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Morphological and molecular evidence supports the first occurrence of two fishes, *Siganus sutor* (Valenciennes, 1835) and *Seriolina nigrofasciata* (Rüppell, 1829) (Actinopterygii: Perciformes), from marine waters of Odisha coast, Bay of Bengal, India

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Abstract

Marine ecosystems provide a wide variety of diverse habitats that frequently promote migration and ecological adaptation. The extent to which the geographic distribution of marine organism has reshaped by human activities remains underappreciated. The limitations intrinsic to morphology-based identification systems have engendered an urgent need for reliable genetic methods that enable the unequivocal recognition of fish species, particularly those that are prone to overexploitation and/or market substitution. In the present study, however, an attempt has been taken to identify two locally adapted fish species, *Siganus sutor* (Valenciennes, 1835) and *Seriolina nigrofasciata* (Rüppell, 1829) of order Perciformes, which happens to be the first record in Odisha coast, Bay of Bengal. The diagnostic characteristics of *Siganus sutor* are: dorsal fin XIII-10, anal fin VII-9, pectoral fin 15, pelvic fin II-3, while that of *Seriolina nigrofasciata* dorsal fin VI-I-35, anal fin I-17, pectoral fin 16, pelvic fin 5. All COI barcodes generated in this study were matched with reference sequences of expected species, according to morphological identification. Bayesian and likelihood phylogenetic trees were drawn based on DNA barcodes and all the specimens clustered in agreement with their taxonomic classification at the species level. The phylogeographic studies based on haplotype network and migration rates suggest that both the species were not panmitic and the high-frequency population distribution indicates successful migration. The result of this study provides an important validation of the use of DNA barcode sequences for monitoring species diversity and changes within a complex marine ecosystem.

Key words: *Siganus sutor*, *Seriolina nigrofasciata*, morphological characteristics, DNA Barcoding

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1 Introduction

Every species occupies an ecological niche or range whose boundaries reflect the breadth of the environmental conditions that the species can tolerate. Intolerance to extreme environmental conditions can lead to specie dispersal within and beyond their geographic range (Spicer and Gaston, 1999). It is clear that all species do not harbor the adaptive potential to cope with the rapid environmental changes within their native range (McCarty, 2001; Hoffmann et al., 2003) and evidence shows that changes in species distribution is frequently associated with climate change (Stewart and Lister, 2001; Davis et al., 2005). However, migration of species into new areas is often associated with entrance into novel habitats where the selective regimes are likely to differ from that they have experienced before (Davis et al., 2005; Phillips et al., 2006).

Most marine fish populations have traditionally been regarded as large panmictic entities with high connectivity due to the apparent lack of geographical barriers, high dispersal capabilities, and slow genetic drift as a result of large effective population size (DeWoody and Avise, 2000; Waples and Gaggiotti, 2006; Allendorf et al., 2010). However, this assumption is challenged by an increasing number of genetic studies reporting high levels of local adaptation in marine fish populations despite substantial gene flow (Nielsen et al., 2009; Clarke et al., 2010; Limborg et al., 2012; Therkildsen et al., 2013; Milano et al., 2014).

The Bay of Bengal is the largest triangular basin in the Indian Ocean. It is not only the largest basin but also have one of the most unique marine ecological systems in the world. Its unique ecological system makes it more vulnerable, susceptible and prone to the advent of incipient species, compared to other ecosystems. In other hand, climatic changes and other regulatory factors responsible for species dispersal, has accelerates the rate of introduction of non-native marine organisms mostly from tropical and sub-tropical regions into the Bay of Bengal (Barik et al., 2018).

A variety of methods have been used for the identification of

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fish species. The first indication of its existence is typically the observation of phenotypic traits such as growth, fecundity, behavior and most importantly species identification. There is a growing body of quantitative research detailing morphological differences between populations of locally adapted species. In addition, experimental measures of differentiation may not have the resolution to detect the subtle difference between the populations, or may not measure the particular trait on which adaptation is acting (Tepolt, 2015). Thus, it is essential to critically evaluate methods for determining both the identification of species and species boundaries.

DNA barcoding happens to be a rapid and reliable technology to identify species based on the sequence of short standard DNA region(s) that are universally present in the target lineages and have sufficient sequence variation to recognize species (Hebert et al., 2003). The sequence of the "Folmer fragment" (Folmer et al., 1994), a polymorphic part of the mitochondrial cytochrome oxidase subunit I gene (COI/COX1), can be used to identify closely related species as well as higher taxa in animal phyla.

The extant species of Siganidae are morphologically a very uniform group of coral-reef fishes of order Perciformes and are known by rabbitfishes or spinefoots. For example, they all exhibit uniformity in those phenotypical characters (i.e., dorsal fins with 13 spines and 10 rays and anal fins with 7 spines and 9 rays) which the systematics of fishes usually rely on. Fishes of this family are regarded as important components in coral reef communities as primary consumers because Siganid fishes are active herbivores. Although the members of Siganidae family are demarcated by different characters like the arrangement of spines (Johnson and Gill, 1998), these are also grouped in three different clades depending upon the body structure such as deep bodied species, tenuous bodied species, and streamlined, spindle-shaped species. Furthermore, for species identification color pattern among the members of cognate species of Siganidae are of prime importance and is the universal meristic character. The distribution pattern of family Siganidae is restricted to the Indian Ocean and East Andaman Sea, comprising of 29 nominal species in a single genus, *Siganus* (Froese and Pauly, 2017). While the family Carangidae is the most diverse radiation of marine fishes composed of 146 valid species in 30 genera (Eschmeyer and Fong, 2017). *Seriolina nigrofasciata* (Valenciennes, 1835) commonly named as black-banded trevally, is the sole member of genus *Seriolina*, native to the Indian Ocean, the western Pacific Ocean and the Atlantic coast of South Africa. However, genus *Seriolina* has close morphological relationships with genus *Seriola*.

