen was preserved in 10 % formalin solution. In the laboratory, the specimen was identified based on the morphological features described in relevant literature[6-8] and following Fishbase[9]. The voucher specimen was registered and deposited in the National Repository of the Estuarine Biology Regional Centre, Zoological Survey of India, Gopalpur–on–Sea, Odisha, India.

Material examined

EBRC/ZSI/F12409, one specimen, collected from Gopalpur fish landing centre, Odisha, India (19°15′36.08″ N; 84°54′47.90″ E), Date: 9th November 2020.

Molecular analysis

About 20 mg of pectoral fin tissue sample was collected by using sterilized scissors and washed thoroughly with distilled water to avoid contamination. DNA isolation was carried out by the salting out method[10]. The concentration of the extracted genomic DNA from the sample was measured using a Qubit 4 Fluorometer, yielding a value of 45.1 ng/μL. The DNA samples were then stored at –20 °C until further use. COI gene amplification was carried out using an ABS (Applied Biosystem) Thermo Fisher Scientific PCR machine with a final reaction volume of 25 μL. The reaction mix consisted of 9 μL Nuclease-free water, 12.5 μL of 2X Hi G9 Taq PCR Master Mix, 0.5 μL of the forward primer Fish F1 (5'-TCAACCAACC ACAAAGACATTGGCAC-3'), 0.5 μL of the reverse primer Fish R1 (5'-TAGACTTCTGGGTGGC CAAAGAATCA-3')[11] and 2.5 μL of the extracted DNA template. The PCR thermo-cycling conditions were as follows: an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 54 °C for 30 sec, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. The reaction was then held at 4 °C indefinitely according to Mohapatra *et al.*[12]. The unidirectional sequencing of the obtained amplified DNA product was done using the Sanger sequencing method. The sequence assembly was performed using BioEdit version 7.2. The consensus COI gene sequence was submitted to NCBI to obtain the accession number. The NCBI accession number of the submitted gene sequence and the number of base pairs are MW881247 and 629, respectively. For the confirmation of the morphological identification, the particular COI gene sequence was compared with other conspecific and congeneric genes retrieved from GenBank during BLAST. The multiple alignment of the similar COI gene sequences obtained from NCBI, BOLD database (Table S1), pairwise evolutionary distance (Kimura 2 Parameter), and Maximum Likelihood (ML) tree analysis using Kimura 2-parameter model[13] were carried out using the software MEGA 10(ref. 14). Bootstrap analysis was carried out using 1000 replications for the verification of robustness of the internal node of the ML tree. The species delimitation analysis was carried out from the above-mentioned sequences using the online ASPA (Assemble Species by Automatic Partitioning) web browser (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html #) based on the default parameters[15,16].

Results

The collected specimen was initially identified as *P. pessuliferus* (Fowler, 1904) (Perciformes: Serranidae: Epinephelinae) based on morphological characters, which was later confirmed through molecular analysis.

Morphometric characters

The morphometric measurements of the species are provided in Table 1. Body moderately elongated, robust and laterally compressed; depth nearly 3 times in Standard Length (SL); pre-anal length 1.7 times in SL; pre-dorsal length 2.9 times in SL; head length 3.1 times in SL; pre-orbital length 2.9 times in Head Length (HL); Inter Orbital Space (IOS) without scales and the IOS 5.1 times in HL; upper jaw length and lower jaw length 2.5 and 2.3 times in HL, respectively; eye diameter is 6.6 in HL (Fig. 1). Ventral side of body clearly more convex than dorsal profile; dorsal head profile convex; eye positioned near the dorsal contour of head. Mouth superior, large, oblique and protractile with a big gap between both jaws; canine teeth present on both jaws; lower jaw longer. Caudal fin emarginated; caudal peduncle length is more than its depth. The lower limb of first gill arch contains 10 developed gill rakers; gill raker at the angle is shorter than gill filament (Fig. 2). Pre-opercular edge serrated. Both cycloid and ctenoid scales present on the body; cycloid scales are mostly on tail, chest, pre-dorsal and pre-pelvic region and the

