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Molecular phylogeny reconstruction and biogeographic pattern of Rays (Elasmobranchii: Myliobatiformes) from Indian coastal waters

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In the present study, the phylogenetic relationship among Myliobatiformes was reconstructed from the Indian waters based on the cytochrome c oxidase subunit I (*COXI*) gene. Overall, 307 sequences (18 collected from Mandapam fish landing centres, Tamil Nadu, and 289 from the previous literatures) from 34 species of Myliobatiformes were clustered under two major phylogenetic clades. The families Mobulidae, Rhinopteridae, Plesiobatidae, and Gymuridae were monophyletic, while the families Myliobatidae and Dasyatidae were polyphyletic. Further, the genera *Aetomylaeus* (Myliobatidae) and *Pateobatis* (Dasyatidae) show polyphyly by showing deep genetic divergence in the phylogenetic tree. Based on the phylogenetic tree analysis, *Himanutra uarnak* appears to be *H. tutul* in the Indian waters. Similarly, *Neotrygon indica* should be consistently used instead of *N. kuhlii*, as Indian specimens forms a distinct subclade within *N. kuhlii* species complex. In this study, it is also observed that several entries in the NCBI GenBank are erroneous; thus, an updation of data is recommended based on the present study. The biogeographic patterns revealed *H. tutul*, *Maculabatis gerrardi*, *Brevitrygon imbricata*, and *Gymnura poecilura* from the Indian coast form a separate haplotype compared to other geographical areas (Indo-west Pacific). In addition, *G. poecilura* and *B. imbricata* were genetically divergent between east and west coast populations of India, indicating a possibility of cryptic species.

[**Keywords**: Haplotype network, Indian coast, Marine rays, Phylogeny, Ray's taxonomy]

Introduction

Batoid rays of order Myliobatiformes form a major component of the elasmobranch fisheries in India[1]. The fishing of rays in India is generally non-targeted and are mostly caught as by-catch in trawl fishing. However, in recent years, direct fishing in some areas has also been reported due to the increasing demand for international shipment owing to their biomedical values[2]. The landing of rays in India has increased significantly during the past few decades[2]. The east coast contributes ~70 % of rays landing in the country, while the west coast contributes the remaining ~30 %[refs. 2,3].

The order Myliobatiformes consists of ~239 valid species globally, belonging to 12 families, namely Zanobatidae, Hexatrygonidae, Dasyatidae, Urotrygoniade, Gymnuridae, Plesiobatidae, Urolophiade, Aetobatidae, Myliobatidae, Rhinopteridae, Mobulidae, and Potamotrygonidae. Only the family Potamotrygonidae consists of freshwater and marine rays, while other families have marine and estuarine species[4]. Among other families, Dasyatidae is the most diverse, with ~97 valid species under four subfamilies, namely, Dasyatinae (35 species), Neotrygoninae (17 species), Urogymninae (39 species) and Hypolophinae (6 species)[4]. Altogether, 51 marine and estuarine species of rays in the order Myliobatiformes belonging to 8 families, namely Hexatrygonidae (1 species), Dasyatidae (30 species), Gymnuridae (3 species), Plesiobatidae (1 species), Aetobatidae (3 species), Myliobatidae (4 species), Rhinopteridae (2 species), Mobulidae (7 species) are reported from the Indian coastal waters[1,5-15]. A list of valid species from the Indian waters is given in Table 1.

The myliobatoid rays have distinct morphological characteristics such as a flattened body, enlarged pectoral fins fused to the head, and ventrally placed gill slits. Identification up to the family level or, in some cases, up to the genus level is easier; however, the species level identity possesses problems because of similar morphological features and cryptic species complex[1,16]. Species identity is essential to assess their natural stocks and population biology so that necessary steps can be taken for their conservation

Table 1 — Status of rays of order Myliobatiformes from Indian coastal waters (NA = Not available *COXI* sequence). Numerical values (1-5) in the superscript represents erroneous *COXI* sequences in the NCBI GenBank

Family	Common name	Genus	Total species	Species reported from Indian waters	*COXI* gene sequence available	Revised Status based on *COXI* gene phylogeny in the present study
Hexatrygonidae	Sixgill stingrays	1	1	*Hexatrygon bickelli*	No	NA
Dasyatidae	Stingray	16	31	*Brevitrygon imbricata*	Yes	Yes
				Brevitrygon walga	Yes	Yes
				Hemitrygon bennetti	Yes	Yes
				Himantura leoparda	Yes	Yes
				Himantura uaranak[1]	Yes	No
				Himantura tutul[1]	Yes	Yes
				Himantura undulata	Yes	Yes
				Maculabatis arabica	No	NA
				Maculabatis bineshi	No	NA
				Maculabatis gerrardi	Yes	Yes
				Megatrygon microps	Yes	Yes
				Neotrygon indica[2]	Yes	Yes
				Neotrygon kuhlii[2]	Yes	No
				Pastinachus ater	Yes	Yes
				Pastinachus gracilicaudus	Yes	Yes
				Pastinachus sephen	Yes	Yes
				Pateobatis bleekeri	No	NA
				Pateobatis fai	Yes	Yes
				Pateobatis jenkinsii	Yes	Yes
				Pteroplatytrygon violacea	Yes	Yes
				Taeniura lymma	No	NA
				Taeniurops meyeni	Yes	Yes
				Urogymnus asperrimus	Yes	Yes
				Urogymnus granulatus	Yes	Yes
				Maculabatis pastniacoids	Yes	Yes
				Himantura marginata	No	NA
				Telatrygon crozieri	No	NA
				Bathytoshia lata[3]	Yes	No
				Pateobatis uarnacoides	Yes	Yes
				Himantura fava	No	NA
				Urogymnus polylepis	Yes	Yes
Gymnuridae	Butterflyrays	1	3	*Gymnura tentaculata*	No	NA
				Gymnura poecilura	Yes	Yes
				Gymnura zonura	Yes	Yes
Plesiobatidae	Deepwater stingrays	1	1	*Plesiobatis daviesi*	Yes	Yes
Aetobatidae	Pelagic eagle rays	1	3	*Aetobatus flagellum*	Yes	Yes
				Aetobatus ocellatus	Yes	Yes
				Aetobatus narinari[4]	Yes	No
Myliobatidae	Eagle rays	2	4	*Aetomylaeus maculatus*	Yes	Yes
				Aetomylaeus milvus	No	NA
				Aetomylaeus nichofii	No	NA
				Aetomylaeus vespertilio	Yes	Yes
Rhinopteridae	Cownose rays	1	2	*Rhinoptera javanica*[5]	Yes	No