In the present study, an attempt has been taken to elucidate the identification of two marine fishes on the substructure of morphological diagnostic characters (i.e., both morphometric and meristic), such as total and standard length, number of fins and fin-rays, etc. and putative molecular features (operational taxonomic units, evolutionary species, and phylospecies) delimitated utilizing different approaches on the substratum of a single-gene marker (i.e., the mitochondrial cytochrome oxidase subunit I – COI/COX1).

2 Materials and methods

2.1 *Fish sampling and preservation*

Fish were caught by fishermen in the nearby coastal waters of the Bay of Bengal (19.26°N, 84.86°E), Odisha coast (Fig. 1), during the post-monsoon period of year 2016–2017. Three fish specimens of order Perciformes were collected from fishermen. In all cases, the fishes were dead when available for taxonomy and genetic studies. Lateral muscle tissue and fin clips from the right-hand side were removed from each individual and stored in 95% ethanol under deep freezing condition for DNA extraction. All the

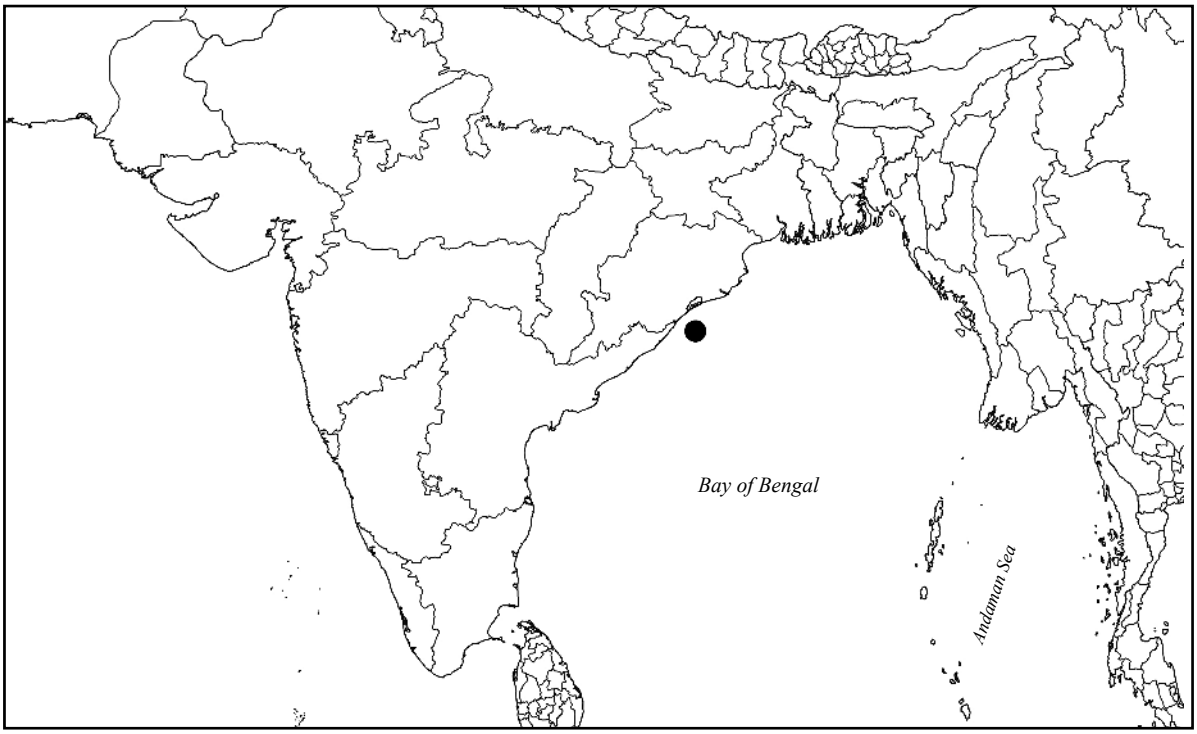

Fig. 1. Geographical site of collection of fish species. The spot (black) in the northwester Bay of Bengal shows the site of collection of *Siganus sutor* and *Seriolina nigrofasciata*.

specimens were vouchered and stored in –20°C for further morphological studies. After identification, all the specimens were formalin fixed and preserved in 70% ethanol for long term storage at Department of Zoology, Berhampur University, Odisha.

2.2 *Taxonomic identification*

Specimens were categorized systematically based on the taxonomic characters available from the original description as well as subsequent re-descriptions and taxonomic reviews. The morphometric variables were measured and were confirmed by comparing with the described taxonomic keys available in the leading taxonomic guides, e.g., Commercial Sea Fishes of India (Talwar and Kacker, 1984). However, the samples were designated into the respective species as per the authoritative taxonomic keys and species nomenclature follows the Catalog of Fishes (Eschmeyer et al., 2017).

