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Morphological and molecular evidence for the first records and range extension of two marine fish species *Pomadasys andamanensis* and *Siganus fuscescens* to Odisha Coast, Bay of Bengal

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Abstract

Two specimens are from each of *Pomadasys andamanensis* (Mckay and Satapoomin) and *Siganus fuscescens* (Houttuyn) were collected from Gopalpur-on-sea, Odisha coast, Bay of Bengal. A study on conventional taxonomy interestingly demonstrates not only the first record of appearance of both the species, but also their inclusion to their respective species on the Odisha coast, Bay of Bengal. It was further, strengthened by molecular analysis through DNA barcoding which showed high confidence sequence similarity in their species identification. Moreover, the congruent clustering of both the species according to their morphological identification, strongly support the species identification through DNA barcoding. Above all, the generated time tree with regards to their origin largely agrees with other recent reports based on mitochondrial loci analysis indicates middle to early Miocene sub-epoch for *Pomadasys andamanensis* and for *Siganus fuscescens* it occurred sometimes in the late Pleistocene epoch. The migration of these reef-associated fishes is probably for their specific attraction to reef region of Bay of Bengal or/and ecological disturbances in their native region. The overall outcomes confirmed the first ever extensive range of occurrence of these two marine fish species on the Odisha coast, Bay of Bengal.

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Introduction

Biological diversity is rapidly inundating worldwide with unprecedented rates mostly due to human activities (Hubert and Hanner, 2015). Determining the extent to which unprecedented globalization and intensification of human-related threats affect biodiversity, either through the loss of species at particular sites or through changes in range size, requires accurate data on the species distribution (Gaston and Blackburn, 2000). Thus, precise taxonomic identification and delimitation of species is highly necessary for conservation and sustainable exploitation of natural resources and also paramount prerequisites to population genetic, physiological and ecological studies (Butlin *et al.*, 2009). On the other hand, it is also important to know whether economic benefit estimates are stable over time, thus accurate identification of species is additionally consequential for their protection (Lew and Wallmo, 2017).

It is imperative that the ichthyofauna of Bay of Bengal have been well studied for effective conservation and resource management. The Bay of Bengal is the largest marine ecosystem of the world, this pleasant environmental condition, seems to be responsible for introduction of large no of non-native invasive species (NIS). The new alien species are invaded to Bay of Bengal, because of growth, development, reproduction and exploiting the environment for further establishment of their population. Latest record shows that, the existence of invasive species such as *Ulua mentalis, Pinjalo pinjalo, Tylosurus crocodilus*, *Cephalopholis formosa*, and *Myripristis jacobus* to Bay of Bengal at different time period (Barik *et al.*, 2018a, b, c; 2021).

Introduction of several types of marine aquatic noninvasive species may lead to declines or even extinctions of native species; create disturbances in marine ecosystems, increase the transmission of viruses and pathogens, and create significant damage to the flow of the food-chain (Simberloff *et al.*, 2013). Concerns over marine and coastal ecosystems, NIS are being invaded to a new environment because of various human activities such as fisheries, shipping, ornamental and live seafood trades, opening and construction of canals, climate change, habitat modification and aquaculture sites, Marinas may act as hotspots for several aquatic marine biological invasion species and promote further establishment of NIS (Occhipinti-Ambrogi and Savini, 2003; Molnar *et al.*, 2008; Williams *et al.*, 2013). Successful establishment of NIS is due to the species characteristics such as broad range of physiological tolerance, rapid growth, polyphagy, high dispersal ability, high genetic variability, high phenotypic plasticity and human association have been put forward for expanding their range in a new habitat (Chan and Briski, 2017).

Once alien species arrived to a new habitat, these nonnative species must overcome all the physical barrier of geography and survive all the environmental conditions and establish a self-sustaining population (Blackburn *et al.*, 2011). Marine Ecosystem that are tending to susceptible invasion of NIS invading the native habitat, have several criteria of environmental condition such as; few natural enemies, low species diversity, high environmental heterogeneity, a history of habitat disturbances (Levine *et al.*, 2004; Fridley *et al.*, 2007; Melbourne *et al.*, 2007; Herborg *et al.*, 2007; Clark and Johnston, 2011). In addition to that several evolutionary processes such as; genetic drift, adaptation, genetic bottleneck effect, selection and admixture can strongly influence the successful establishment of NIS and helps in proliferation inside a new environment (Sakai *et al.*, 2001; Lee, 2002; Roman and Darling, 2007).