Table 1 — Morphometric detail of *Plectropomus pessuliferus* collected from Gopalpur fish landing centre, Odisha

Morphometric measurements	***P. pessuliferus* (EBRC/ZSI/F 12409)**	**Morphometric measurements**	***P. pessuliferus* (EBRC/ZSI/F 12409)**
Total length (TL in cm)	39.6	***% of SL***	
Standard length (SL in cm)	32.7	Pectoral fin length	14.9
% of SL		Longest anal fin ray length	13.7
Pre-anal length	57.4	Longest pectoral fin ray length	14.9
Pre-dorsal length	34.5	Longest pelvic fin ray length	14.3
Dorsal fin Base Length (DBL)	37.3	***% of HL***	
Anal fin Base Length (ABL)	14.3	Sub-orbital depth	20.3
Pectoral fin Base Length (P1BL)	6.4	Sub-orbital width	20.3
Pelvic fin Base Length (P2BL)	5.8	Snout length	31.1
Caudal fin Base Length (CBL)	13	Eye diameter	15.09
Pectoral fin length	16.2	Pre-orbital length	33.9
Body width	12.8	Post-orbital length	50.9
Body depth	33.6	Inter orbital space	19.8
Head Length (HL)	32.4	Upper jaw length	39.6
Caudal peduncle length	17.1	Lower jaw length	43.3
Caudal peduncle depth	13.7	Gill raker length at angle	6.6
Dorsal spine III, IV length	8.2	Gill filament length at angle	8.4

Fig. 1 — Lateral view of fresh specimen of *Plectropomus pessuliferus*

rest of the body is covered with ctenoid scales. Nostrils set in a shallow groove in front of eye. Dorsal fin single, originates above middle of the pectoral fin; spines of dorsal fin strong in comparison to anal fin spines.

Colour

The collected specimen has orange-brown ground colouration with scattered dark-edged blue spots, mostly larger than twice the size of the nostrils, on body and head except for the ventral portion (Fig. 1); ventral side whitish in colour. Few midbody spots are vertically elongated. All fins have dark-edged blue spots except for the pectoral fin. Head and fins are darker than the body colour.

Distribution

Previously, the species was known to be distributed from the Red Sea, Indo-West Pacific: (East Africa, Mozambique, Madagascar, and Saint Brandon's Shoals, east to Fiji and Tonga[3,4,17]). The current study added a new distributional record from peninsular India (Fig. 3).

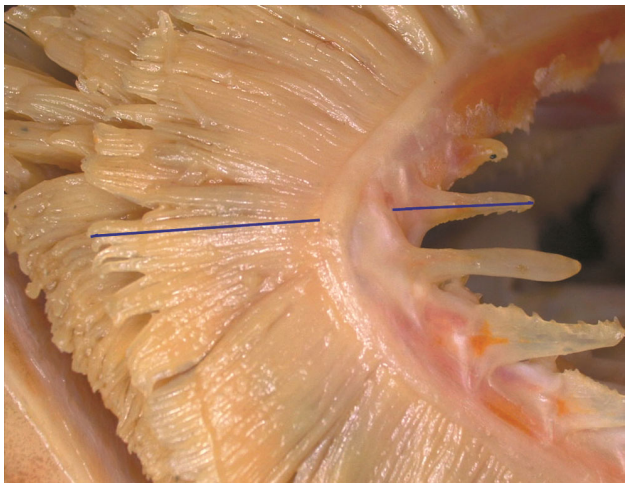


Fig. 2 — The image showing gill raker at angle is shorter than gill filament

Molecular characterisation

The COI gene sequence of the recently studied species *P. pessuliferus* shows 96 – 100 % similarity percentage and 97.5 – 100 % identity percentage when compared with other sequences in NCBI. The nine conspecific gene sequences of *P. pessuliferus* from India, Bangladesh and Australia retrieved from NCBI and BOLD database shows the 0.4 – 0.8 % of K2P distance from the currently studied species