(*contd.*)

Table 1 — Status of rays of order Myliobatiformes from Indian coastal waters (NA = Not available *COXI* sequence). Numerical values (1-5) in the superscript represents erroneous COXI sequences in the NCBI GenBank (*contd.*)

Family	Common name	Genus	Total species	Species reported from Indian waters	*COXI* gene sequence available	Revised Status based on *COXI* gene phylogeny in the present study
				Rhinoptera jayakari	Yes	Yes
Mobulidae	Devil rays	1	7	*Mobula alfredi*	No	NA
				Mobula birostris	Yes	Yes
				Mobula kuhlii	Yes	Yes
				Mobula japonica	Yes	Yes
				Mobula tarapacana	Yes	Yes
				Mobula thurstoni	Yes	Yes
				Mobula eregoodoo	No	NA

and management[17,18]. Due to confusion and inconsistency in the species identification, several un-described species go unnoticed, resulting in poor reporting on catch, exports, and management of rays fishery at the species level[8].

In recent years, molecular and genetic data have been extensively used for species-level identification and to ascertain the species delimitation among morphologically similar organisms[19,20]. In particular, DNA barcoding enables rapid and accurate species identification and differentiates cryptic lineages[21,22]. Several genetic studies have resulted in the description of previously unreported species as well as resolving cryptic species complex, thereby increasing local diversity across the world[16,19,23-27]. Approximately 655 nucleotide fragments of mitochondrial gene cytochrome c oxidase subunit I (*COX1*) amplified using FISH F1 and FISH R1 primers[22] have been considered suitable to provide quick and reliable confirmation of ray's species[24]. As a result, *COX1* has been used either solely or in combination with other markers to study molecular taxonomy, phylogenetic relationships among taxa, and biogeographic distribution patterns of rays, worldwide[10,22,24,28-31].

Description of rays from India has a long history[8]. However, most of the species described by early Ichthyologists have been either synonymized or considered invalid at present due to inability to re-examine the samples as they were not preserved properly in the national collections[8]. Exhaustive geographical sampling and molecular studies can validate additional unrecognized species[32]. In India, a molecular approach has been undertaken to identify ray species from the Indian coastal waters using the *COX1* gene in the last decade[6,7,9-12,26,27,33,34]. Pavan-Kumar *et al*.[12] described a novel species *Neotrygon indica* from the Gulf of Mannar, Tamil Nadu, which was later reported from different locations on the east coast of India[13]. The study by Bineesh *et al*.[10] covered wide geographical locations for collecting rays' samples (10 fish landing centres across India) and sequenced the *COXI* gene from 161 Myliobatiformes samples. However, molecular studies of Myliobatiformes from Indian coastal waters are hitherto focused only on specific genus and lack detailed analysis such as updated taxonomic status, phylogenetic relationship among taxa, and biogeographic patterns. Hence, this study aim to fill these prevailing gaps. In the present study, the Myliobatiformes species were collected and sequenced from the Mandapam fish landing centres in Tamil Nadu and compiled almost all mitochondrial genes published so far for Myliobatiformes from the Indian coastal waters. Further, a robust phylogeny is built, updated valid species names, and constructed a phylogeographic network analysis of selected species to observe major genetic structuring among the populations.

Material and Methods

Sampling

A total of 307 sequences of rays belonging to the order Myliobatiformes reported from the Indian coastline are utilized in the present study. This includes 18 individuals of rays sampled from the fish landing centres of Mandapam (9°16'36.99" N 79°9'15.48" E), Tamil Nadu, from July 2019 to January 2020, and 289 individuals from previous published literatures[6,7,-13,26,27], and the NCBI GenBank nucleotide sequence database till 30th October 2020. For the specimens collected in the present study,

tissue samples from the tail region were collected and preserved in 95 % ethanol for molecular identification. The specimens' preserved tissue samples are deposited as voucher materials at the National Zoological Collections of Marine Biological Research Centre (MBRC), Zoological Survey of India, Chennai. All individuals and their gene accession used in the present study are listed in Table S1.

DNA extraction, PCR and sequencing

Total genomic DNA was extracted using OMEGA BIO-TEK E.Z.N.A. Blood & Tissue DNA Kit, USA following the manufacturer's protocol. PCR amplification was performed for the *COXI* gene using FishF1 (5'-TCAACCAACCACAAAGACA TTG GCAC-3') and Fish R1 (5'-TAG ACTTCTGG GTGGCCAAAG AATCA-30) primers[22]. A total of 25 µl reaction mixture containing 12.5 µL 2X PCR master mix (Ampliqon, Denmark), 2.5 µL each of the two primers (10 nM), 2.5 µL of template DNA (10-20 ng), and nuclease-free water to make a final volume of 25 µL was taken. PCR conditions were as follows: initial denaturation at 95 ℃ for 10 min, 35 cycles of 95 ℃ for 45 sec, 50 ℃ for 45 sec, and 72 ℃ for 45 min, and final extension at 72 ℃ for 10 min. PCR products were then visualized on 1 % agarose and products with the high-intensity band were purified using EXOSAPIT and sequenced on ABI Prism 3730 Genetic Analyzer (Thermo Fisher Scientific, USA) based on BigDye Terminator Chemistry. Sequence chromatograms were then visualized, edited, and contigs were prepared using BioEdit[35] for phylogenetic and biogeographic pattern analysis.

Molecular phylogeny

COXI gene sequences generated in the present study and those retrieved from the previous published literature and NCBI GenBank nucleotide sequence databases, were aligned using the ClustalW web server (https://www.ebi.ac.uk/Tools/msa/clustalo/). All the species under the order Myliobatiformes were included as ingroup terminal, whereas four species belonging to three sharks (*Carcharhinus amblyrhynchos* (EF609308), *C. plumbeus* (EU398639), *Iago* sp. and one guitarfish (*Glaucostegus granulatus*) were included as outgroups terminal. Cytochrome oxidase subunit I (*COXI*) sequences for *G. granulatus* and *Iago* sp. were generated in this study from specimens collected from the fish landing centre at Mandapam, Tamil Nadu, India and submitted in NCBI GenBank database having accession number MT317247 (*Iago* sp.) and MT317248 (*G. granulatus*).

