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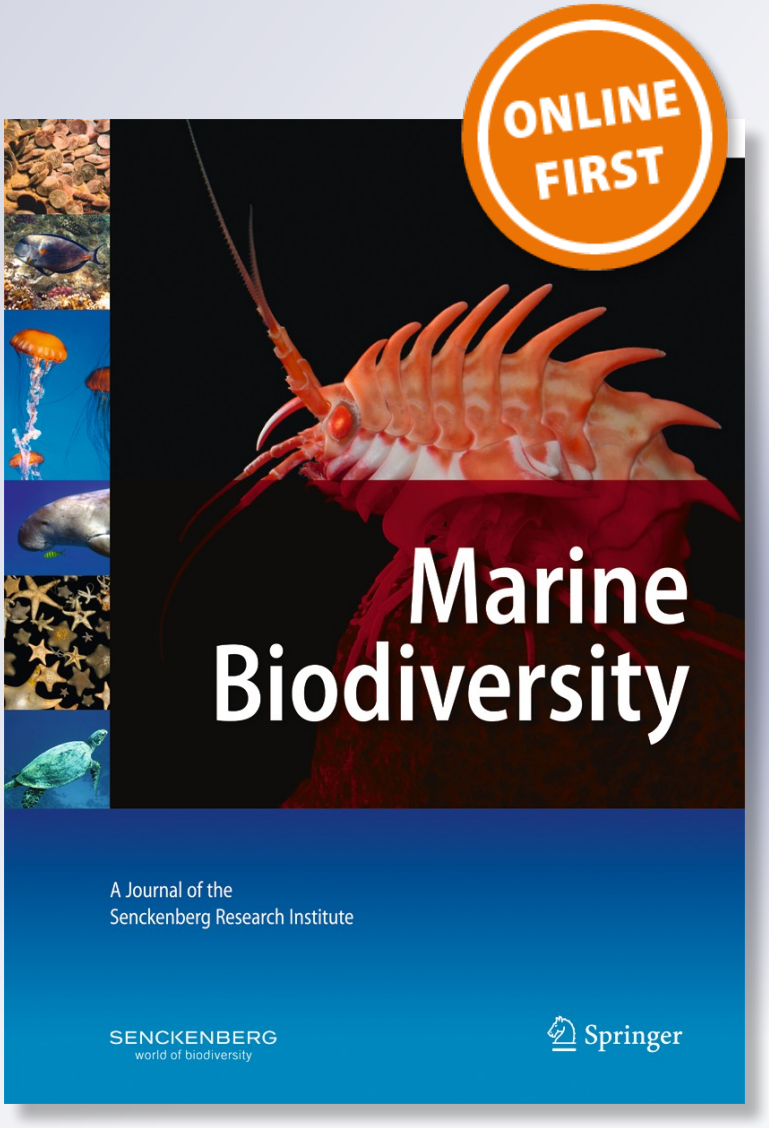

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A molecular analysis of selected marine fishes from the southwest coast of India for species delineation

C. Lavina-Vincent[1] · Supriya Dutta[1] · Gaurav Banerjee[2] · R. Saravanan[3] · V. Sachithanandam[4] · P. Krishnan[2]

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Abstract In this study, mitochondrial genes 16S and cytochrome C oxidase I (COI) were used as taxonomic markers for the molecular analysis of selected fishes from the southwest coastal waters of India, viz., *Sillago sihama* (Forsskål, 1775), *Mene maculata* (Bloch & Schneider, 1801), *Seriolina nigrofasciata* (Ruppell, 1829), and *Minous dempsterae* (Eschmeyer, Hallacher & Rama-Rao, 1979). Both the gene fragments showed diagnostic differences and divergence within each taxon. The mean nucleotide diversity (Pi) among all the species was estimated as 0.083. The average transition and transversion ratio was 1.63. The average K2P distance of species within genera was estimated as 13.71 %, whereas it was 20.22 % among species within families. The variation was more among the species within families than among the congeneric individuals. The study on the phylogenetic relationships among the species revealed distinct clusters in concurrence with the taxonomic status of the species. The study also suggests that the mtDNA 16S (rRNA) and COI genes can be used as barcodes for the identification of fish species based on sequence information.