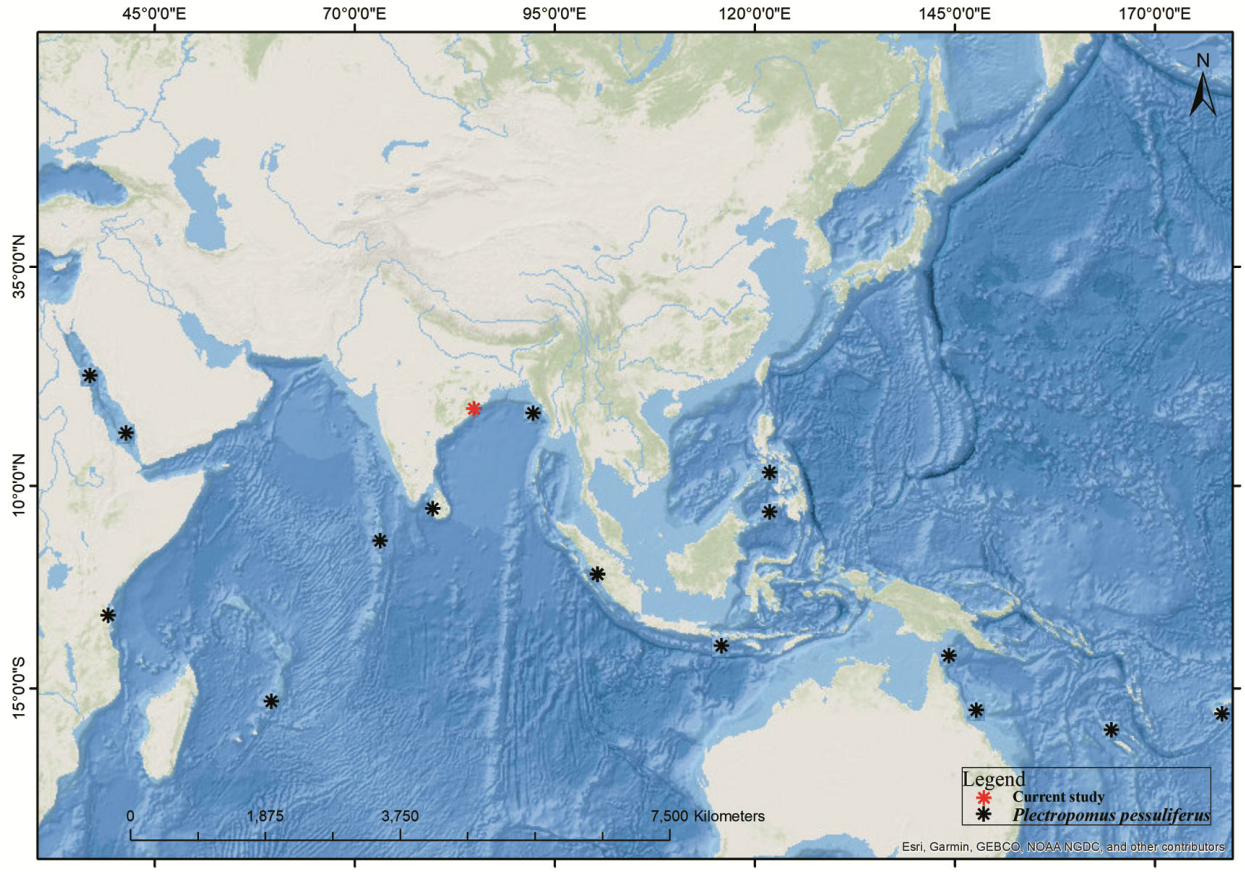