2.3 *Extraction of DNA and quantification*

Total genomic DNA was isolated from the stored muscle tissue according to the salting out method (Sambrook and Russell, 2001) with some minor modifications. The concentration and purity of the extracted DNA was estimated using NanoDrop Lite spectrophotometer (Thermo Scientific, USA). Extracted DNA was stored at –20°C until further use.

2.4 *PCR amplification and sequencing*

The extracted DNA from all specimens were used as a template for multiplex PCR amplification with oligonucleotide primers reported in Ivanova et al. (2007), targeting the 5′ region of mitochondrial COI gene (Table 1). The amplification was performed in 25 μL reaction mixture of 100 ng template DNA, 10 μmol/L of each specific primer, 10 mmol/L of dNTPs mix, 10% Trehalose, 1.0 unit of DreamTaq DNA polymerase (Thermo Scientific, USA) and 1× PCR assay buffer containing 20 mmol/L $MgCl_2$. The PCR conditions were initial denaturation at 95°C for 2 min, followed by 35 cycles of 30 s at 94°C, 30 s at 54°C, 60 s at 72°C and a final extension at 72°C for 10 min. The PCR products were visualized by electrophoresis on 1.5% agarose gel containing ethidium bromide (10 mg/mL). Prior to sequencing PCR products were purified with PCR purification kit (Qiagen, USA) following the manufacturer's protocol and the most intense purified products were sequenced commercially with the primers for M13-tailed PCR products (Messing, 1983) (Table 1).

2.5 *Sequence data analysis*

Prior to analysis, electropherograms were base-called using PHRED and only the sequences with Phred scores more than 20 (i.e., 99% correct base calling) were considered for further analysis. The PCR amplified products as well as their corresponding DNA sequences were larger than 650 bp. The noisy sequences were trimmed at both ends using Geneious R8 software (Biomatters, NZ). In some cases of discrepancy, both the sequences were reviewed manually and quality value of the sequences were considered to determine the most likely nucleotide using the software Chromas version 2.6.4 (Technelysium, Tewantin, Australia). The protein-coding sequences were translated to amino acid and no stop codons were found. BLASTN program implemented in NCBI (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov) web server, was used to ascertain if the sequence was of the locus targeted (Altschul et al., 1990) and the fragment showing 100% alignment with no gap or indel (insertion/deletions) was selected. The assembled sequences were aligned using MAFFT v 7.22 (Katoh and Standley, 2016). Ambiguous sites from the alignment matrices were trimmed with *trimAl* using the heuristic algorithm *automated1* (Capella-Gutiérrez et al., 2009), which uses the distribution of gaps and similarities to determine the thresholds for removing the poorly aligned sites of an alignment. The alignment conservation and confidence score was calculated using GUIDANCE2 server (http://guidance.tau.ac.il/ver2/). The sequences were submitted to NCBI and BOLD databases and accession numbers as well as BINs were obtained.

2.6 *Phylogenetic analysis*

Phylogenies were next reconstructed using Bayesian inference (BI) and maximum likelihood (ML) approaches. Bayesian phylogenetic analysis was conducted with MrBayes 3.2.6 (Ronquist and Huelsenbeck, 2003) using COI sequences from 51 individuals of 16 species of family Siganidae and 29 individuals of 11 species of family Carangidae. Three partitions (first, second and third codon positions) were set in the dataset, assuming that functional constraints on sequence evolution are similar within codon positions. The general time reversible model with assumption that some sites are invariable and the others variable following a discrete gamma distribution (GTR+I+Γ; Yang, 1994) was selected as the best fit model of the nucleotide substitution by Model Test version 3.7 (Posada and Crandall, 1998) for all partition. We used the default values for the temperatures of chain heating and the number of Metropolis-coupled Markov chain Monte Carlo chains for the replicate runs with 5 000 000 generations. We ensured a minimum value of 100 for the effective sample sizes of each parameter. A total of 50% majority-rule consensus phylograms and posterior probabilities were obtained with a burnin fraction of 25%. The parameters used in MrBayes as follows: "lset nst=6", "rates=invgamma (I+Γ)", "mcmc ngen= 5 000 000", "samplefreq=100", "nchains=4", and "prset ratepr=variable". ML analyses were performed using RAxML-HPC (Stamatakis, 2006). The best-fitting model of evolution, GTR+G+I, was determined with Akaike Information Criterion (AIC) as implemented in the R package Phangorn (Schliep, 2011). A total of 1 000 ML bootstrap replicates were performed.

The phylogenetic positions of the two sampled species in cognation to other species of family Siganidae and Carangidae re-

Table 1. PCR primers used for the DNA barcoding

Primer	Primer sequence (5′ to 3′)	mtDNA target	Amplicon size/bp	Reference
Fish DNA barcoding primer cocktail (C_FishF1t1/C_FishR1t1) (ratio 1:1:1:1)				
VF2_t1	TGTAAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC	Cytochrome c oxidase 1 (COI) gene	652	Ivanova et al. (2007)
FishF2_t1	TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC			
FishR2_t1	CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA			
FR1d_t1	CAGGAAACAGCTATGACACCTCAGGGTGTCCGAARAAYCARAA			
M13F*	TGTAAAACGACGGCCAGT			Messing (1983)
M13R*	CAGGAAACAGCTATGAC			

Note: COI amplification in our study was done by the use of Fish cocktail primers. * Sequencing primers for M13-tailed PCR products.

spectively were investigated using comprehensive mitochondrial sequence datasets. The sampled specimens were invariably sequenced and their congeneric sequences were acquired from the database (GenBank) to examine the caliber of intraspecific variation. The GenBank accession numbers along with respective species names of the developed as well as acquired sequences are mentioned in the figures representing phylogenetic analysis.