Aligned sequences were visualized using BioEdit[35] for necessary modifications and were uploaded on W-IQ-TREE web server[36] (http://iqtree.cibiv.univie.ac.at) for maximum likelihood phylogenetic tree construction. The Hasegawa-Kishino-Yano (HKY) evolutionary model was found to be the best-fit substitution model on this dataset. The calculated parameters were as follows: state frequencies (empirical counts from alignment) pi(A) = 0.2488, pi(C) = 0.269, pi(G) = 0.1763, pi(T) = 0.3058, substitution rate parameter, A-C: 1.0000, A-G: 4.9512, A-T: 1.0000, C-G: 1.0000, C-T: 4.9512, G-T: 1.0000, and uniform rate of heterogeneity. The robustness of the ML tree was analyzed by reiterating the observed data using an ultrafast bootstrap approximation for 1000 generations[37].

Genetic divergences

The pairwise genetic divergences within species and between species of Myliobatiformes from the Indian waters were calculated using the Kimura 2-parameter distance model[38] as implemented in MEGA 7[(ref. 39)].

Haplotype network analysis

Templeton, Crandall, and Sing (TCS) haplotype network[40] analysis was performed using PopART[41,42] (also available online at http://popart.otago.ac.nz) on the nucleotide sequence matrix of the *COXI* gene. Networks were generated for four species (*Brevitrygon imbricata*, *Gymnura poecilura*, *Maculabtis gerrardi*, and *Himantura tutul*) with sequences generated in the present study as well as previously reported from different geographical locations, obtained from the NCBI GenBank. A list of individuals and their GenBank accession numbers are given in Table S2. Before TCS haplotype network analysis, the sequence matrix was trimmed to a core length. This software also provided statistics on Nucleotide diversity, number of segregating sites, number of parsimony informative sites, and AMOVA.

Results

Molecular phylogeny

A total of 18 samples sequenced in the present study belong to 4 families and 9 species of the order Myliobatiformes, namely *Aetobatus ocellatus* (3 nos.), *Rhinoptera jayakari* (1 no.), *Neotrygon indica* (4 nos.), *P. atrus* (1 no.), *Maculabatis gerrardi*

(4 nos.), *Maculabatis pastinacoides* (1 no.), *Gymnura poecilura* (1 no.), *Brevitrygon imbricata* (2 no.), and *Himantura tutul* (1 no.). These *COXI* gene sequences were submitted in NCBI GenBank with accession numbers MT308592 - MT308609. In addition, 289 *COXI* gene sequences were compiled from previous literature and public databases belonging to 39 taxa. However, based on *COXI* gene phylogeny, found the presence of only 34 species (Table 1).

The maximum likelihood phylogenetic tree based on 307 *COXI* gene sequences could divide the sequences into two major clades (Fig. 1). First major clade (Clade I) includes species from the families Mobulidae (5 species), Rhinopteridae (1 species), Plesiobatidae (1 species), Gymnuridae (2 species), and Aetobatidae (2 species). These species were clustered into a single monophyletic clade and were strongly supported by the high bootstrap values from ML analysis. In clade I, polyphyl in the family Myliobatidae was observed as two species, *Aetomylaeus maculatus* and *A. vespertilio* were clustered into separate subclades. *Aetomylaeus maculatus* formed a separate sister subclade to members of the family Aetobatidae and Gymnuridae, while *A. verpertilio* formed a sister subclade to the members of the family Dasyatidae and Plesiobatidae, suggesting the possibility of polyphyletic nature within the genus *Aetomylaeus*. Likewise, the genus *Megatrygon*, a member of the family Dasyatidae formed a separate sister subclade to the family Plesiobatidae and Myliobatidae. The other members in the clade I belong to the family Dasyatidae, such as *Taeniura*, *Hemitrygon*, *Pteroplatytrygon* and *Neotrygon*, which formed a sister subclade within the clade I supported by strong bootstrap values from ML analysis (Fig. 1a).

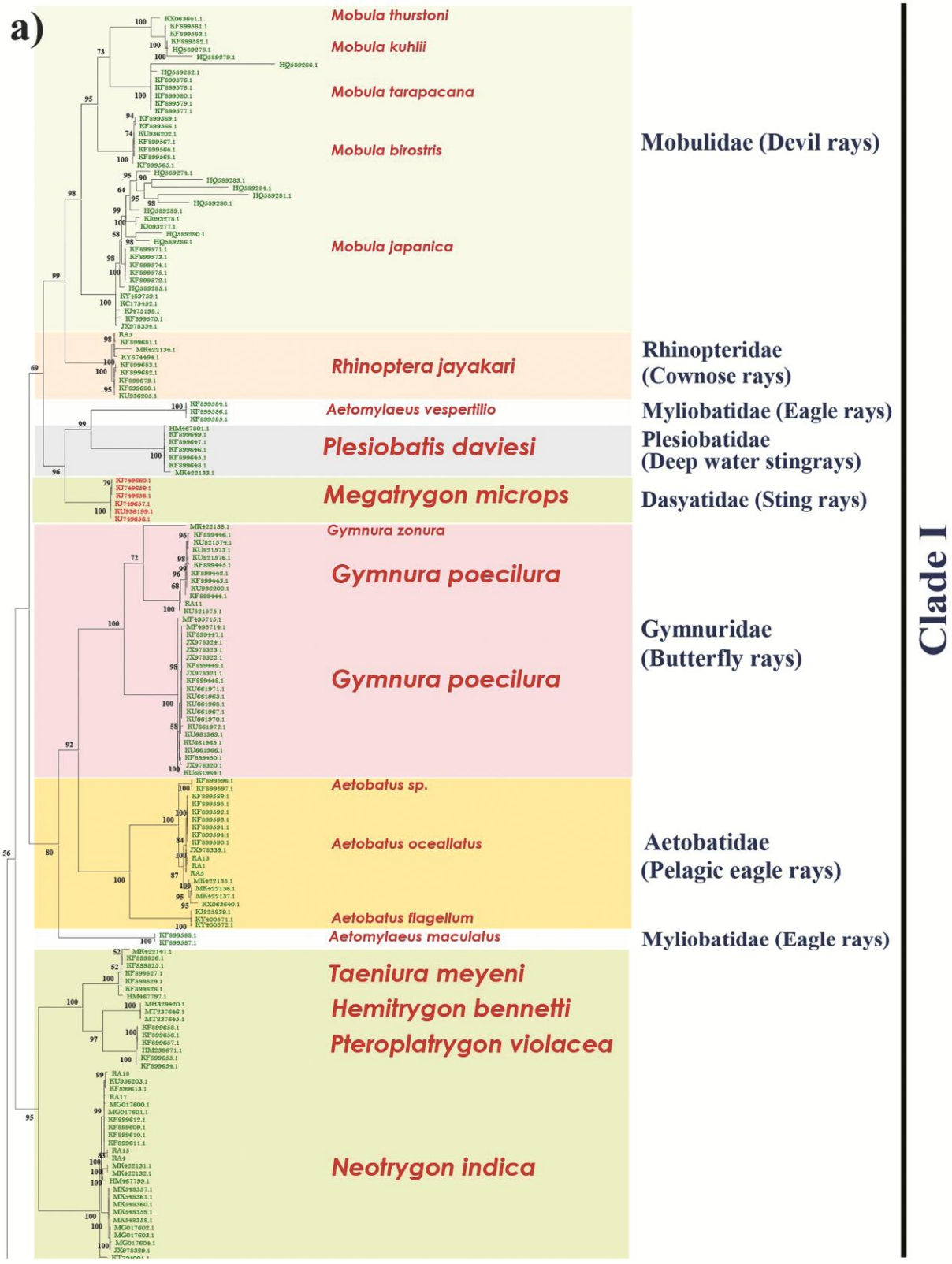
In the second major clade (clade II), members of the family Dasyatidae (16 species) were found with strongly supported bootstrap values from ML analysis. The genus *Brevitrygon*, *Urogymnus*, *Maculabatis*, *Himantura*, and *Pastinachus* formed a monophyletic subclade within clade II (Fig. 1b). Three species of the genus *Pateobatis* formed separate subclades based on *COXI* gene phylogeny indicating the polyphyletic nature of the genus. Species *Pateobatis jenkinsii* clustered within a sister subclade to the genus *Brevitrygon*; *P. uarnacoides* formed a sister subclade to the genus *Maculabatis*; and *P. fai* formed a separate sister subclade to other members of the family Dasyatidae. In addition, several species were observed misidentified or mislabelled in the public databases. Therefore, the species list is updated for the Indian region based on the *COXI* gene phylogeny (Table 1).