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✉ V. Sachithanandam
pondiunisachin@gmail.com

✉ P. Krishnan
krishnanars@yahoo.com

[1] PRIST University, Thanjavur 613 007, Tamil Nadu, India

[2] Fisheries Science Division, CARI, P.B. No. 181, Port Blair 744 101, Andaman and Nicobar Island, India

[3] Research Centre, Central Marine Fisheries Research Institute, Mangalore 575 001, India

[4] Department of Ocean Studies and Marine Biology, Pondicherry University, Port Blair 744 112, Andaman and Nicobar Islands, India

Keywords 16S rRNA · COI sequence · DNA barcoding · India

Introduction

The major part of the Earth's biodiversity is yet to be explored. There are approximately 1.7 million named species and biologists have estimated that some 8 million species have not yet been described (Stockle and Hebert 2008). It is evident that there is an erosion of the Earth's biodiversity and that one-third of the inhabitants are facing extinction in the coming decades. Thus, cataloguing Earth's remarkable biodiversity before much of it disappears has become the prime target of biologists and taxonomists worldwide (Hubert et al. 2008). DNA barcoding is a technique used for identifying organisms based on short standardized fragments of genomic DNA. Barcoding will help people to quickly and cheaply recognize known species and retrieve information about them (Hubert et al. 2005; Ward et al. 2005). This will speed up the cataloguing for millions of species yet to be named. For animals, mitochondrial genes like 16S, cytochrome C oxidase I (COI), etc. are ideal gene targets to be used as DNA barcodes (Saccone et al. 1999). Currently, 430,000 barcodes representing about 50,000 species (30 % of all known species) have been collected (Silva-Brandao et al. 2009; International Barcode of Life, iBOL 2010).

Fishes are a highly diverse group of vertebrates; the identification of fish species through DNA barcoding will provide new perspectives in ecology and systematics of fishes (Hubert et al. 2008; Ward et al. 2005, 2009). There are approximately 32,100 extant fish species worldwide (as at August, 2011, http://www.fishbase.org), which constitutes about 50 % of all vertebrates. DNA barcodes have been obtained for 6000 species of fishes, including 400 species from New Zealand,

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207 species from Australia, 250 species from South Africa, and 100 species from Pacific Canada (Ward et al. 2009).

The coastal waters of India are known for their rich and diverse fish species (Venkataraman and Wafar 2005). Tropical fishery in India is so diverse that there are several misnomers among the fishes identified, and certain similar species are recorded as similar/same for even scientific purposes. An exploratory survey along the southwest coastal waters of India revealed the presence of certain new species recorded akin to their nearest generic counterparts, but appearing distinct for a classical taxonomist. Similarity in the external morphology of the sillaginid fishes has led to great confusion in their identification and many true species have been concealed in the synonymy of wide ranging species (McKay 1992). Since barcoding of fishes can clearly distinguish them, the study was carried out for barcoding of these fish samples. In this study, two mitochondrial genes, 16S rRNA and COI, are used as taxonomic markers for DNA barcoding and phylogenetic analysis of selected fishes from the southwest coastal waters of India.

Materials and methods

Study area

A major fish landing center along the southwest coast of India at Mangalore was the primary study area. This coastal stretch is located along the southwest coast of India, between latitudes 12°48′00″N and 13°21′00″N and longitudes 74°36′00″E. and 74°55′00″E. This dynamic coastal stretch with a well-defined Malabar upwelling zone is characterized by diverse fishing and industrial activities. This region is very dynamic, with tidal currents and seasonal wind-driven current systems. The seasonal heavy precipitation is carried into the Arabian Sea through numerous streams and rivers, in addition to the land runoff. These characteristics likely influence the fishery potential in the region.