2.7 *Genetic diversity*

In order to derive haplotype diversity, both overall diversity comparison and pairwise comparison between locations were carried out. A median-joining network (Bandelt et al., 1999) was constructed in PopART v1.7 (http://popart.otago.ac.nz) to determine the genealogical relationships among haplotypes. Mobile species subjected to genetic statistical differentiation tests often to fail to display minor amount of population subdivision even if they exist (Palumbi and Warner, 2003). Therefore, we used Spatial Analysis of Shared Alleles (SAShA) (Kelly et al., 2010) implemented in MATLAB environment. SAShA generates the observed distribution of geographic distances of each haplotype, as well as a null distribution generated from the same data. SAShA tests for a significant deviation between the arithmetic mean of the observed distance distribution (ODD) and that of the expected distance distribution (EDD). An ODD significantly less than EDD indicates that alleles are under-distributed, and therefore gene flow restricted. We tested for significance of the difference between ODD and EDD using 10^4 permutations.

Population pairwise estimates of migration rates based on the mtDNA (COI gene) sequence data were calculated under the "isolation with migration" model implemented in IMa (Hey and Nielsen, 2007). This model does not assume the migration-drift equilibrium, and is thus appropriate for recently diverged populations that share haplotypes because of both gene flow and the retention of ancestral polymorphism. Initial runs were carried out to set upper limits for parameter prior. Three independent runs were carried out for 50 million generations, with a burn-in of 5 million generations and sampling every 100 generation. Convergence of parameter distributions was confirmed by examining effective sample sizes and concordance between runs. Genealogies from the three runs were combined in a single final run, and the peaks of the marginal posterior distributions were estimated for the migration parameters m_1 and m_2. The numbers of migrants per generation (N_m) were calculated as $N_m = (\theta M)/4$, where θ indicates effective population size and M indicates corresponding migration rate (Marko and Hart, 2011).

3 Results

Fish species belonging to the families Siganidae and Carangidae were collected and identified on the basis of classical morphotaxonomy and further confirmed by molecular tool using DNA barcoding. In this study, we are able to record for the first time two different fish species such as; two specimens of *Siganus sutor* (Valenciennes, 1835) and one specimen of *Seriolina nigrofasciata* (Rüppell, 1829) of order Perciformes, from Odisha coast, Bay of Bengal. The morphometric as well meristic data of both the species were presented in Table 2.

Shoemaker spinefoot, *Siganus sutor* were collected in adult stage by gillnets. The species is endemic to the Western Indian Ocean. It is resilient with a minimum population doubling time of less than 15 months. *Siganus sutor* spawn year-round with two

Table 2. Comparative morphometric measurements and meristic counts of the two species under study

Morphological parameters	*Seriolina nigrofasciata*		*Siganus sutor*	
	Present study	Species studied by Paxton et al. (1989)	Present study	Species studied by Woodland (1990)
Total length (TL)/mm	164	153	170	158
Standard length (SL)/mm	135	130	145	134
Measurements in %SL				
Fork length	109	105	110	114
Head length	29.62	26.93	25.52	23.88
Pre-anal length	70.37	66.15	51.72	47.76
Pre-dorsal length	37.04	29.23	28.27	22.38
Pre-pelvic length	32.6	26.92	31.72	30.6
Pre-pectoral length	30.38	26.92	24.82	24.62
Body depth	32.6	27.69	35.17	44
Dorsal fin length	57.77	–	66.2	–
Head length (HL)	27.4	26.92	25.51	23.88
Measurements in %HL				
Eye diameter	32.44	20	32.43	31.25
Pre-orbital length	43.24	28.57	43.24	37.5
Snout length	27	–	35.13	–
Caudal height	54	–	94.6	–
Meristic parameters	***Seriolina nigrofasciata***		***Siganus sutor***	
	Present study	Species studied by Paxton et al. (1989)	Present study	Species studied by Woodland (1990)
Dorsal spines	VI+I	VI+I	XIII	XIII
Dorsal soft rays	35	34	10	10
Anal spines	0+I	0+I	VII	VII
Anal soft rays	17	16	9	10
Pectoral fin rays	16	–	15	–
Pelvic fin rays	5	–	II+3	–
Caudal fin rays	18	–	18	–

peaks occurring one to two months after each monsoon starts (Ntiba and Jaccarini, 1992). It lives in shallow coastal waters to a depth of 50 m. Body laterally compressed, oval and a very small terminal mouth. The dorsal fin with XIII strong spines and 10 soft rays; preceded by a short, sharp, forwardly projecting spine. Anal fin with VII strong spines and 9 soft rays. A unique feature of this family which help in accurate identification of species is the presence of pelvic fins with II spines (one strong and one outer) with 3 soft rays in between.

Similarly, the blackbanded trevally, *Seriolina nigrofasciata* is the only species representing the genus under family Carangidae. Body color is olive green to brown above, grading to silver below; 5–7 dark oblique dark bands and blotches on upper body. Dorsal and caudal fins are spinous and dark brown to black in color; soft dorsal and soft anal fins are dusky brown. The dorsal fin with VII or VIII short spines (weak spines), followed by I spine and 30 or 37 soft rays. Anal fin with I detached spine, followed by I spine and 15–18 soft rays.