During recent centuries, a no. of nonnative marine fishes are invaded into Bay of Bengal causing community shift in their native habitat. This community shift results alter in species composition, which can indirectly change the structural properties of marine habitat. This change in species composition will provide information about ecological disturbance in both native and nonnative habitat (Scheffer *et al.*, 2001; Scheffer and Carpenter, 2003). In order to find out the amount of change in species composition in a

certain habitat, accurate and proper identification of fish species is a prime important work. Earlier studies show that, there are several methods are developed for species identification such as; classical morphotaxonomy, commercial technologies such as immunological assay and cytotaxonomy (Phillips and Ráb, 2001). Frequent change in phenotypic characters, relative costlier process and comparatively lack of expert knowledge are known to be the main drawbacks of earlier studies for species identification. In the recent past, DNA barcoding method has successfully implemented as a robust molecular tool for more accurate species identification (Hebert *et al.*, 2003; Frézal and Leblois, 2008; Leray and Knowlton, 2015). Earlier studies have already proven that mitochondrial cytochrome oxidase-I (COI) is a highly conserved gene used as a barcode marker for most animal species identification (Hebert *et al.*, 2003). The COI-based DNA Barcoding is the most authenticate and versatile method for species identification and have the ability to analyze high rates of sequence changes accompanied with intraspecific divergence at species level (Ivanova *et al.*, 2012; Vences *et al.*, 2012).

Haemulidae is one of the ten diverse, widespread and conspicuous families within the largest sub-order of teleost fishes, the Percoidei (Nelson *et al.*, 2016). They are commonly called grunts, because of their ability to create uproarious sounds by rubbing their pharyngeal teeth together (Burkenroad, 1930). Haemulids have a tendency to congregate during the day and afterward spread out for scavenging around night. The family contains about 145 extant species currently classified in 19 nominal genera (Forese and Pauly, 2017) and grouped into two sub-families i.e. Haemulinae and Plectorhinchinae. The Haemulidae species are morphologically diversified fishes with wondrous and changeable coloration and inhabit the coastal waters in tropical, sub-tropical & temperate inshore reef areas of Atlantic, Indian and Pacific Ocean.

On the other hand, Rabbit fish (Family Siganidae that only include the genus *Siganus*) are morphologically very uniform group under global fish diversity of coral reefs of order Perciformes (Oh *et al.*, 2007). The members of this family Siganidae are also known as spinefoot, demarcated by different characters like the arrangement of spines (Johnson and Gill, 1998) and exhibits uniform phenotypic characters (i.e. dorsal fins with 13 spines and 10 rays and anal fins with 7 spines and 9 rays). Fishes of the family siganids are the primary consumers of coral reefs and act as an active herbivore, exhibits important component in coral communities. The distribution pattern of family Siganidae is restricted to the Indian Ocean and East Andaman Sea, comprising of 29 nominal species (Froese and Pauly, 2017).

Herein we report recent biological invasion of two marine fishes namely banded grunter *Pomadasys andamanensis* and mottled spinefoot *Siganus fuscescens* from Odisha coast, Bay of Bengal, applying the identification of diagnostic morphological and meristic features and subsequently corroborated by DNA barcoding data using single gene marker mitochondrial cytochrome oxidase subunit-I (COI).

Materials and methods

Study area and fish sampling

During a scientific research expedition, two specimen of marine fish species namely *Pomadasys andamanensis* (Family Haemulidae) and *Siganus fuscescens* (Family Siganidae) were caught with gill nets by fishermen in the nearby coastal waters of the Bay of Bengal (Lat 19.26° N and Long 84.86° E), Odisha coast, India (Fig. 1). Immediately, these fish specimens were transported to laboratory under freezing condition for identification. The whole specimens were photographed and vouchered for morpho-taxonomy studies.

Preservation and taxonomic identification

In all cases, the fishes were dead when available for taxonomy and genetic studies. All the Vouchered specimens were stored in -20° C for future morphological studies. After identification all the specimens were fixed with formalin and preserved in 70% ethanol for long term storage.