Collection of samples and DNA isolation

The collected specimens were species-level taxonomical identification through nomenclature done following the Food and Agriculture Organization (FAO) Fish Identification Sheets (FAO 2002, 2004) and local identification keys were used. Based on the classical taxonomical nomenclature, the four fish samples were identified as: (i) *Sillago sihama*, (ii) *Mene maculata*, (iii) *Seriolina nigrofasciata*, and (iv) *Minous dempsterae*. Approximately 50–100 mg of muscle tissue samples from four fishes (for each species, four to five specimens were used for replicates) were collected from the southwest coastal waters of India and preserved in 95 % ethanol at ambient temperature. DNA was isolated from 10 mg of muscle tissue samples using the AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen Biosciences, USA). The isolated genomic DNA was checked in 0.8 % agarose gel and its concentration estimated using a UV spectrophotometer (Bio-Rad, USA).

Amplification and sequencing of DNA

The 16S rRNA mitochondrial gene was amplified using the primer pair (Ivanova et al. 2007) 16Sar (5′-AAAC GCCT GTTT ATCA AAAA CAT-3′) and 16Sbr (5′-AAAC CGGT CTGA ACTC AGAT CACGT-3′). The primer pair COI-1FF2d (5′-AAAT TCTC CACC AACC ACAA RGAY ATYGG-3′) and COI-1FR1d (5′-AAAC ACCT CAGG GTGT CCGA ARAA YCARAA-3′) was used to amplify the COI gene. Twenty microliters of the polymerase chain reaction (PCR) cocktail contained 13.3 μl of nuclease-free sterile water, 2 μl of 10× Taq buffer (Tris with 15 mM $MgCl_2$), 2 μl of dNTP mix (2.5 mM of each dNTP), 1 μl of each primer (0.3 μM), 0.6U of Taq polymerase, and 0.5–1 μl of DNA template for each gene. The PCR thermal regime consisted of an initial step of 2 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 40 s at 52 °C, and 1 min at 72 °C, with a final extension step of 10 min at 72 °C for both

Table 1 Summary of the parameters analyzed

Name of the species	Gene	Average nucleotide composition (%)				Average transitional pairs (si)	Average transversional pairs (sv)	Ratio R = si/sv	Overall mean distance ± SE (K2P %)
		A	T	C	G				
Sillago sihama	16S	29.2	23.0	24.8	23.0	46	37	1.26	26.6 ± 0.004
	COI	24.2	29.1	28.2	18.4	66	49	1.35	15.2 ± 0.020
Mene maculata	16S	28.4	25.3	28.8	17.5	76	85	0.90	13.9 ± 0.065
	COI	23.8	29.2	28.6	18.4	54	37	1.46	0.7 ± 0.012
Seriolina nigrofasciata	16S	28.4	25.3	28.8	17.5	76	85	0.90	12.3 ± 0.067
	COI	23.8	29.4	27.9	18.9	69	40	1.70	4.3 ± 0.014
Minous dempsterae	16S	29.2	24.4	26.9	19.5	61	63	0.98	31.4 ± 0.036
	COI	24.2	29.9	27.4	18.5	71	46	1.54	17.7 ± 0.015

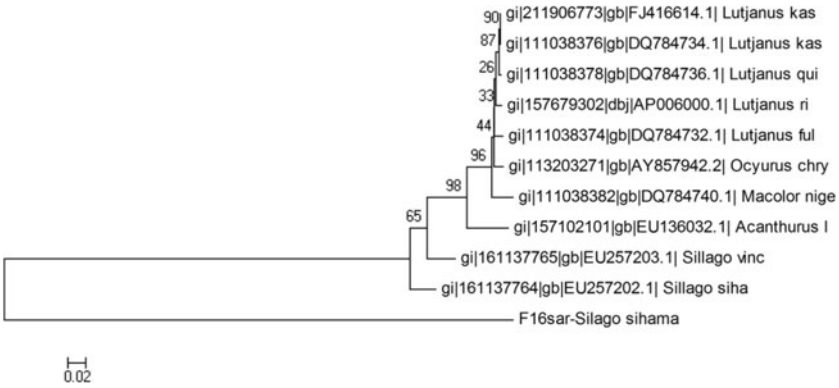

Fig. 1 Neighbor-joining tree of the 16S rDNA gene nucleotide sequences of *Sillago sihama* derived using the K2P distances analyzed. The numbers at the nodes are bootstrap values based on 1000 replicates. The *scale bar* represents an interval of the Tamura–Nei genetic distance

