RESEARCH ARTICLE

DNA BARCODING AND BIOINFORMATICS APPROACHES OF NEWLY RECORDED *Halichoeres timorensis* FROM GULF OF MANNAR, SOUTHEAST COAST OF INDIA

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ABSTRACT

Taxonomic description and DNA barcoding of a newly recorded Wrasse fish species, *Halichoeres timorensis* (Perciformes: Labridae) collected from Keelakarai region in Gulf of Mannar, Southeastern India. The mitochondrial cytochrome oxidase subunit I gene (mtCOI) with 656 bp region were sequenced for phylogenetic analysis. Recent studies have revealed that many marine fishes are closely associated with coral reefs. During the present investigation, mt COI gene sequence was used to identify the marine fish *Halichoeres timorensis*. This is the first report for the availability of this species in Gulf of Mannar, Bay of Bengal and the mt COI sequence was deposited in BOLD database and also in Gen Bank (Accession No: KF422721). The Neighbor-joining method was used for phylogenetic analysis that confirmed the analyzed species were dichotomy relationship with its ancestor species. The pairwise genetic distance calculated the species with eight different closely related species ranged from 0.095 to 0.280%. The length of open reading frame (ORF) was 252. This is the piece of evidence to assist in gene prediction. This study highlights the power of molecular method for species identification and need for an extensive, systematic molecular account of its existing marine ornamental fish biodiversity.

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INTRODUCTION

The world ornamental fish trade is about 4.5 billion US$ and India's export earnings through ornamental fish is about 0.5 million US$. Like the freshwater ornamental fishes, the marine ornamental fishes show tremendous amount of variations in colour pattern. The aquarists and scientists are puzzled by the different colour patterns that may occur in the single species of reef fish. Variations in pattern and the intensity of colors are influenced by a number of ecological factors including the depth, kind of substrate, turbidity and the time of day. Colour intensity variation related to depth is common in many fishes, particularly among the parrot fishes, butterfly fishes, damselfishes and angelfishes, etc. In Gulf of Mannar, a total of 113 marine ornamental finfish species under 24 families viz. Acanthuridae, Balistidae, Chaetodontidae, Haemulidae, Labridae, Pomacanthidae, Pomacentridae, Scaridae and Syngnathidae, have been recorded and their biodiversity and standing stock biomass also assessed (Venkataramani, 2004; Venkataramani *et al*., 2005).

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The diversity, distribution and abundance of reef fishes in Gulf of Mannar was made by many workers (Muthy, 1969; Patterson Edward and Asir Ramesh, 1997; Dorairaj, 1998; Rajagopal and Dhanya Sethnarayanan, 2006; Sudeepta Biswas *et al*., 2012; Murugan and Namboothri, 2012; Rejitha, and Madhusoodanan Pillai 2014; Murugan *et al*., 2014). The wrasse fishes coming under the family Labridae are one of the most conspicuous elements of the coral reef community and are very closely related to parrot fishes in their color pattern. There is no report about the availability of *Halichoeres timorensis* in Gulf of Mannar region. Hence the present study is the first report of *Halichoeres timorensis* in Gulf of Mannar region and its genetic analysis using mtCOI.

DNA barcoding is a concept in which a short nucleotide sequence of mitochondrial genome will act as a DNA barcode for species identification of eukaryotes, in particular, animals and it is proven to be a rapid tool for precise identification of biological specimens. 650 nucleotide bases of 5′ cytochrome c oxidase subunit I gene (COI) have been accepted as a universal barcode to delineate animal life of this planet. In such circumstances, DNA barcodes are helpful for (Packer *et al*., 2009) rare and common species coexist and share a similar appearance, a frequent problem in larval identification for many fish taxa.