Specimens were categorized systematically based on the taxonomic characters as outlined in Commercial Sea fishes of India (Talwar and Kacker, 1984) and reconfirmed following the taxonomic keys and species nomenclature outlined in Catalog of Fishes (Fricke *et al.*, 2022, available at: http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp).

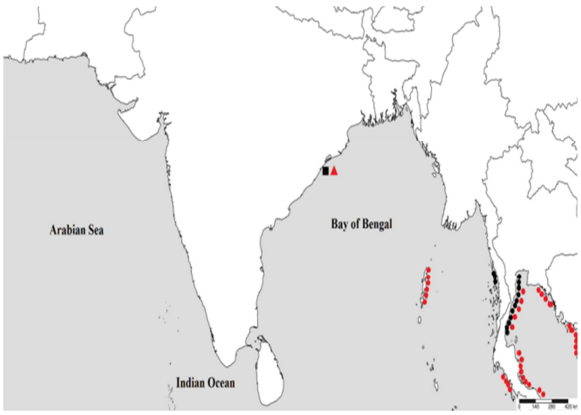

Fig. 1. Distribution map of *Pomadasys andamanensis* and *Siganus fucesence*. The circles (Black and Red colour) showing the natural/previous site records of *Pomadasys andamanensis* and *Siganus fucesence* respectively. While the rectangle and the triangle in Northwest Bay of Bengal show the new site record of these fish

Molecular analysis: DNA extraction, PCR amplification and sequencing

Total genomic DNA was isolated from the preserved muscle tissue according to the salting out method (Sambrook *et al.*, 2001) with some minor modifications. The concentration and purity of the extracted DNA was analyzed through using Nano Drop Lite spectrophotometer (Thermo Scientific, USA). Further, the concentrations of the DNA samples were adjusted to 100 ng/µl by diluting with ultrapure water and the diluted DNA was evaluated using 1.2% agarose gel electrophoresis and was stored at -20° C until further use. The PCR amplification was carried out for ~ 655 bp of the 5´ end of mitochondrial cytochrome c oxidase subunit I (mtCOI) gene, using earlier described standard primes (Table 1). In brief, the PCR was carried out containing 100ng of template DNA, 0.2 µM of each primer, 0.2 mM of dNTPs mix, 0.6 U of *Taq* DNA polymerase and 1x PCR assay buffer in a total volume of 50 µl. The thermal regime for the mtCOI gene amplification was one cycle of 2 m @ 94°C followed by 35 cycles * (30 s @ 95°C, 40 s @ 52°C, 1 m @ 72°C), and final one cycle of 10 m @ 72°C. The quality of the amplified product was verified using 1.2% agarose gel and subsequently cleaned by using QIAGEN PCR purification kit following the manufacturer's protocol. Finally, the cleaned PCR products were outsourced for sequencing.

Table 1. Primer details used in this study to generate COI gene barcode sequences

Primers for COI gene amplification	References
FishF1-5′TCAACCAACCACAAAGACATTGGCAC3′ FishF2-5′TCGACTAATCATAAAGATATCGGCAC3′ FishR1- 5′TAGACTTCTGGGTGGCCAAAGAATCA3′ FishR2-5′ACTTCAGGGTGACCGAAGAATCAGAA3′	Ward *et al.*, 2005

Phylogenetic analysis

Prior to data analysis, the trace files were handled and filtered by considering various parameters. The resultant DNA sequences were found to be larger than 650 bp and were submitted to NCBI (National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/). To create a phylogenetic relationship among and between species, the generated nucleotide sequences were used for phylogenetic reconstruction by using distance-based neighbor-joining (NJ) approach.

Prior to the analysis all the nucleotide sequences (both generated and acquired) were tested for substitution saturation and also for redundancy using METAPIGA 3.01 (Helaers and Milinkovitch, 2010). The Kimura-two parameter (K2P) often considered as a standard for DNA barcode data analysis was also calculated using Mega X (Kumar *et al.*, 2018). In NJ phylogenetic relationship, the nodes were supported with 1000 bootstrap replicates.