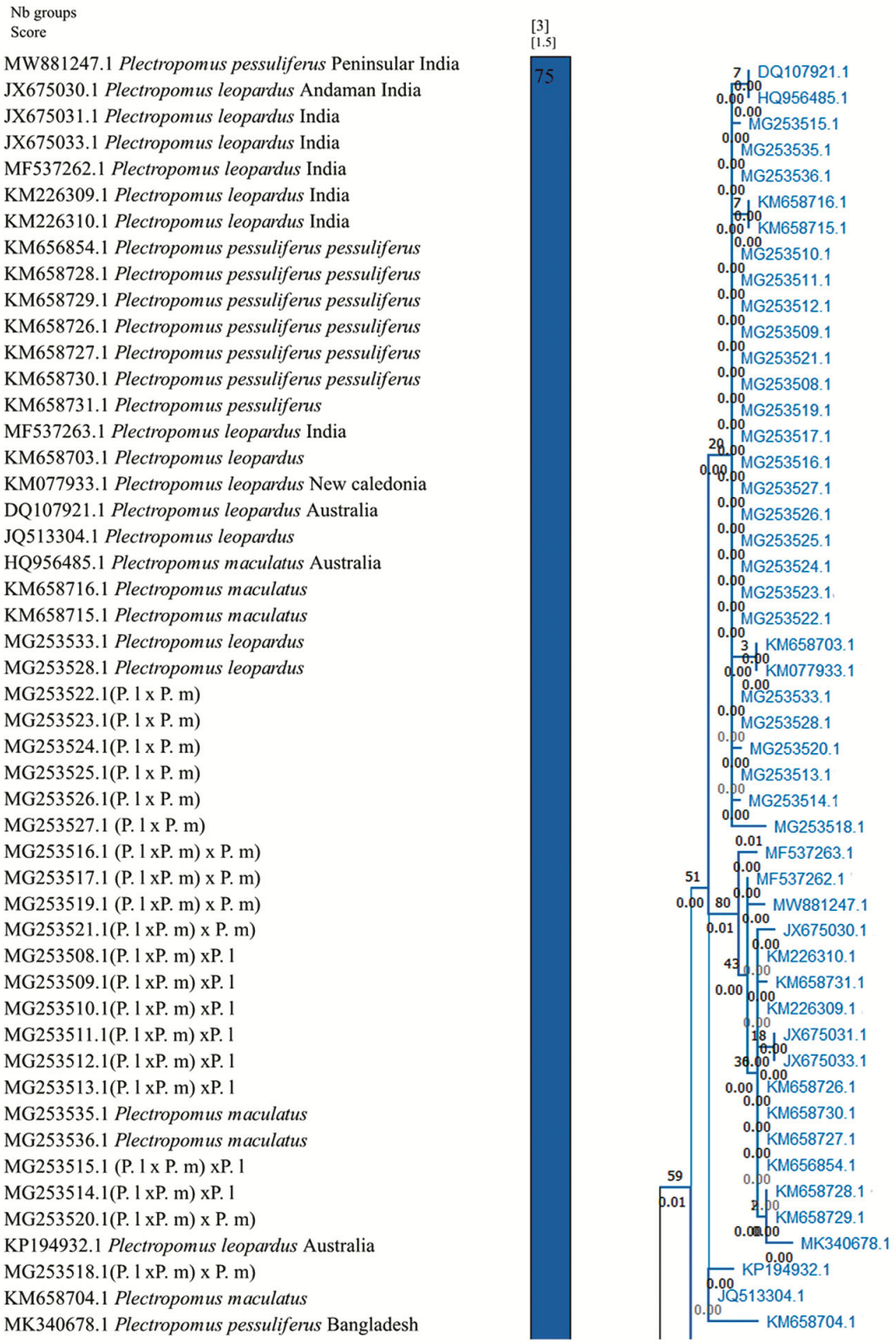