Furthermore, the overall pairwise genetic divergence within species varied from 0 to 6.86 % with a minimum intra-specific genetic distance of 0.0 % in *Aetobatus flagellum*, *Aetomylaeus vespertilio*, *Aetomyleus maculatus*, *Gymnura zonura*, *Hemitrygon bennetti*, *Himantura undulata*, *Megatygon microps*, *Mobula thurstoni*, *Pastinachus gracilicadus*, *P. sephen*, *Urogymnus asperrimus*, *U. granulatus*, and *U. polylepis*; and a maximum intraspecific genetic distance of 6.86 % in *Gymnura poecilura.* The pairwise genetic divergence between the species varied from 5.87 to 26.3 %, with a minimum distance between *Pastinachus atrus* and *P. gracilicadus* and a maximum distance between *Aetobatus ocellatus* and *Aetomylaeus vespertilio* (Table S3).

Haplotype diversity

Since the results suggested more diverged clades in certain species, the objective was to describe the population genetic structure of the entities within the clades. To distinguish among the different scenarios above (phylogeny and genetic distance), the level of haplotype diversity among the populations of different geographic locations was explored assuming that they form genetically dissimilar haplotypes and was implemented through PopART.

In *Himantura tutul*, using 645 aligned sites, nine different haplotypes were found among 55 individuals belonging to different geographic locations. Out of 11 sequences from the Indian waters, three different haplotypes were found to form a separate cluster from the West Pacific (Indonesia and Malaysia) sequences. One sequence from Zanzibar (East Africa) is observed to be clustered with the Indian haplotypes (Fig. 2). In *Gymnura poecilura*, using 651 aligned sites, 26 different haplotypes were found among 62 individuals belonging to different geographical locations. The haplotype network denoted major population genetic structuring in *G. poecilura* and formed a separate cluster between India's east coast and west coast populations (Fig. 3). The east coast populations formed a network with the individuals from Bangladesh, and the west coast populations formed a network with the individuals from Qatar and Saudi Arabia. Likewise, the Indo-West-Pacific populations, such as Indonesia and Malaysia, formed a separate cluster in the haplotype network. However, the


a)
Mobula thurstoni
Mobula kuhlii
Mobula tarapacana
Mobula birostris
Mobula japanica
Mobulidae (Devil rays)
Rhinoptera jayakari
Rhinopteridae (Cownose rays)
Aetomylaeus vespertilio
Myliobatidae (Eagle rays)
Plesiobatis daviesi
Plesiobatidae (Deep water stingrays)
Megatrygon microps
Dasyatidae (Sting rays)
Gymnura zonura
Gymnura poecilura
Gymnura poecilura
Gymnuridae (Butterfly rays)
Aetobatus sp.
Aetobatus oceallatus
Aetobatus flagellum
Aetobatidae (Pelagic eagle rays)
Aetomylaeus maculatus
Myliobatidae (Eagle rays)
Taeniura meyeni
Hemitrygon bennetti
Pteroplatrygon violacea
Neotrygon indica
Clade I

(*contd.*)