Molecular dating and relaxed-clock partitioning

Molecular dating for divergence time estimation of both the species were calculated using RelTime with Dated Tips (RTDT) algorithm implemented in MEGAX (Kumar *et al.*, 2018). The generated mitochondrial COI gene sequences of both the species along with other retrieved sequences (from NCBI gene bank) of same and closely related species were taken into consideration for the divergence time analysis. As the RelTime with Dated Tips (RTDT) method only requires the minimum and/or maximum boundaries of calibration, we choose the fossil evidence time as boundaries from the original studies such as fossil evidence of the genus *Cosmoptychius Striatus*. Wardie Shales, Lower Oil Shale Group, Scotland (Dineley and Metcalf, 1999) for both species. Based on the assumption that, the equal rates of evolution were not testable in between the in-group and out-group sequences (Kumar *et al.*, 2018), the out-group clade was automatically removed during the analysis.

Results

Specimens examined

During a periodic investigation of marine fish and fauna of Gopalpur-on-sea, Odisha coast, Bay of Bengal, we encountered two fish species belongs to the family Haemulidae and Siganidae. Later on, both the fish samples, were morphologically confirm to their respective species level as *Pomadasys andamanensis* (Fig. 2) and *Siganus fuscescens* (Fig. 3).

Fig. 2. *Pomadasys andamanensis*, Voucher Q216, 75 mm SL, Gopalpur coast, Northwest Bay of Bengal, Odisha, India

After thorough examination and analysis of earlier records, we confirmed that both the species are not native to Odisha coast of Bay of Bengal. Thereby, we are able to report the arrival of both the species for the first time from Gopalpur-on-sea, Odisha coast, Bay of Bengal.

Morphological taxonomy

Andaman Grunter, *Pomadasys andamanensis* (Mckay and Satapoomin, 1994) were collected in adult stage by gillnets. The *P. andamanensis* belongs to haemiludae is a reef-associated fish bears some of the general morphological characters of this family such as Oblong, compressed, perch like fishes with a maximum length of 75cm, chin with 2 pores anteriorly and, in all but 1 genus, a median groove, No teeth on roof of mouth and posterior margin of suborbital not exposed, with some special characters such as silvery white with 4 horizontal black or dark brown stripes on dorsal half of body; anal fin with a dark brown streak or blotch covering anterior two thirds of soft-rayed portion. The measured total length (TL) and standard length (SL) of the collected specimen was 210 mm and 75 mm respectively. The head length (HL) to snout length (SL) ratio of the identified specimen was measured to be 4.3.

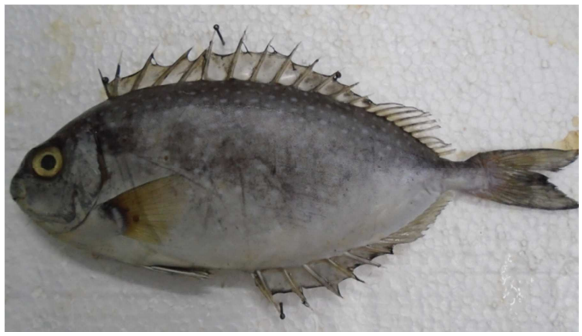

Fig. 3. *Siganus fucesence*, Voucher Q274, 164 mm SL, Gopalpur coast, Northwest Bay of Bengal, Odisha, India

On the other hand, mottled spinefoot, *Siganus fuscescens* (Houttuyn, 1782) is also a reef-associated fish belong to family Siganidae, endemic to the Western Indian Ocean. The *S. fuscescens* bears few general morpho-characters such as; very small mouth with terminal pattern, non-protrusible jaws, and a short sharp dorsal spine projecting forwardly, with some special characters such as; presence of 4 to 6

rows of spots between first spine of dorsal fin and lateral line. The measured total length (TL) and standard length (SL) of the collected specimen was 197 mm and 164 mm respectively. The head length (HL) to snout length (SL) ratio of the identified specimen was measured to be 4.4. The details of morphometric and meristic characters of both the species are described in (Table 2).