primer pairs. The PCR products were checked in 2 % agarose gel along with a ladder. The amplified PCR product fragment was excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen, USA). The purified PCR products were sequenced by a commercial sequencing service provider (Synergy Scientific Pvt. Ltd., India) using both forward and reverse primers.

Sequence analysis

The 16S and COI gene sequences obtained were compared with similar sequences from the NCBI with the help of the BLASTn tool (Altschul et al. 1990). The sequences of the top 20 fishes reference sequences retrieved from those distributed in tropical and subtropical waters were selected for further analysis based on the product of query coverage and maximum identity percentage. The sequences were aligned using CLUSTALW for multiple sequence alignment. The sequence distances between the species were estimated using MEGA4.1 (Molecular Evolutionary Genetic Analysis version 4.0) (Tamura et al. 2007). The pairwise evolutionary distance was determined by the K2P distance model (Kimura 1980). Neighbor-joining (NJ) trees of the K2P distances were plotted to provide a graphical representation of the patterning of divergence between the species (Saitou and Nei 1987). These were bootstrapped with 1000 replications in MEGA4 to provide percentage bootstrap values for branch points.

Results

Based on the classical taxonomical nomenclature, the four samples were identified as: (i) *Sillago sihama*, (ii) *Mene maculata*, (iii) *Seriolina nigrofasciata*, and (iv) *Minous dempsterae*. The 16S rDNA and COI genes were partially sequenced for 4–6 specimens for the studied species. The results obtained are presented for the four samples in which the genetic differentiation for the 16S and COI genes are inferred separately.

Sillago sihama

Nine fish species belonging to five different genera and three families were analyzed for the 16S gene, while three fish species belonging to different genera were analyzed for the COI gene. The average nucleotide frequencies were 28.3 % (A), 25.1 % (T), 29.6 % (C), and 16.9 % (G) for the 16S gene and 23.7 % (A), 29.2 % (T), 28.3 % (C), and 18.7 % (G) for the COI gene (Table 1). The average transitional pairs (si) were 139, whereas the average transversional pairs (sv) were 118 for the 16S gene, with an average ratio (R) of 1.18 (si/sv). For the COI gene, the transitional pairs (si) were 53 and transversional pairs (sv) were 33, with an average ratio of 1.6. The average evolutionary divergence distance for the 16S gene was 26.1 % and that for the COI gene was 15.2 %. The NJ tree of the 16S and COI gene sequences using K2P distances clearly differentiated the species of the same genera

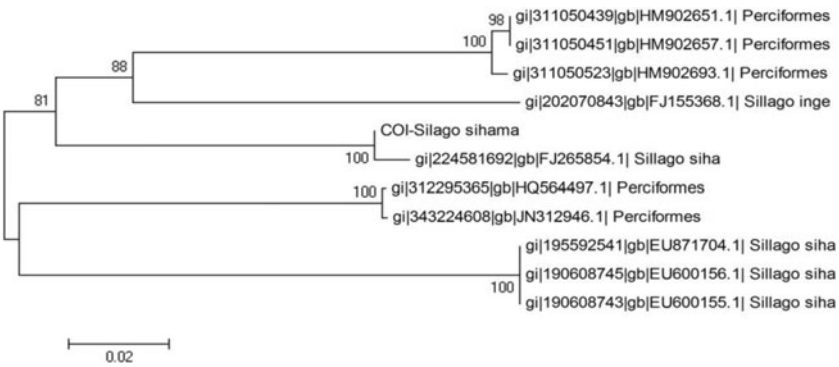

Fig. 2 Neighbor-joining tree of the COI gene nucleotide sequences of *Sillago sihama* derived using the K2P distances analyzed. The numbers at the nodes are bootstrap values based on 1000 replicates. The *scale bar* represents an interval of the Tamura–Nei genetic distance