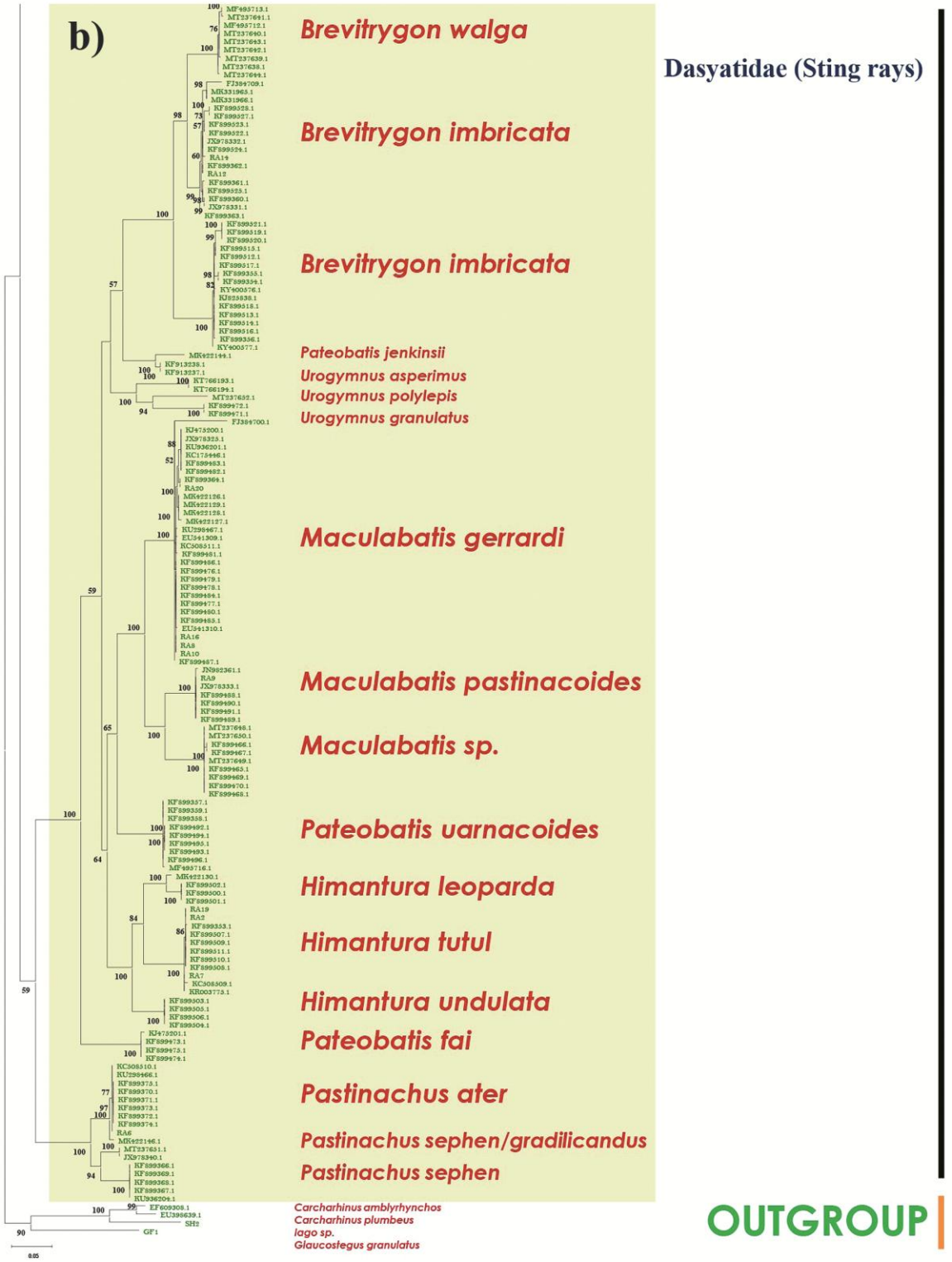


Fig. 1 — Maximum-likelihood phylogenetic tree based on partial *COXI* gene sequences from 307 individuals: (a) Clade I, and b) Clade II and Outgroup. The numbers above and below the branches indicate bootstrap values based on ML. GenBank accession numbers are given in the tree

number of singletons on both the east and west coast of India were 6 and 5, respectively (Fig. 3).

Among the sequences of *Brevitrygon imbricata*, 20 different haplotypes were found from 42 individuals using 617 aligned sites. Individuals of *B. imbricata* also formed a separate haplotype network between the populations of west and east coast of India. A single individual from the Bangladesh has paired with populations from the east coast. In addition, a few individuals from Saudi Arabia and Kuwait formed a cluster with west coast populations. Four singletons were observed each in west and east coast populations (Fig. 4). Among *Maculobatis gerrardi*, 16 different haplotypes were observed from 46 individuals using 638 aligned sites. The Indian Ocean populations formed a separate network including Bangladesh, Myanmar, and South Africa, from the West-Pacific populations such as Taiwan, Malaysia and Indonesia (Fig. 5). Overall, 6 singleton sites were observed in the Indian Ocean populations. Lastly, the nucleotide diversity, parsimony informative sites, segregating sites and AMOVA with significance for *H. tutul*, *G. poecilura*, *B. imbricata*, and *M. gerrardi* is given in Table 2.

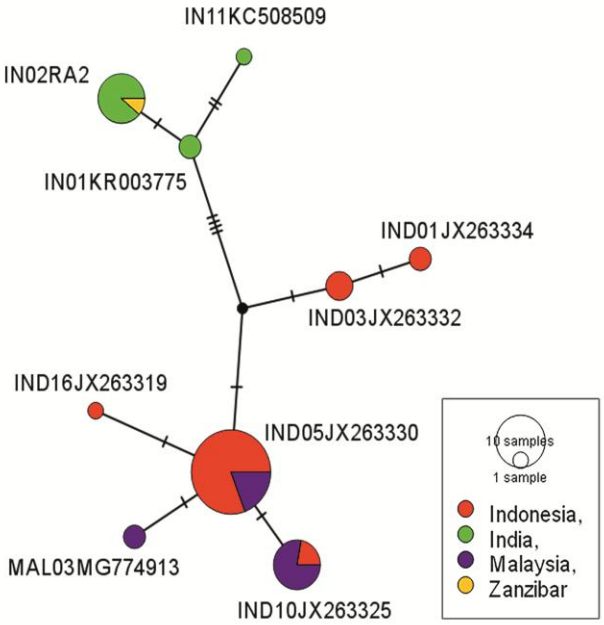


Fig. 2 — Haplotype diversity among the populations of different geographic locations of *H. tutul* using TCS network

Discussion

The study represents a detailed molecular analysis of Myliobatoid in the Indian coastal waters using *COXI* gene sequences from 307 sequences, including 18 sequences generated in the present study and 289 sequences obtained from previously published literature and public databases such as NCBI GenBank. For the first time, an attempt has been made to resolve the current species diversity in India, nomenclature anomalies, phylogenetic relationship, and biogeographic patterns of the order Myliobatiformes using *COXI* gene.