COI barcodes were recovered for a total of three specimens (two from *Siganus sutor* and one from *Seriolina nigrofasciata*) from the Siganidae and Carangidae families respectively. No insertions/deletions, heterozygous sites or stop codons were observed, supporting the view that all of the amplified sequences constitute functional mitochondrial COI sequences. BLAST outcomes of all nucleotide sequences succeeded to identify sequence similarity of all the species under study. One sequence identified more than one species (species identity of 94%–100%) while the other two sequences identified a single species (species identity of 99%–100%). As expected, transitions were more frequent than transversions (ratio=3.36), a plot of transition and transversions versus genetic distance also confirmed that substitutions had not reached saturation (not shown). Thus, transitions and transversions were equally weighted in our phylogenetic analysis. The COI sequence analysis of *Siganus sutor* revealed the average nucleotide frequencies as 27% (A), 27.1% (T), 23% (C) and 22.9% (G). Similarly, in *Seriolina nigrofasciata* the nucleotide frequencies are 22.5% (A), 30.4% (T), 26.8% (C) and 20.3% (G). The mean nucleotide diversity (Pi) between the two species was estimated as 0.366 6.

The Kimura-2-parameter model is recommended by the Consortium for the Barcode of Life (CBOL) for calculating genetic distance (Kimura, 1980; Shen et al., 2016). In this study, the Kimura-2-parameter model was used to calculate the genetic distances at the intraspecific and intragenus levels for both *Siganus sutor* and *Seriolina nigrofasciata*. The K2P distances of the COI sequence at intraspecific level for *Siganus sutor* ranged from 0% to 2.0%. The genetic distances between species ranged from 0% to 2.8%, with an average of 0.27%. While that of *Seriolina nigrofasciata*, the K2P distance at intraspecific level ranged from 0% to 0.07% and the genetic distance between species ranged from 0% to 0.3% with an average of 0.21%.

Phylogenetic analysis resulted in a well-resolved hypothesis of relationships at the species level. In both, Bayesian phylogenetic and maximum likelihood analysis, new sequences from the two species grouped in different clusters. Besides, where applicable, sequence from the same species (newly obtained in this work and retrieved from NCBI) grouped together, showing homology and more or less conspecific distances between them. *Siganus canaliculatus* and *Siganus sutor* seems to be in one cluster due to their evolutionary closeness (Fig. 2) while *Seriolina nigrofasciata* forms a cluster with genus *Seriola* as these two genera are morphologically very close to each other (Fig. 3). Furthermore, sequences from the same species retrieved from NCBI grouped together in the same cluster. Nevertheless, some species were found mixed. The phylogenetic trees generated through Bayesian and maximum likelihood analysis shows same topology. Sequences from above specimens of *Siganus sutor* and *Seriolina nigrofasciata* were submitted to the NCBI GenBank Barcode database and Barcode of Life Database (BOLD) with accession and BIN numbers KY634867, KY634862, BOLD: ACR6962 and KY634861, BOLD: AAB8502 respectively.

The haplotype network for both the species (Fig. 4) has an overall complex pattern of star-like elements. No evident geographic structure could be depicted from this network, i.e., no discernable association between certain haplotypes and locations can be observed. There was one high frequency, widespread haplotype at the center of both the network, radiated by some small low frequency derived haplotypes, a pattern of population expansion or a selective sweep. The difference between the overall observed distance distribution (ODD) and the expected distance distribution (EDD) of shared alleles generated by SAShA suggest that both the species were not panmictic (ODD=893 km, EDD=1 217 km, p<0.001) (Fig. 5).

Levels of gene flow between pairs of populations revealed by the IMa analysis were generally very high (six migrants per generation) indicating the migration of both the species to their new location. Migration at rates lower than 0.01 migrants per generation had little impact, whereas one migrant per generation tended to lead to the inference of one species. The impact of migration is affected not only by the number of immigrants (N_m) but also by the population size: at the same N_m, a smaller population size (N) means a larger proportion of immigrants (m).

4 Discussion

Dispersal is an essentially imperative and dynamic process, and an increasing figure of evidence recommends that it may play a vital role in marine invasion on various levels. While, migration to local environment have significance importance on the generation and maintenance of biodiversity (Levene, 1953; Gavrilets, 2003), the contraction and expansion of species geographic ranges (Kirkpatrick and Barton, 1997) and evolutionary dynamics of species interactions (Kaltz and Shykoff, 1998). Under any form of anthropogenic change, species will have to move, adapt or die. Progress in genetic studies of adaptation until recently had been constrained by the lack of resolution and absence of genomic perspective. Genetic tools can give us crucial insights into these processes. At the largest scale, molecular tools can identify cryptic species and their introductions, as in this study.

The present study is based on first occurrence of two fish species *Siganus sutor* (Valenciennes, 1835) and *Seriolina nigrofasciata* (Rüpell, 1829) from Odisha coast, Bay of Bengal and exploration of this migration using DNA barcoding. DNA barcoding uses a genetic marker (often a single gene) to assign an individual to a particular known species. It has also been suggested that barcoding can be used to identify unknown species based on the expectation that interspecific genetic divergence considerably exceeds intraspecific variation to form a clear “barcode gap”. In addition to that, these fish species have never been reported earlier, hence this happens to be the first record from Odisha coast, Bay of Bengal. Three fish specimens: two *Siganu sutor* and one *Seriolina nigrofasciata*, were assessed in this work using COI barcodes from a sub-area of the coast.