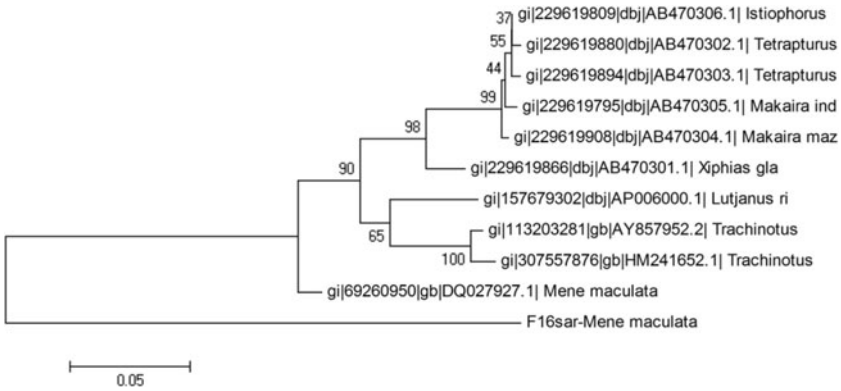

Fig. 3 Neighbor-joining tree based on the 16S rDNA gene of *Mene maculata* derived using the K2P distances analyzed. The numbers at the nodes are bootstrap values based on 1000 replicates. The *scale bar* represents an interval of the Tamura–Nei genetic distance

into distinct clusters (Figs. 1 and 2). From these analyses, it can be observed that the 16S rRNA references sequence is not available in the public domain, such as GenBank. However, barcoding references sequences can be found in the NCBI and BOLD systems.

Mene maculata

Fishes belonging to seven genera and five families were analyzed for the 16S gene and the COI gene was analyzed in fishes belonging to the Menidae family. The average nucleotide frequencies for the 16S gene were 27.5 % (A), 25.5 % (T), 30.4 % (C), and 16.7 % (G), while those for the COI gene were 23.2 % (A), 28.7 % (T), 29.4 % (C), and 18.7 % (G). The average transitional pairs (si) were more than the average transversional pairs (sv) for both genes. The values si = 3 and sv = 2 for the COI gene were comparatively lower than si = 509 and sv = 327 for the 16S gene. The overall average divergence distance was high for 16S (13.7 %) compared to COI (0.7 %). The NJ tree for the 16S and COI genes clearly depicted the species of different families by separate clusters (Figs. 3 and 4).

Seriolina nigrofasciata

The fish sequences analyzed for the 16S and COI genes belonged to the genera *Seriola*. The nucleotide frequencies of the sequences analyzed were 25.5 % (A), 24.0 % (T), 26.1 % (C), and 24.4 % (G) for the 16S gene and 23.2 % (A), 29.7 % (T), 27.5 % (C), and 19.7 % (G) for the COI gene. The average transitional and transversional pairs for the 16S gene were same, i.e., si = 25 and sv = 25. The average ratio (R = 3.16) of transitional pairs (si = 20) and transversional pairs (sv = 6) was very high for the COI gene. The mean congeneric divergence distance estimated for the 16S gene was 12.3 % and that for the COI gene was 4.2 %. The NJ tree derived using the K2P distance for the 16S and COI genes had only 2–3 major branches/nodes, which clearly indicated the similarities between the sequences analyzed (Figs. 5 and 6).

Minous dempsterae

The fishes belonging to six genera and five families were analyzed for the 16S gene. The COI gene of the fish sequences analyzed belonged to the orders Perciformes and Scorpaeniformes. The nucleotide composition for the 16S gene was estimated as 29.4 % (A), 24.8 % (T), 28.9 % (C) and 6.9 % (G) and the nucleotide composition for the COI gene was 25.5 % (A), 30.4 % (T), 26.1 % (C) and 18.0 % (G). The average transitional pairs and transversional pairs for the 16S gene were si = 248 and sv = 212, whereas for the COI gene, the average values were si = 59 and sv = 39, respectively. The average evolutionary divergence for the 16S gene was