Traditional morphology-based taxonomic procedures are time consuming and therefore a multidisciplinary approach to taxonomy that includes morphological, molecular and distributional data are essential. Indeed, the typical COI gene is a candidate for DNA barcoding which is well constrained amino acid sequence and thus broad applicability of primers also restricts its information content at deeper phylogenetic levels (Russo *et al.,* 1996; Zardoya and Meyer, 1996; Naylor and Brown, 1997; Hebert *et al.,* 2003). Finally, while apparently engaging, the term DNA barcoding is unfortunate, as it implies that each species has a fixed and invariant characteristic like a barcode on a supermarket product. The current available records of about 41771 fishes, representing 6566 fish species on the Barcode of Life Data System, BOLD (Ratnasingham and Hebert, 2007). This technique will speed up the discovery of species yet to be named. Thus this technology will provide vital news toll for appreciating and managing the species biodiversity in the earth. DNA barcode is an efficient method for species-level identification of many marine organisms in worldwide. Many benefits of DNA bar coding for species identification. A certain fragment of the mitochondrial gene CO1, coding for the enzyme cytochrome oxidase, has risen to remarkable status in recent years. It became commonly known and used as "DNA Barcode" for the identification of animal species. The efficiency of the method hinges on the degree of sequence divergence among species and species-level identifications (Ward *et al*., 2005).

Traditionally Labridae fishes are identified based on visible morphological, meristic, and anatomical characters. Many time taxonomic ambiguities exist due to morphological and meristic similarities. DNA barcoding can provide accurate species identification by using the genetic variation that is found between species. Analysis of short, standardized genomic regions (DNA barcodes) can discriminate morphologically recognized animal species (Hebert *et al*., 2002, & 2003). In particular, the mitochondrial CO1 gene can serve as a uniform target gene for a bio identification system. Application of this technique can provide valuable information for species identification to complete the taxonomic data and phylogeny. The present study has constructed a first DNA barcode sequence database for Timor wrasse (*H. timorensis*) species of Keelakarai region in Gulf of Mannar.

MATERIALS AND METHODS

The collection of Timor wrasse, *H. timorensis* species belonging to the family Labridae was made from Anaipar island of Keelakarai, Gulf of Mannar, Southeastern India (9° 09' N Lat. 78° 42' E Long) using fish traps. The photographs were taken immediately and the tissue part of the fishes were dissected and stored at -20ºC. We choose a target segment of DNA in the cytochrome oxidase subunit I. We developed DNA barcoding protocols to identify marine fish species within several groups (COI) gene for PCR amplification.

Laboratory analysis

DNA extraction and PCR Amplification

Total DNA was extracted from the 100mg of muscle tissue by the standard proteinase-K/ phenol-chloroform-isoamyl alcohol method (Sambrook *et al*., 2001). A 656 bp section of the mitochondrial (mt) DNA genome from the COI gene was amplified using published universal primers of Forward FISH F1 (5' – TCAACCAACCACAAAGACATTGGCAC – 3') and Reverse FISH – R1 (5' – TCGACTAATCATAAAGATAT CGGCAC – 3') (Ward *et al*., 2005). The polymerase chain reaction (PCR) components were prepared 25 µl reaction as follows: PCR buffer (10X), $MgCl_2$ (25mM), dNTP (10m), Forward Primer (30ng/l), Reverse Primer (30ng/µL) 1µl, Taq polymerase (3U), Ultra-pure water, DNA (100ng/µl). The thermal cycler program were as follows, in 94ºC for 5min the Taq polymerase were initially denatured followed by 35 cycles of denaturation for 30sec at 94ºC, annealing at 54ºC for 40 sec and extension at 72ºC for 1 min, After the completion of 35 cycles a final elongated extension step of 7min at 72ºC was performed. The PCR amplified products were tested in 1.5 % Agarose gel, visualized and photographed using GELSTAN Gel Documentation System. Finally the amplified product was sequenced in both directions using Sanger dideoxy method in commercial lab (Eurofins, Bangalore). The sequencing was done both in the forward and reverse directions. Sequences were deposited in the Barcode of Life Data System, BOLD (Ratnasingham and Hebert, 2007), in the public project: Reef associated ichthyofauanl diversity (BDUMS).