In the current study, we evaluated different plausible phylogeographic scenario to explain the identification, migration and adaptation of both the species at the new geographical location. Although, the sample size is little less (because of first record

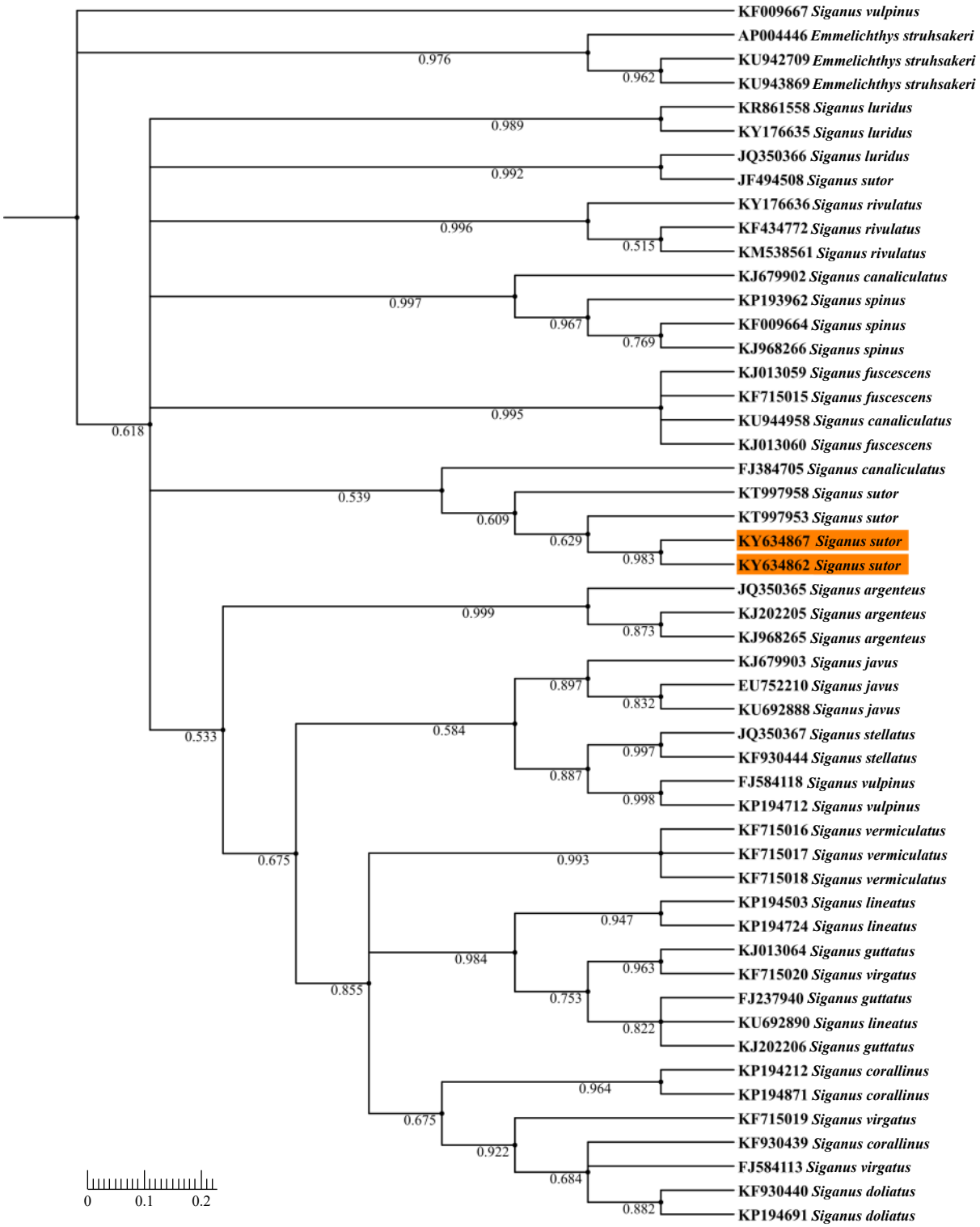

Fig. 2. Phylogenetic analysis of *Siganus sutor* using the maximum likelihood method based on GTR+I+Γ model. The bootstrap consensus tree inferred from 1 000 replicates and less than 50% bootstrap replicates are collapsed. The sequences with colored background were generated in this study.

from Odisha coast in this report) in the present investigation, we tried to confirm the local adaptation of the two fishes in the Bay of Bengal, through genetic diversity study. From the haplotype network one can clearly infer that there is no geographical structure. Pure models of panmixia, secondary contact, and presence of a phylogeographic break do not seem to explain the results obtained while the distance distribution of shared alleles and the high frequency of populations indicates successful migration and local adaptation of both the species. By any sensible species concept, the two populations should be considered one species