Fig. 3 — Specimen collection locality of current study along with the distribution of *P. pessuliferus*

P. pessuliferus. The other gene sequences of *P. leopardus* and *P. maculatus* from India, Australia, Indonesia, Philippines, Vietnam and Malaysia retrieved from the same databases shows 1.5 – 5 % of K2P distance from current study. Some exceptions were also seen during genetic analysis. Some gene sequences of *P. leopardus* and *P. maculatus* also show a negligible K2P distance *i.e.*, 0.3 – 0.9 %. In the ML tree analysis, many sequences of *P. leopardus*, *P. maculatus* and *P. marisrubri* form a distinct clade with strong bootstrap support of 72 – 100 %. However, the clade formed (with moderate bootstrap support of 55 %) by the sequences of the species *P. pessuliferus*, also contained few sequences of *P. leopardus*, *P. maculatus* and many hybrid sequences of both species (Fig. 4).

Species delimitation analysis

Species delimitation using the ASAP (Assemble Species by Automatic Partitioning) method is a process used in molecular biology to identify and distinguish species based on genetic data. This method is particularly useful for species that are difficult to differentiate morphologically. ASAP attempts to partition the sequences into groups called Molecular Operational Taxonomic Units (MOTUs), which are representatives for species. The algorithm tests various ways to split the sequences into different partitions and calculates a score for each possible partitioning scenario. The score reflects the likelihood that the partitioning represents real species boundaries. Lower scores indicate better partitioning, meaning the scenario is more likely to represent true species delimitations. The algorithm selects the partitioning with the best score, ideally representing the most likely species groups. The ASAP resulted in the partitions with Nb group/ partition with ASAP score of 1.5 – 14 and probable inclusion of 2 – 31 species. The best ASAP resulted partition here is ASAP score of 1.5 and Nb groups 3 (Fig. 4). As per Puillandre *et al.*[15], the Nb group with the lowest ASAP score will provide better species delimitation and boundaries. In connection with this, the partition with ASAP score of 1.5 indicates that the inclusion of


Nb groups
Score
[3]
[1.5]
75
MW881247.1 Plectropomus pessuliferus Peninsular India
JX675030.1 Plectropomus leopardus Andaman India
JX675031.1 Plectropomus leopardus India
JX675033.1 Plectropomus leopardus India
MF537262.1 Plectropomus leopardus India
KM226309.1 Plectropomus leopardus India
KM226310.1 Plectropomus leopardus India
KM656854.1 Plectropomus pessuliferus pessuliferus
KM658728.1 Plectropomus pessuliferus pessuliferus
KM658729.1 Plectropomus pessuliferus pessuliferus
KM658726.1 Plectropomus pessuliferus pessuliferus
KM658727.1 Plectropomus pessuliferus pessuliferus
KM658730.1 Plectropomus pessuliferus pessuliferus
KM658731.1 Plectropomus pessuliferus
MF537263.1 Plectropomus leopardus India
KM658703.1 Plectropomus leopardus
KM077933.1 Plectropomus leopardus New caledonia
DQ107921.1 Plectropomus leopardus Australia
JQ513304.1 Plectropomus leopardus
HQ956485.1 Plectropomus maculatus Australia
KM658716.1 Plectropomus maculatus
KM658715.1 Plectropomus maculatus
MG253533.1 Plectropomus leopardus
MG253528.1 Plectropomus leopardus
MG253522.1(P. l x P. m)
MG253523.1(P. l x P. m)
MG253524.1(P. l x P. m)
MG253525.1(P. l x P. m)
MG253526.1(P. l x P. m)
MG253527.1 (P. l x P. m)
MG253516.1 (P. l xP. m) x P. m)
MG253517.1 (P. l xP. m) x P. m)
MG253519.1 (P. l xP. m) x P. m)
MG253521.1(P. l xP. m) x P. m)
MG253508.1(P. l xP. m) xP. l
MG253509.1(P. l xP. m) xP. l
MG253510.1(P. l xP. m) xP. l
MG253511.1(P. l xP. m) xP. l
MG253512.1(P. l xP. m) xP. l
MG253513.1(P. l xP. m) xP. l
MG253535.1 Plectropomus maculatus
MG253536.1 Plectropomus maculatus
MG253515.1 (P. l x P. m) xP. l
MG253514.1(P. l xP. m) xP. l
MG253520.1(P. l xP. m) x P. m)
KP194932.1 Plectropomus leopardus Australia
MG253518.1(P. l xP. m) x P. m)
KM658704.1 Plectropomus maculatus
MK340678.1 Plectropomus pessuliferus Bangladesh
DQ107921.1
HQ956485.1
MG253515.1
MG253535.1
MG253536.1
KM658716.1
KM658715.1
MG253510.1
MG253511.1
MG253512.1
MG253509.1
MG253521.1
MG253508.1
MG253519.1
MG253517.1
MG253516.1
MG253527.1
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MG253518.1
MF537263.1
MF537262.1
MW881247.1
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KM226310.1
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JX675031.1
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KP194932.1
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KM658704.1

Fig. 4 - Contd...