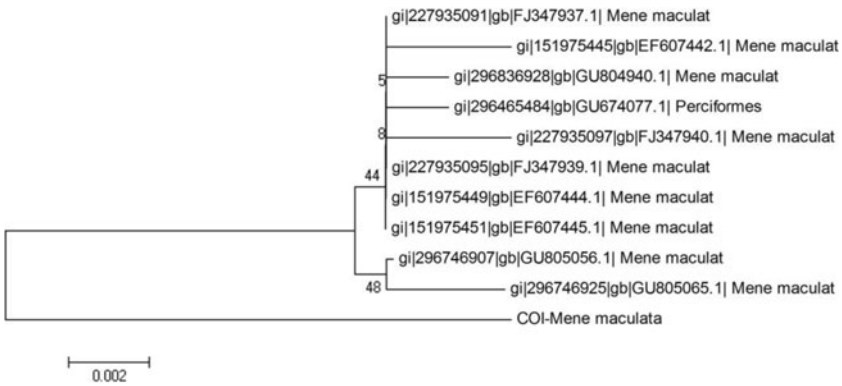

Fig. 4 Neighbor-joining tree based on the COI gene of *Mene maculata* derived using the K2P distances analyzed with reference sequences retrieved from GenBank. The numbers at the nodes are bootstrap values based on 1000 replicates. The *scale bar* represents an interval of the Tamura–Nei genetic distance

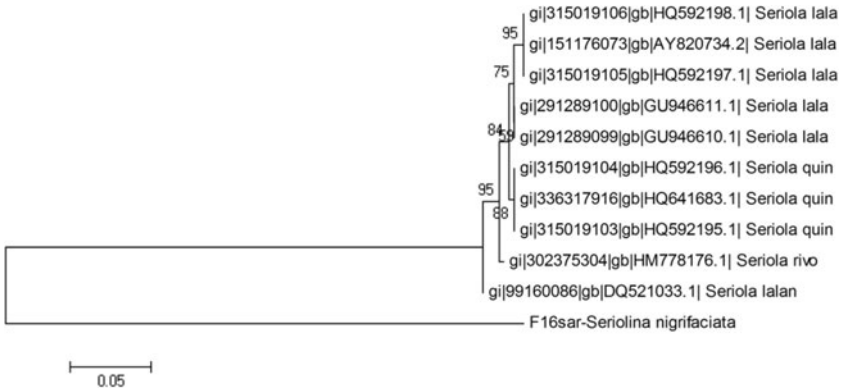

Fig. 5 Neighbor-joining tree based on the 16S rDNA gene of *Seriolina nigrofasciata* derived using the K2P distances analyzed with reference sequences from GenBank. The numbers at the nodes are bootstrap values based on 1000 replicates. The *scale bar* represents an interval of the Tamura–Nei genetic distance

31.3 % and that for the COI gene was 17.4 %. The NJ tree of the 16S gene clearly depicted the species in the same genera into distinct clusters (Fig. 7). The NJ tree of the COI gene revealed four clusters, of which the first and second clusters were formed by Scorpaeniformes, while the third and fourth clusters were formed by Perciformes (Fig. 8).

Discussion

This study reveals that the mitochondrial genes 16S and COI can be used as barcodes for identifying fish species. The universal primers amplified nearly 98 % of the target region in all the species, generating two barcodes each for the four species. Based on the genetic differentiation, the four taxa sampled in this study correspond with an interpretation as four distinct species. Both 16S and COI fragments show diagnostic (fixed) differences, and divergence within each taxon. The 16S and COI sequences clearly discriminated the taxonomic status of all four species examined. In this study, the mean nucleotide diversity (Pi) among all the species was estimated as 0.083. The average transition and transversion ratio was 1.26. The average K2P distance of species within genera was estimated as 13.71 %, while it was 20.22 % among species within families. The variation was more among the species within families than among the congeneric individuals. In the largest barcoding study on marine fishes, the average divergence was 9.9 % between congeneric species, while the mean divergence among species within families was 15.5 % (Ward et al. 2005). Based on the barcoding study on Indian marine fishes, the average distance between congeneric species was 6.60 % and 9.91 % for confamilial individuals (Lakra et al. 2011; Sachithanandam et al. 2012). For freshwater fishes, the average distance between congeneric species was 8.3 % and among species within families it was 15.4 % (Hubert et al. 2008), almost the same as marine fishes. A steady increase of genetic variation through the increment of taxonomic levels was observed, supporting a marked change of genetic divergence at the species boundaries.