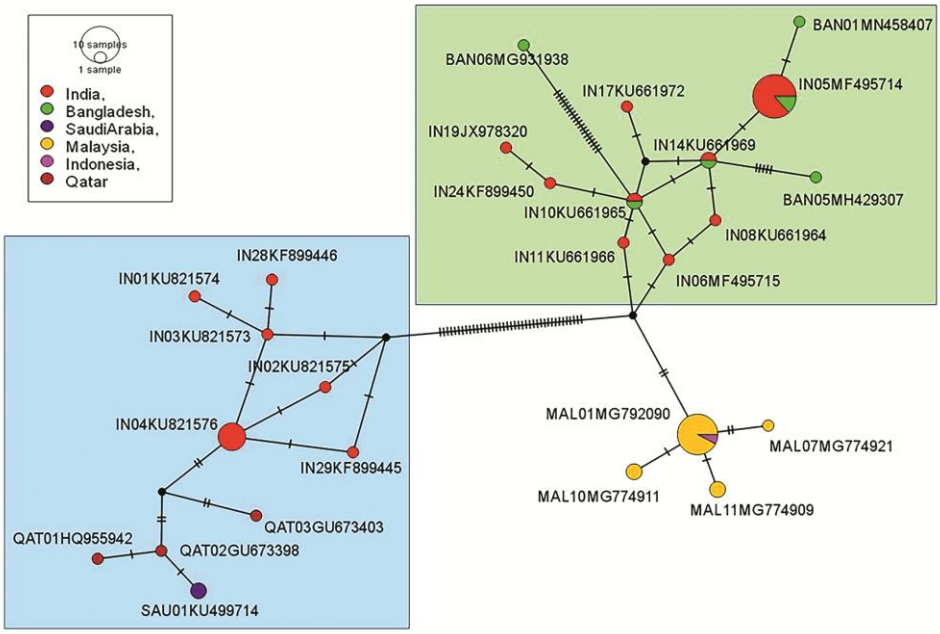


Fig. 3 — Haplotype diversity among the populations of different geographic locations of *G. poecilura* using TCS network

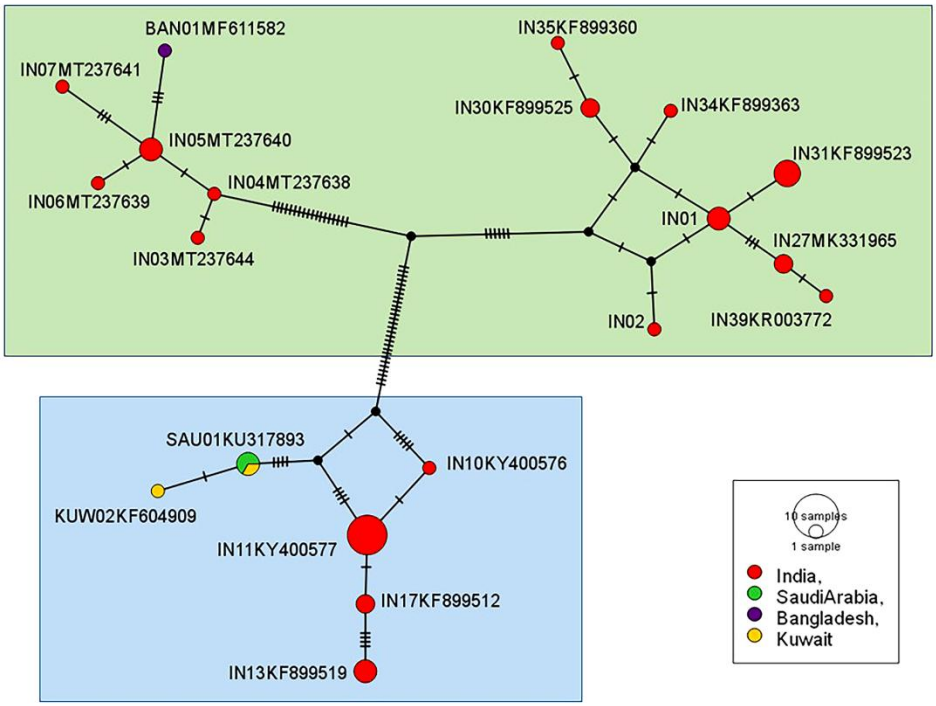


Fig. 4 — Haplotype diversity among the populations of different geographic locations of *B. imbricata* using TCS network

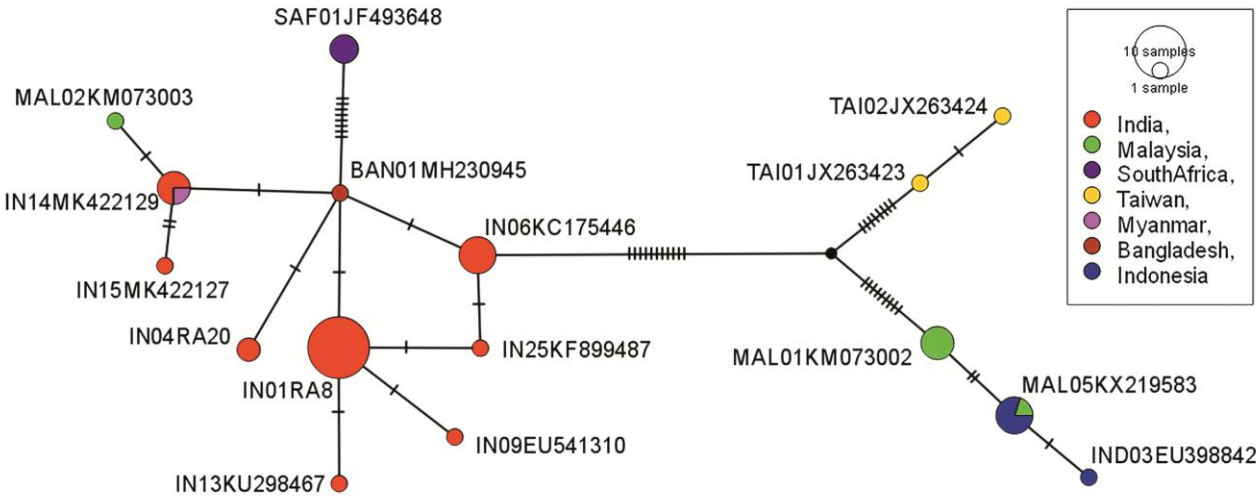


Fig. 5 — Haplotype diversity among the populations of different geographic locations of *M. gerrardi* using TCS network

Table 2 — Nucleotide diversity, parsimony informative sites, segregating sites and AMOVA with significance for *H. tutul*, *G. poecilura*, *B. imbricata*, and *M. gerrardi*

	Nucleotide diversity, PI sites, Segreagating sites and AMOVA				
	Nucleotide diversity	Parsimony informative sites	Segregating sites	AMOVA (φST)	Significance (φST)
H. tutul	3.87	10	13	0.877	0.001
G. poecilura	0.036	54	81	0.375	0.001
B. imbricata	0.47	71	79	0.071	0.019
M. gerrardi	0.014	38	43	0.864	0.001