Table 2. Detailed morphometric and meristic characters of *Pomadasys andamanensis* and *Siganus fucesence*

Morphometric characters (mm)	*Pomadasys andamanensis*	*Siganus fucesence*
Total length (TL)	210	197
Standard length (SL)	75	164
Morphometric measurements (% SL)		
Fork length (FL)	264	112
Pectoral fin length	70.6	17
Pelvic fin length	52	13.4
Anal fin length	33.3	41.4
Dorsal fin length	121.3	67.6
Head length (HL)	72	23.7
Pre-dorsal length	101.3	26.8
Pre-anal length	156	51.2
Pre-pectoral length	73.3	22.5
Pre-pelvic length	85.3	30.4
Body depth	98.6	35.3
Caudal height	50.6	24.3
Dorsal fin height	40	11.5
Anal fin height	49.3	8.5
Peduncle depth	29.3	6.0
Caudal fin length	57.3	23.7
% of HL		
Eye diameter	29.6	33.3
Snout length	16.6	15.3
Pre-nasal length	27.7	33.3
Inter-orbital width	25.9	35.8
Meristic features (Numbers)		
Dorsal fin rays	XII,15	XIII, 10
Pectoral fin rays	17	15
Anal fin rays	III, 8	VII, 9
Caudal fin rays	17	16
Pelvic fin soft rays	I+ 5	III, 2
Caudal peduncle scales	22	-
Pored lateral-line scales	49-51	-
Scales above and below lateral line	7/14	-

Phylogenetic analysis

The COI barcode sequences of both the species were generated. The length of edited barcode generated from both the species, such as *Pomadasys andamanensis* and *Siganus fuscescens* was 682bp and 664bp respectively with >98% homology score in BLAST search engine. The COI sequence analysis of *Pomadasys andamanensis* revealed the average nucleotide frequencies as 22.3% (A), 29.7% (T), 29.5% (C) and 18.5% (G). Similarly, in *Siganus fuscescens* the nucleotide frequencies are 24.2% (A), 29.1% (T), 28% (C) and 18.7% (G). None of the generated COI sequences were found to be tagged with nuclear signatures in the form of indels and heterozygous sites.

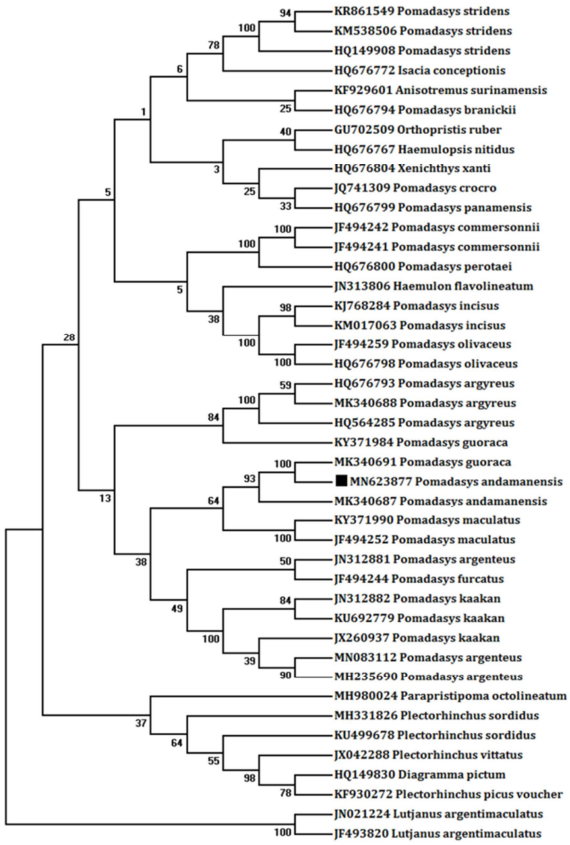

Fig. 4. Neighbor-joining phylogenetic tree for *Pomadasys andamanensis*. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Accession no. MN623877 (Solid Rectangle marked) generated in this study.