The barcode analyses primarily seek to delineate species boundaries at the COI locus for the assignment of unknown individuals to known species. Unsuspected diversity and overlooked species are often detected through barcodes analyses, sometimes spectacularly (Meyer and Paulay 2005; Kerr et al. 2007). It has been shown that lineages diversify more quickly within species than between species (Pons et al. 2006). The branch length between species tends to be much deeper than between conspecific individuals, leading to a gap in the distribution of the pairwise distance between conspecific individuals and between species that has been referred to the barcoding gap (Meyer and Paulay 2005). The NJ tree revealed an identical phylogenetic relationship among the species. The phylogenetic relationship among the species was clearly established though 16S and COI sequences and similar

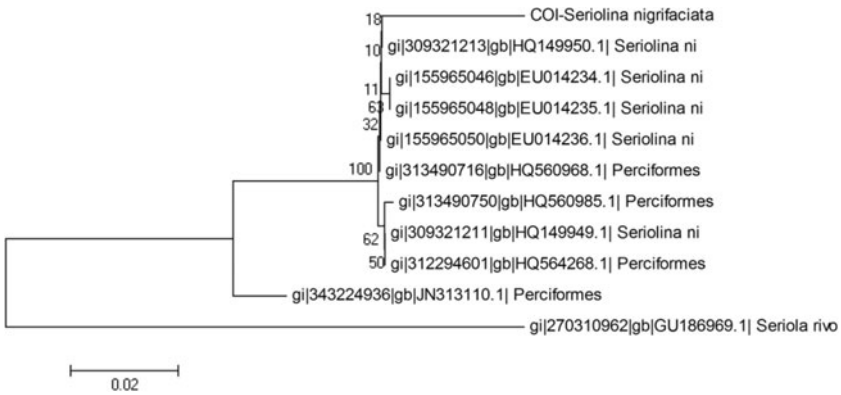

Fig. 6 Neighbor-joining tree of the mtDNA COI gene of *Seriolina nigrofasciata* derived using the K2P distances analyzed with reference sequences from GenBank. The numbers at the nodes are bootstrap values based on 1000 replicates. The *scale bar* represents an interval of the Tamura–Nei genetic distance

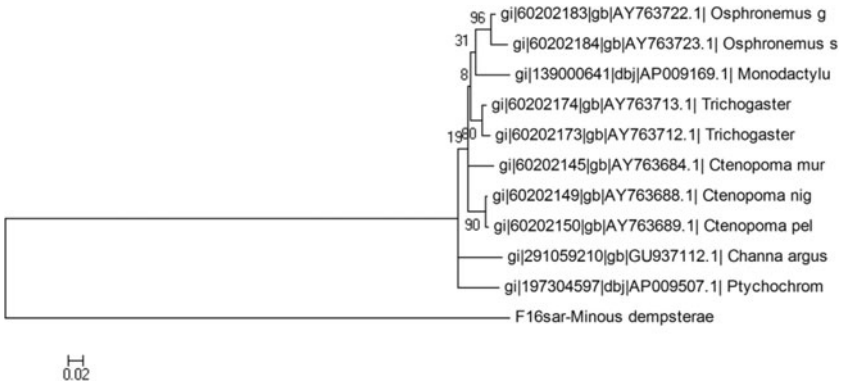

Fig. 7 Neighbor-joining tree of the 16S rDNA gene nucleotide sequences of *Minous dempsterae* derived using the K2P distances analyzed with reference sequences from GenBank. The numbers at the nodes are bootstrap values based on 1000 replicates. The *scale bar* represents an interval of the Tamura–Nei genetic distance

species were clustered under the same nodes, while dissimilar species were clustered under separate nodes. Congeneric species always clustered together and, in most cases, so did the confamilial species.