Taxonomic ambiguities

Major issues in the species delineation of the order Myliobatiformes include morphological similarities, cryptic species complex, and widespread species with intraspecific geographical variations[24]. Mitochondrial *COXI* gene-based barcoding and phylogenetic approach has been effective in the ray's taxonomic identification and species delineation[10,24,29]. However, DNA barcoding has brought another problem in the form of deposition of non-verified sequences with incorrect, inconsistent, duplicate, or outdated/ unaccepted names in public databases such as NCBI GenBank. Indeed, it has been observed that several entries in the NCBI GenBank were erroneous, *e.g*. *Himantura uranak* (Gene accession no. FJ384700, EU541309), *Pateobatis bleekeri* (KC508511), and *Megatrygon microps* (EU541310) were clustering with *M. gerrardi* in the *COXI* based phylogenetic tree. Thus, it could be a case of misidentification. Similarly, sequence(s) submitted as *Urogymnus aperimus* (KC508509), *Himantura leoparda* (KF899353) and *Aetobatus narinari* (KR003775) were found to be clustered with *Himantura tutul*. Three specimens of *Bathytoshia lata* (KJ825838, JX978331, JX978332) from the NCBI GenBank appeared to be *Brevitrygon* spp. Upon closer examination into the genus *Himantura*, it has been found that previously reported *Himantura uarnak* from the Indian coast is clustering with recently reported species, *H. tutul*[34]. Hence, it is believed that the species with the name *H. uarnak* reported in the Indian waters may be *H. tutul*. Similarly, in Neotrygon *kuhlii* species complex, all the individuals clustered together in one subclade of *N. indica*[12]; thereby indicating the presence of only one single species in this species complex in the Indian waters. Further, a single sequence of *Aetobatus narinari* (JX978339) has clustered within the subclade of *A. ocellatus* and a single sequence of *Rhinoptera javanica* (KU936205) clustered within subclade of *R. jayakari*, suggesting misidentification in these two species. As the information on the voucher materials is not available (either not deposited or maintained at the museums), the species identity cannot be ascertained by re-examining the samples morphology. Overall, out of 39 species for which *COXI* genes were available, it is strongly argued only 34 species of Myliobatiformes are present in the Indian coastal waters (Table 1).

Phylogenetic relationships among the Indian Myliobatiformes

Utilizing *COXI* gene phylogeny, two major clades were observed within the Order Myliobatiformes. The major Clade I consist of monophyletic clades of the families Mobulidae, Rhinopteridae, Plesiobatidae, and Gymuridae; and polyphyletic clades of families Myliobatidae and Dasyatidae (Fig. 1). Among the Mobulidae, 5 species namely *M. thurstoni*, *M. kuhlii*, *M. tarapacana*, *M. biostris*, *M. japanica* formed a separate subclade. However, in the subclade of *M. japonica*, deep genetic divergence among the sequences was observed, and it is suspected that the reasons could be the bad quality of sequences deposited under accession numbers HQ series (HQ589274.1, HQ589280.1, HQ589281.1, HQ589283.1, HQ589284.1, HQ589285.1, HQ589286.1, HQ589289.1, HQ589290.1). The poorly aligned positions and highly divergent regions were found in multiple sequence alignment with closely related species. Further, the location of the sample collection was not provided, besides, it was mentioned under the miscellaneous features of sequences in the NCBI GenBank labelled as "similar to cytochrome c oxidase subunit I". However, it may also be possible that these sequences may belong to different geographical locations as *M. japanica* exhibits circum-global distribution[30,43].

In clade I, another interesting observation was the polyphyletic nature of the family Myliobatidae as *Aetomylaeus maculatus* did not cluster with its congeneric species *A. vespertilio*. This observation is supported by the fact that the genetic divergence between these two species was 24.7 %[(ref. 10)]. However, Naylor *et al*.[44] did not find the polyphyletic nature of *Aetomylaeus* spp. using additional/or another marker. Hence, to ascertain this claim, further phylogenetic studies utilizing multilocus markers or a phylogenomic approach is much warranted[45]. The family Gymnuridae formed a monophyletic clade represented by two species, namely *G. zonura* and *G. poecilura*. *Gymnura poecilura* showed significant divergence among the populations forming two subclades. It indicates the possibility of geographical separation between the east and west coast of India, which was evident from recent studies[11]. In the family Aetobatidae, subclades of *Aetobatus* sp. (2 sequences) was observed which is not yet identified and could represent a novel species[10]. Further, a separate subclades of *A. ocellatus*, and *A. flagellum* was also observed in this study. Based on the phylogeography

studies, it is reported that *A. ocellatus* has formed two distinct subclades belonging to the Indian population and Indo-West Pacific populations[46,47]. In addition, the results of present study indicate that there could be two divergent populations of *A. ocealltus*, each belonging to east and west coast. However, it requires deep phylogenetic studies involving more samples and molecular markers to establish the claims.

The *COXI* gene phylogeny indicated the polyphyletic nature of the Dasyatidae family as few members segregated in major clade I, while others were in clade II. *Megatrygon microps* formed a separate subclade which was distantly placed from the members of the subfamily Dasyatinae (family Dasyatidae). Similar observations were made in the previous studies, suggesting the possibility of having unique characters distant from other members of the subfamily Dasyatinae. It was also hypothesized that this species may belong to a new undescribed family[29]. Further, to strengthen the argument of polyphyly in the family Dasyatidae, several genera were observed closely, including *Taeniura*, *Hemitrygon*, *Pteroplatytrygon* and *Neotrygon* clustered within the major clade I, while other genera such as *Brevitrygon*, *Urogymnus*, *Maculabatis*, *Himantura*, *Pastinachus* and *Pateobatis* formed a separate major clade II (Fig. 1). This observation agrees to the previous reports on the phylogeny of the order Myliobatiformes based on *COXI* and NADH2 gene sequences[24,29,44,48].