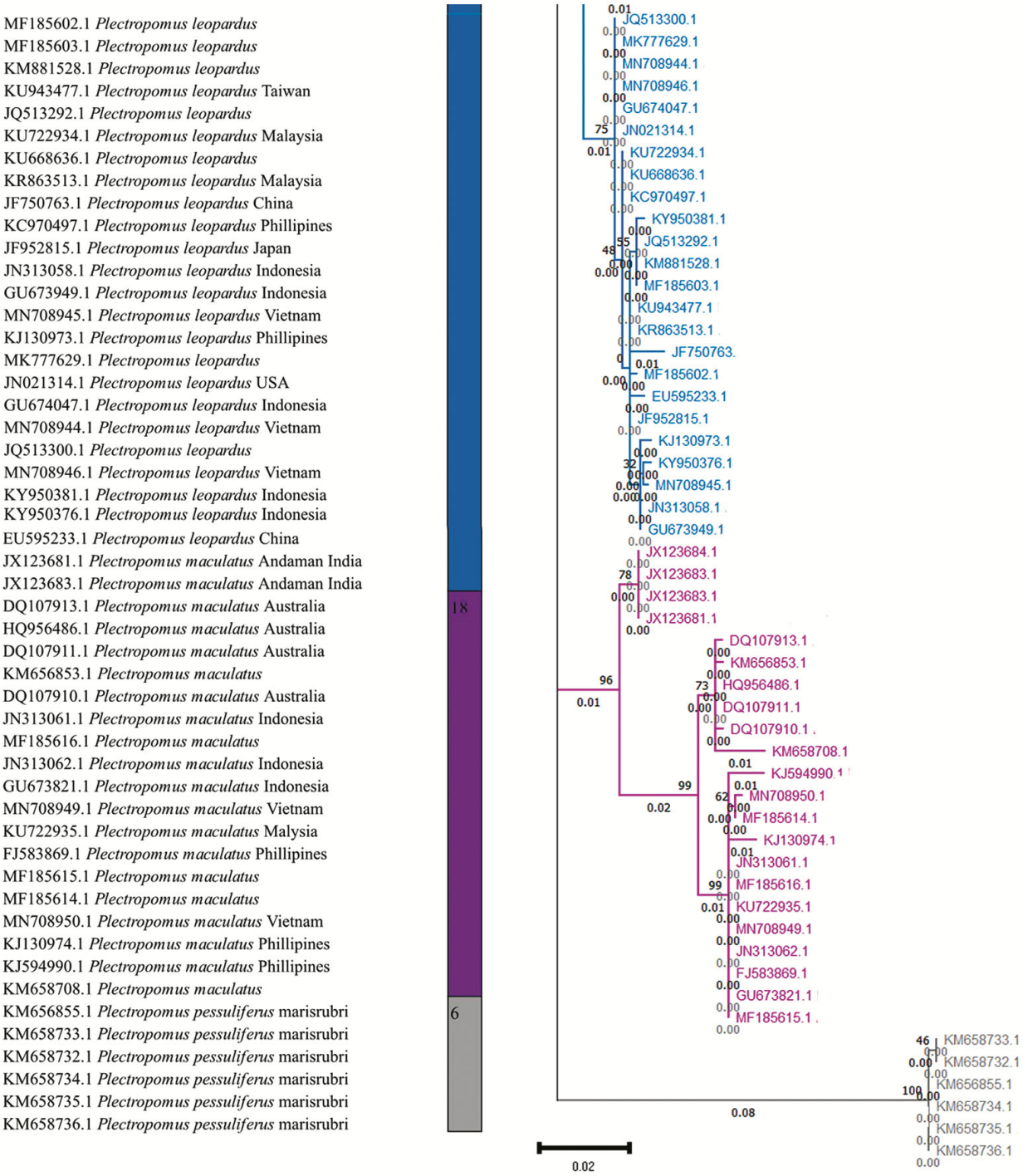

Fig. 4 — Species delimitation analysis of *P. pessuliferus* and *P. leopardus* (blue), *P. maculatus* (pink), *P. pessuliferus marisubri* (light gray), and best fit maximum likelihood tree of *Plectropomus pessuliferus* with its conspecific and congeneric specimens