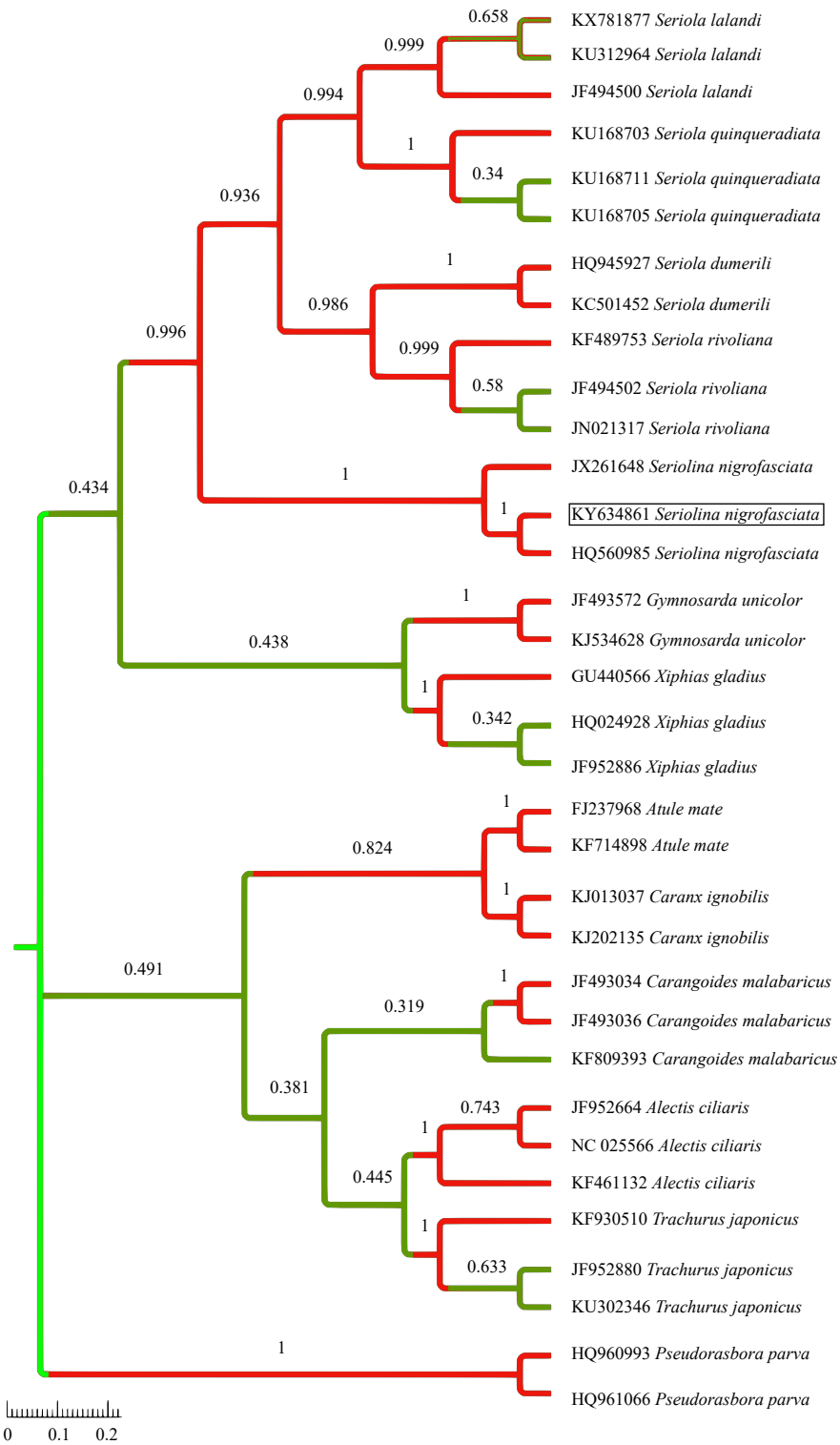

Fig. 3. Phylogenetic analysis of *Seriolina nigrofasciata* using the Maximum Likelihood method based on GTR+I+Γ model. The bootstrap consensus tree inferred from 1 000 replicates and less than 50% bootstrap replicates are collapsed. The sequences within the rectangle box were generated in this study.

when the migration rate and level of hybridization within the population is very high. Our simulation also demonstrates that the method is very unlikely to be misled to infer separate species if samples are taken from distant localities of one species with a wide geographical distribution and experiencing isolation by distance. For this purpose, a model of migration (e.g., Hey, 2010) is useful for estimating parameters such as migration rates when such migrations are known to occur.

Another question is whether a single DNA segment is sufficient for species delimitation and whether the current recommendation of sampling 5–10 individuals (Hajibabaei et al., 2007) is adequate. Our results suggest that one single gene locus may