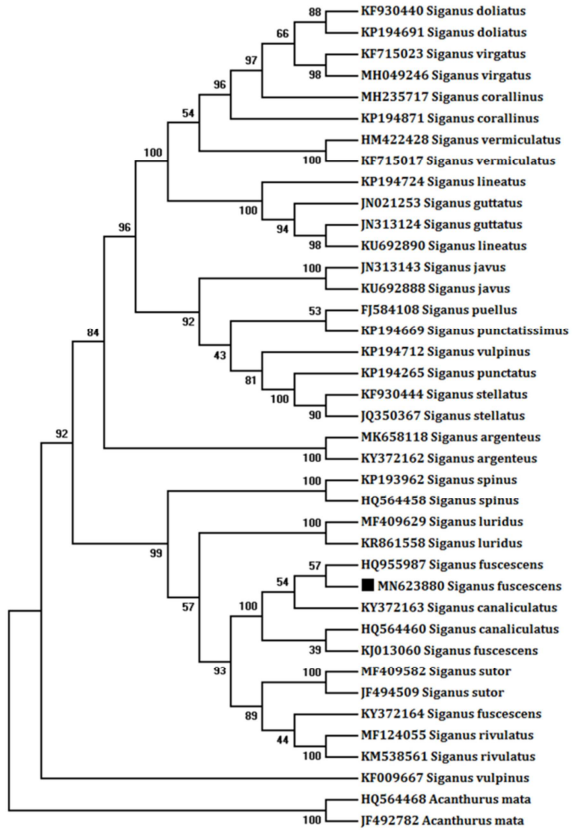

Fig. 5. Neighbor-joining phylogenetic tree for *Siganus fucesence*. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Accession no. MN623880 (Solid Rectangle marked) generated in this study

The distance-based neighbor-joining (NJ) phylogenetic analysis was carried out by using K2P distances of the COI gene sequences of both the species along with all downloaded sequences from GenBank. The NJ tree of *P. andamanesis* (GenBank accession number MN623877) successfully clustered together with other specimens of *P. andamanesis* suggesting a monophyletic lineage (Fig. 4). Similarly, the NJ tree for *S. fuscescens* (GenBank accession number MN623880) also showed same type of tree topology by forming a clade with other specimens of *S. fuscescens* (Fig. 5).

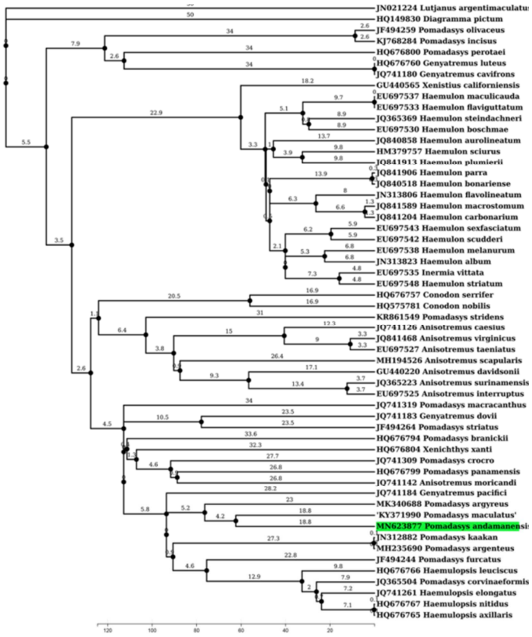

Fig. 6. Time-calibrated phylogenetic tree of *Pomadasys andamanensis* and related lineages with fossil calibration data applied to root. A timetree inferred using the Reltime method and the General Time Reversible model. The timetree was computed using 1 calibration constraints. The estimated log likelihood value is -9437.11. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3981)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 32.18% sites). The figures on the branches represents the time (in Mya) of different nodes

Molecular dating analysis

Divergences with the well-established genera *Pomadasys* and *Siganus* were estimated to have occurred in the early Cenozoic era in the Tertiary period and Pleistocene epoch. The separation of *Pomadasys* clade from the common ancestor was estimated to occur in the upper Cenozoic era approximately 34.04 Mya (95% confidence intervals) and that of the species *Pomadasys andamanensis* was estimated to occur in the early Miocene sub-epoch approximately 18.82 Mya (95% confidence

intervals) (Fig. 6). While, the separation of *Siganus* clade and that of *Siganus fuscescens* from the common ancestor were estimated to occur in the late Pleistocene epoch approximately 26.36 Mya (95% confidence intervals) and 0.47 Mya (95% confidence intervals) respectively (Fig. 7).

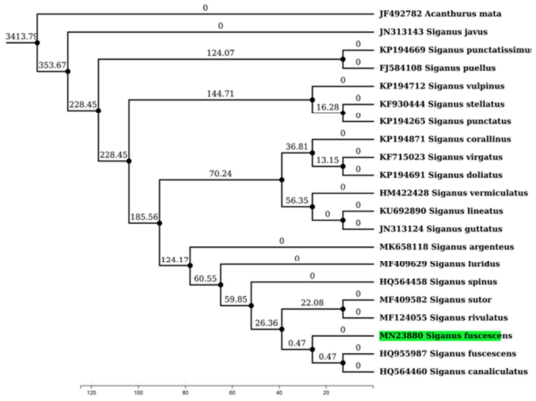

Fig. 7. Time-calibrated phylogenetic tree of *Siganus fucesence* and related lineages with fossil calibration data applied to root. A timetree inferred using the Reltime method and the General Time Reversible model. The timetree was computed using 1 calibration constraints. The estimated log likelihood value is -2966.98. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2993)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 36.30% sites). The figures on the branches represents the time (in Mya) of different nodes.