three categories of fishes can be identified as *P. marisrubri*, *P. maculatus*, and a species complex that includes *P. leopardus*, *P. pessuliferus*, and their hybrids. Since the third group represents a species complex, a detailed study is recommended to resolve the taxonomic uncertainties within the *Plectropomus* group in the Indo-West Pacific region. However, there is no distinct species boundary among *P. leopardus*, *P. maculatus* (sequence uploaded from the Andaman region, India), *P. pessuliferus* and the sequences of

the hybrids of *P. leopardus*, *P. maculatus* shown in the above-mentioned partition. Further, *P. maculatus* reported from other regions such as Indonesia, Philippines, Vietnam, Australia and Malaysia have a clear-cut demarcation. The species *P. marisrubri* also shows a clearly distinct partition.

Discussion

The species *P. pessuliferus* was earlier recorded from the neighbouring areas of the Bay of Bengal, particularly from Andaman and Nicobar Islands[18], Sri Lanka[7], Bangladesh[19] and Myanmar[20]. However, there is no valid record from Peninsular India yet[21]. This species is known to have a distribution from East Africa in the west to Fiji and Tonga in the east[17], however, there is no such evidence of its continuous distribution in the above-mentioned range. In the northern Indian Ocean, the species is known from Maldives, Sri Lanka, Bangladesh, Myanmar, Sumatra (Indonesia), and the Andaman Islands (India)[22]. Heemstra & Randall[7] have clearly stated that *P. maculatus* is known only from the tropical western Pacific and reports of this species formerly listed from the western Indian Ocean[23] are based on misidentified *P. pessuliferus.* With this background, the report of *P. maculatus* by Jones & Kumaran[24] from Lakshadweep islands is to be treated as *P. pessuliferus*, and hence, the inclusion of this species from Laccadives (Lakshadweep) in Choat *et al.*[21]. Thus, the listing of *P. maculatus* in Rajan *et al.*[25] is to be corrected as *P. pessuliferus.* Therefore, till date, *P. pessuliferus* has been known to occur in coral reef regions of India, *i.e.* from Lakshadweep and Andaman Islands, but never recorded from the potential fishing zone of the Indian Peninsula. Hence, the present record forms its first report from peninsular India.

Eight valid species of the genus *Plectropomus* are recognised globally, of which five species have been reported in the reef areas of Lakshadweep and the Andaman Islands. These include *P. areolatus* (Rüppell, 1830), *P. laevis* (Lacepède, 1801), *P. leopardus*, *P. maculatus*, and *P. pessuliferus*[21]. Although other species are distributed in the far west or far east, their distribution is discontinuous, and they are rarely encountered. The closest recorded occurrence of *P. pessuliferus* is from St. Martin's Island in Bangladesh, approximately 800 km to the north, while its easternmost record is from the Andaman Islands, and its southernmost record is from Sri Lanka. All *Plectropomus* species typically inhabit reef lagoons and seaward reefs[7]. They are rare, mostly solitary, and often remain hidden under reef ledges or ridges, making them largely inaccessible to fishers. The presence of a ridge at a depth of 30 meters off Gopalpur may offer a suitable habitat for this species.