The cryptic species complex of *Neotrygon* spp. was resolved based on COI phylogeny that resulted in eleven parapatrically - distributed lineages, each classified into a valid species[12,25,28,48]. The Indian Ocean mask ray was identified recently as *N. indica*[12]. Four of the sequences generated in this study (GenBank accession MT308594, MT308604, MT308606-07) clustered with *N. indica* are reported from the Indian coast[12,13]. Also, the genetic divergence among the sequences could be linked with the geographical locations, which was also reported by Kundu *et al*.[13]. Among the *Himantura* species complex, we found only three species from the Indian coastal waters, namely *H. leoparda*, *H. undulata*, and recently reported *H. tutul*. It was observed that the sequences which were reported to be *H. uarnak* in the previous studies[10] are clustered with *H. tutul* in the present (GenBank accession MT308608) and previous study[34]. An earlier report by Arlyza *et al*.[16] on *H. uarnak* species complex has clearly stated the confusion in morphology-based taxonomy. Further, they conveyed the importance of the COI gene in separating the clades of *H. uarnak* complex. However, the possibilities of mitochondrial introgression among *H. uarnak* complex are yet to be documented[16]. Likewise, the recent study on the DNA barcoding of *Himantura* has confirmed *H. tutul* as a nominal species based on an integrative approach (morphology+COI phylogeny)[34]. Additionally, *H. tutul* may have overlapping geographic ranges and shared habitats, and one can suspect other isolating mechanisms to occur as observed for its congeneric *H. uarnak* complex[16]. Therefore, it is sensible to include COI as one of the potential diagnostic characteristics to distinguish *Himantura* spp. complex. Recently, *H. tutul* was also reported from Sri Lankan waters, having a clear distinction from *H. uarnak* based on morphology and NADH2 sequences[49].

Haplotype and biogeography

The population genetic structure of *H. tutul* from India indicated a significant genetic differentiation ($P < 0.001$) pointing towards gene flow and migration among the individuals between the Indian Ocean (India and Zanibar) and West Pacific (Indonesia and Malaysia). This can be supported based on the high nucleotide diversity of 3.87 with an overall φST value of 0.877. However, the results presented should be seen cautiously because of the low sample sizes, *i.e*., only 55 individuals with a limited distribution range. Therefore, it is speculated that the genetic structure of *H. tutul* may not be restricted to the Indian Ocean but will also extend to the Western Pacific, which is under the Indo-Polynesian province[50]. Therefore, extensive sample sizes are necessary, along with multi-locus markers, to build strong evidence on the population structure of *H. tutul* within the restricted range of distribution.

Interestingly, in the case of *Gymnura poecilura* complex, there is a deep genetic divergence between east and west coast populations in the Indian waters. For instance, the individuals from the eastern states of India formed a separate haplotype network with Bangladesh sequences and formed a closer network with the Western Pacific populations (Malaysia+Indonesia). At the same time, the population from the Northern Arabian Sea, including Gulf waters (western states of India+Qatar+Saudi Arabia), formed a separate haplotype network. The population of *G. poecilura* between east and west

coast were separated with several mutations, indicating the possibility of a cryptic species complex. Similar, deep genetic divergence between the east and west coast populations was also observed in *G. poecilura* by Muktha *et al*.[11]. It is possible that the geographical barriers near the Southern India and Sri Lanka such as Ram bridge, together with ancient climatic differences may act as means of isolation concerning *G. poecilura* populations between the Bay of Bengal and Arabian Sea[51]. Likewise, *Brevitrygon imbricata* populations in the east and west coast of India appeared to be diverged with several mutations. Lastly, the *Maculobatis gerrardi* populations also exhibit shared haplotypes within the Indian Ocean (India+Bangladesh+South Africa+Malaysia). However, some of the populations, in particular, Taiwan, East coast of Malaysia and Indonesia, have mixed haplotypes pointing towards the substantial gene flow and mixing of populations within the Indian Ocean and Western Pacific.

A recent description of *Maculabatis ambigua* from the Red Sea formed a sister clade with its closest congener *M. gerrardi* and *M. randalli*. Interestingly, the distribution range of *M. gerrardi* was further east in the Indian Ocean to Northwest Pacific; and of *M. randalli* is in the Persian and Arabian Gulf[48]. This indicates the restricted gene flow within the Red Sea as a possibility of endemism as compared to the other two species, which have substantial gene flow in their distribution range, as observed in the case of coral reef fishes[52]. The biogeography of the elasmobranchs is mainly driven by migration patterns and gene flow[31]. For example, a recent study on the oceanic white tip sharks *Carcharinus longinamus* suggests a homogenous population/single stock along the Indian coast owing to their substantial migration capacity[53]. Similarly, substantial mixing of gene pools and connectivity due to oceanic currents has been studied extensively for coral reef fishes[54]. The present study also observed that the populations of rays are more homogenous if they have been collected only in Southeast and Southwest coast of India *i.e*., Tamil Nadu (in east) and Kerala (in west). One of the possible reasons could be the collection of fish in one part of the coast and landing on the other, which has been commonly reported in the case of elasmobranch fisheries/trade[1]. More samples need to be collected throughout India from the artisanal fishers as they fish in the nearby sea, directly indicating the local population of rays; thereby helping delineate biogeographic patterns and endemism[55]. Further, biogeographic information sheds light on natural habitats, which is essential to take conservation associated steps.

Eventually, it is urged to use the updated/valid species list/nomenclature for the study; as well as to submit at least a part of tissue in the national museum/collection centres as an voucher material. It is also recommended to mention the sample collection location while submitting sequences in the public database (NCBI GenBank). This would be helpful for future researches to explore the possibility of characterizing the cryptic lineages and uncovering putative new species in the Indian Ocean using the molecular taxonomy. Moreover, it is also believed that this study has proven the potential of revisiting rays' phylogeny from the Indian waters, which in turn gain insights into the species validation, the phylogenetic relationship, and biogeographic patterns in understanding the diversity. The limitation of the present study in using only one mitochondrial gene marker (*COXI*) for phylogeny and network analysis is also acknowledged. Adding more nuclear/ mitochondrial markers can further improve our understanding of Myliobatiformes phylogenetic positioning. Nevertheless, the above results can be used as a framework for any effective conservation and management plans concerning ray's population in the Indian waters.

Supplementary Data

Supplementary data associated with this article is available in the electronic form at https://nopr.niscpr.res.in/jinfo/ijms/IJMS_52(02)65-78_SupplData.pdf

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships

that could have appeared to influence the work reported in this paper.

Ethical Statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Specimens were collected from the bycatch of the commercial fishing harbors.

Author Contributions

AK & SP: Field sampling, lab work, data analysis, and corrections in the manuscript draft. AK: Preparation of first draft of manuscript.

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