Discussion

Some crucial factors such as presence of phenotypic plasticity among the species and less expertise taxonomy knowledge mislead the way of morphological species identification process that may result in misidentification. In this regard, molecular taxonomy in the form of DNA barcoding gains immense importance as an effective tool for accurate species identification especially for damaged specimens and/or species consisting of several morphologically distinct characters. In a generalized acceptable term, DNA barcode analyses work quite well in circumscribing potentially recognizable species in various groups ranging from genera to families. DNA barcoding define itself as a DNA fragment commonly shared by different organism having significant interspecies difference. However, DNA barcoding also has some limitations. In some cases, very closely related species may present identical sequences making DNA barcoding ineffectual for accurate species identification.

In this study, we successfully amplified the COI barcode sequences of two fish species that are new to this geographic location. The identification results through DNA barcoding were in agreement with that of the morphological identification. Earlier records have the evident of successful identification of marine ichthyofauna along with the monitoring of non-native species through DNA barcoding approach in other geographic regions (Bingpeng *et al.*, 2018).

The base composition analysis of the COI gene revealed that, AT content is higher than the GC content for both the species. The different codon positions mostly the second and third position were affected by the variation in GC content. In our result the range of variation in codon is highest in the third position in compare to the second position. The variation in codon positions is an indicator of degree of selective constraint. Therefore, the GC content analysis could provide a significant insight into the impact of natural selection on the nucleotides (Clare *et al.*, 2008).

The phylogenetic analysis of both the species through NJ showed the strongest clustering of both the species into respective monophyletic clades, proving the efficiency of DNA barcoding in accurate species delimitation. But occasional misidentification and nucleotide saturation through substitution may sometimes alter the outcome of phylogenetic analysis. To provide more supports to the resultant NJ phylogeny, we reconstructed the phylogenetic tree with maximum likelihood approach.

However, both the phylogenetic analysis ended up with same tree topology. However, some

discrepancies in the phylo-analysis were observed in the species *Siganus fuscescens*. The species forms a mixed clade with *Siganus canaliculatus* due to genetically very closeness and did not form distinct monophyletic clusters and were not clearly separated. Moreover, the morphological taxonomy in the family Siganidae is contentious, and several disputes about species delimitation have arisen.

The relaxed clock molecular dating phylogeny of both the species revealed that the *Pomadasys* genus has radiated from the common ancestor in the 34.04 and that of the *Pomadasys andamanesis* was approximately18.82 Mya. In the other hand, the genus *Siganus* has radiated from the common ancestor in the 26.36 and that of *Siganus fucesence* was approximately 0.47 Mya.

Knowledge of fine-scale patterns of connectivity in migrating organisms also has important implications for the design of marine reserves (Palumbi, 2003; Cowen *et al.*, 2006). This climatic change is impacting the ecology and biogeography of marine fish populations and will continue to do so in the future. Thus, we can expect fish populations in new habitats on a global scale to decline as well as a collapse of many fisheries species (Arvedlund, 2009). An inevitable increase in biological invasions of marine fishes due to globalization is expected in the coming decades, especially in developed countries that already experience a high number of invasion events. To slow down this trend, an increase in our knowledge of potential invasion pathways, the effective storage of interception data, and most importantly the accurate species identification are necessary requirements.

Conclusion

The overall narrated information strongly evident that both the species (*Pomadasys andamanensis* and *Siganus fuscescens*) have significantly broadened their natural and earlier reported geographic range. The northward range expansion holds great biogeographic and conservation significance as both of them are flagship coral reef associated species. The findings mark the first record of these species in Odisha coast, Bay of Bengal region. Although, the single specimens could not predict the settled population structure in this region, but it opens up new possibilities for research and understanding of the ecological dynamics in this area. This first encounter may lead to further exploration and discoveries regarding the behavior and distribution of this species in the Bay of Bengal.

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