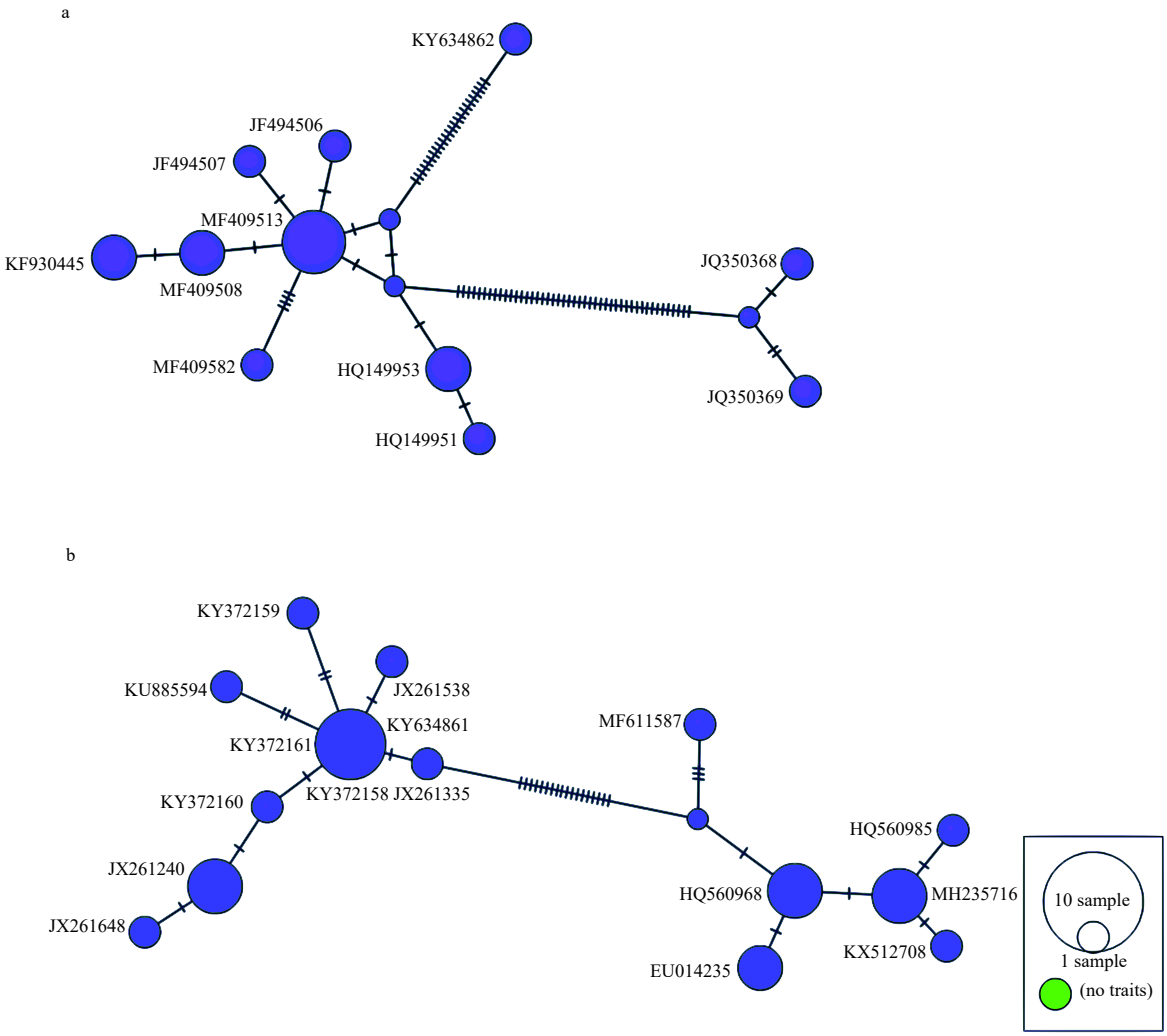

Fig. 4. Median-joining post-processed COI gene haplotype networks for *Siganus sutor* (a) and *Seriolina nigrofasciata* (b). The area of circles is proportional to the frequency of individuals in the sample. Lines are proportional to mutations and dashes along the lines represents the rate of mutations.

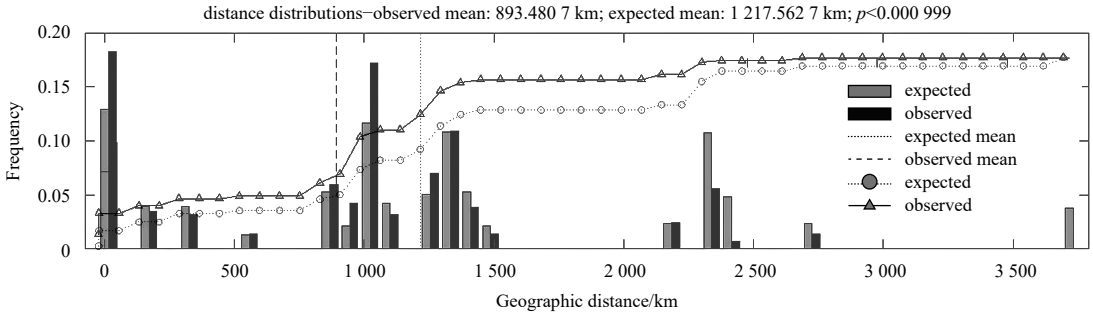

Fig. 5. Spatial analysis of shared COI mtDNA distribution for *Siganus sutor* and *Seriolina nigrofasciata*. The geographic distances observed between co-occurring alleles in the form of histograms and as cumulative frequency plots. The observed and expected mean distances are indicated with vertical lines. Traingles and circles are the cumulative frequency of alleles at increasing distance. The *p*-value is the probability that the observed mean is greater than the expected.

indeed contain enough information to delimit species. However, 15 or more individuals from each species seem necessary if the species divergence is recent, whereas five individuals may be enough for identifying well-diverged species. When it is unfeasible to sample multiple individuals, as with rare or hubert protected species, multiple loci should be used for effective species delimitation. The single gene locus sequence of the same species formed high bootstrap-supported clusters without any overlap between species, even in species within the same genera with exception of the genus *Seriolina*. As *Seriolina* is a monophyletic

genus belong to family Carangidae, it forms cluster with morphologically very close genus *Seriola*. Also, *Siganus canaliculatus* (Park, 1797) forms a cluster with *Siganus sutor* due to their evolutionary closeness (Smith, 1986).

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; Harley 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).

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