There is extensive morphological diversity and drastic variations in colouration patterns in the shallow tropical coral reef fishes[26]. Three species *viz*., *P. maculatus*, *P. leopardus* and *P. pessuliferus*, have overlapping morphological features with some marked differences in the colour pattern, dots in the pelvic and pectoral fins, gill rakers at the angle of the gill arch and dorsal head profile. A comparative morphological feature between these three species is provided in Table 2 for better understanding. Spot patterns are mostly used to identify these species; however, the clear dots pattern is only pronounced in adult individuals, which often leads to misidentification if the collected individuals are juveniles or naturally occurring hybrids[1,2]. The pelvic fin has clear blue spots in the case of *P. pessuliferus*, while *P. maculatus* and *P. leopardus* don't have blue spots on the pelvic fin. *Plectropomus leopardus* can be distinguished from *P. maculatus* by having a gill raker at an angle shorter than the gill filament vs. a gill raker at an angle longer than the gill filament[7]. This claim is clearly evident from the molecular phylogeny analysis and species delimitation analysis.

Further, a sequence of *P. maculatus* (accession number KM658716) with only 0.9 % nucleotide difference in comparison to the recent study and 0.4 – 1 % difference with the other nine sequences of *P. pessuliferus*, may possibly be a misidentified specimen of *P. pessuliferus.* The overlapping species delimitation results of all *P. pessuliferus* and *P. leopardus* species, fewer nucleotide differences, the appearance of these two species in the same clade in the ML tree (Fig. 3), and many similarities in morphological assessment are in agreement with the earlier claim of naturally occurring hybridisation and introgression.

As stated by Frisch & Herwerden[2], sharing overlapping time and space in terms of spawning aggression, the rarity of mates, and monopolisation of dominant males provides an opportunity for natural hybridisation by juxtaposing heterospecific gametes in the individuals of closely related species of the genus *Plectropomus.* Further in agreement with Frisch & Herwerden[2], in this genus of groupers, the

Table 2 — Comparison of *P. pessuliferus* from Gopalpur (Odisha) with congeneric species

Meristic measurements	***P. pessuliferus* (Fowler, 1904) (Current study– EBRC/ZSI/F 12409)**	***P. pessuliferus* (Heemstra & Randall[7])**	***P. maculatus* (Heemstra & Randall[7])**	***P. leopardus* (Heemstra & Randall[7])**
Gill rakers on upper limb	3	1–3	1–3	1–3
Gill rakers on lower limb	10	7–10	6–10	6–9
Gill rakers total	13	7–13	7–13	7–12
Scales on lateral line	102	85 – 104	89 – 99	83 – 97
Pores on lateral line	102	--	--	--
Dorsal fin spines	VIII	VIII	VIII	VIII
Dorsal fin rays	11	10–12	10–12	10–12
Pectoral fin rays	16	14–17	15–16	15–17
Anal fin spines	III	III	III	III
Anal fin rays	8	8	8	8
Gill raker at angle	Shorter than gill filament	Shorter than gill filament	Longer than gill filament	Shorter than gill filament
Pelvic fin blue dot	Pelvic fin with blue spots	Pelvic fin with blue spots	Pelvic fin without blue spots	Pelvic fin without blue spots
Pectoral fin blue dot	Pectoral fin with blue dot more towards base than edge	--	Pectoral fin with blue dot more towards base than edge	--
Dorsal head profile	Clearly convex	Clearly convex	More or less straight	Clearly convex
Blue spots on body	Twice the size of nostrils or more	More than twice the size of nostrils	More than twice the size of nostrils	About the size of nostrils
Mid body spots in adults	Some spots vertically elongate	Some spots vertically elongate	Some spots horizontally elongate	All spots rounded

reproductive isolation may not be complete and natural hybridisation may be a common phenomenon. Therefore, a more specific study focusing on the genus Plectropomus in the Indian Ocean, in the long run, should be undertaken to understand the implication of natural hybridisation for the effective management of the coral trout population.

Supplementary Data

Supplementary data associated with this article is available in the electronic form at https://nopr.niscpr.res.in/jinfo/ijms/IJMS_53(06)459-468_SupplData.pdf

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Conflict of Interest

Authors declare no conflict of interest for the publication of the manuscript.

Ethical Statement

The organisms under the study are not under the scheduled list/protection categories; thus, ethical clearance certification is not applicable.

Author Contributions

SRM, SA, RKB, & PS: Collection, preservation, identification of the species and manuscript preparation; SA: Molecular characterisation; LP, JKS, SSM, & AM: Manuscript preparation and critical analysis.

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