In addition to the species identification, DNA barcoding has been used for the identification of processed fish products (Smith et al. 2008). Using this method would clearly allow the identification of individually isolated freshwater fish eggs, larvae, fillets, and fins, hence providing many news tools useful for the practice of conservation and genetic forensics in these freshwater fishes (Hubert et al. 2008). The identification of several cases of polyphyletic or paraphyletic COI species genealogy further supports the view that an iterative process of DNA barcoding followed by taxonomic analyses using other characters will be a productive way to catalog biodiversity (Kerr et al. 2007; Barber and Boyce 2006); hence, barcoding and morphological analysis should go hand in hand. The entire cataloguing of the fish fauna, which is currently being undertaken by FISH-BOL, will result in a significant improvement of our knowledge concerning the systematics of the fishes of the region and also facilitate monitoring changes in the geographic distribution of species that will probably occur in the future (Hubert et al. 2008). An ideal application of barcoding would be a system in which the sequence variants found within a species group together, excluding all other species, in a cluster diagram based on genetic distance (Ortman et al. 2010). In our study, the "barcoding gap" was obviously detected for all taxa based on COI or 16S sequences, and the same species were clustered under the same nodes by high bootstrap values (Figs. 1,3,5, and 7), which indicated that both COI and 16S could be useful as a biological barcoding tool for distinguishing species within the studied taxa. However, according to our result, one of the advantages of using 16S data for barcoding hydrozoans is that, unlike COI, the sequences are easier to amplify and sequence because they are relatively more conservative, which was consistent with the results of other research (Miglietta et al. 2009; Moura et al. 2011a, b).

Nucleotide sequences without proper validation of the species identity have little application. An example is that the finfish species *Nemipterus mesoprion* has a distribution range along the coast of southeast Asian countries (Froese and Pauly 2012) but many Indian workers have submitted its genetic sequence in GenBank, which is a non-existing fauna along the Indian coast (Russell 1990, 1991). In the current study, the barcodes were developed after proper taxonomic characterization and delineation at the species level. The results from our data are congruent with the taxonomic divisions of the fishes under study, based on morphological characters as reported in the FAO Fish Identification Sheets. This study suggests that the 16S and COI genes can be used for unambiguous delineation of fishes with complex taxonomical features,

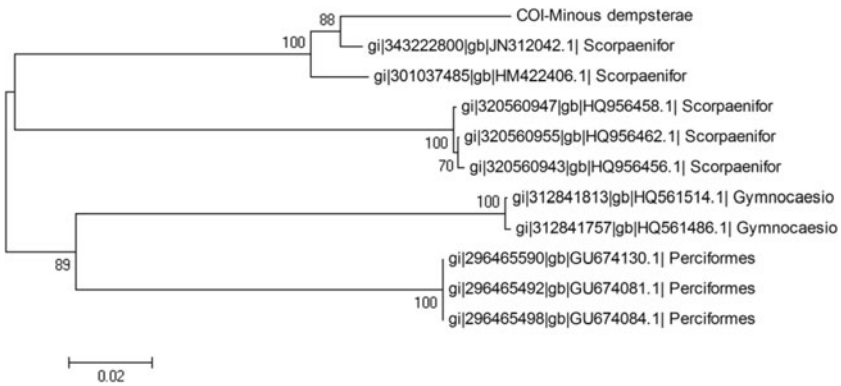

Fig. 8 Neighbor-joining tree of the COI gene of *Minous dempsterae* derived using the K2P distances analyzed with reference sequences from GenBank. The numbers at the nodes are bootstrap values based on 1000 replicates. The *scale bar* represents an interval of the Tamura–Nei genetic distance

as in the case of sillaginid fishes, which are similar externally and can be distinguished based on the swim bladder structure and vertebrae count.

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