Sequencing and Phylogenetic Analysis

DNA sequences were initially aligned using CLUSTAL X (Thompson *et al*., 1994) and default gap, extension penalties were used followed by manual editing using SeqApp 1.9 (Gilbert, 1994). Alignments were adjusted by manually following guidance of Odorico and Miller (1997) and Chen *et al.* (2004). A Blast search was performed on GenBank for each sequence and the matching homologous sequences were retained for subsequent alignment.

Sequences were aligned using Clustal W (Bermington E, Lessios HA) and submitted to BOLD with GenBank under the accession number (KF422721). Specimen voucher DMSBDU - 108. Molecular evolutionary analyses were conducted using MEGA version 5 (Tamura *et al*., 2007). The extent of sequence differences between species was calculated by averaging pair-wise comparison of sequence difference across all individuals. Sequence divergence was calculated using the Kimura 2-parameter (K2P) model (Kimura, 1980) and the mid-point rooted Neighbour-joining (NJ) tree of K2P distances was created to provide a graphic representation of the species divergence (Nei *et al*., 1987). This method was implemented in MEGA version 5 (Kumar *et al*., 2001).

RESULTS AND DISCUSSION

The genus *Halichoeres* contains many species that are difficult to identify morphologically. In the present study, the mtDNA sequences of COI gene of the wrasse fish *H. timorensis* belongs to the family Labridae (Order: Perciformes) were initially compared with same genus of eight different fishes like, *H. argus, H. marginatus, H. bicolor, H. cosmetus, H. trispilus, H. hartulanaus, H. zeylonicus, H. nigrescens.* These are closely related to the *H. timorensis*. It was the first record in Indian waters and also the first submission record of

the mtDNA Cytochrome Oxidase subunit 1(COI) in BOLD database. The GenBank accession number is KF422721and the specimen voucher number is DMSBDU 108. A 650-bp segment of the 5' region of the mitochondrial cytochrome oxidase subunit I gene is currently used for classification of molecular biodiversity. The A, T, G and C contents of the sequence of *H. timorensis* were 23.5%, 31.9%, 17.8%, 26.8% respectively. The GC content was observed at 50% in this species. Other sequence variation was not observed among those individuals.

Systematic account of *Halichoeres timorensis* (Bleeker, 1852)

Halichoeres timorensis belongs to the family Labridae and order Perciformes. Only one specimen was collected in Anaipar island of Keelakarai, Gulf of Mannar, Southeastern India during July-2012 (9° 09' N Lat. 78° 42' E Long). This species was found in a shallow coastal reef, mainly rock substrates with soft coral and algal growth. In the IUCN report, this species is listed as the Least Concern level. The body colored with blue-green, except thorax and abdomen which are pinkish gray. More or less in vertical alignment with the series of blotches on side of body; head with irregular reddish bands and a vertically elongate spot behind the eye. Caudal fin pale with a large blackish area containing pale spots and markings posteriorly in fin and some faint irregular bands basally.

The taxonomic description (cm) of *Halichoeres timorensis* includes the Total length 12.3, Standard length 10.4, Fork length 11.2, Body depth 4.2, Head length 4.3, Post orbital length 2.3, Pre orbital length 1.2, Eye diameter 0.8, 1st dorsal fin base 6.4, Length of pectoral fin 2.3, Anal fin base 1.2. Dorsal-fin origin posterior to point midway between eye and pectoral-fin base; body not as deep and compressed; lower pharyngeal jaw with molariform teeth; Caudal fin slightly rounded; anterior dorsal and anal soft rays longer than posterior rays; pelvic fins of males long, reaching or nearly reaching anus.

Halichoeres timorensis (Bleeker, 1852)

- *Halichoeres timorensis* Bleeker, 1862, Atlas Ichthyologique, Vol.1, p. 120.
- *Halichoeres timorensis*: Ukkrit Satapoomin, 2011, The Fishes of South Western Thailand, 70: Phuket Marine Biological Centre, Res. Bull. 70: 29-77.

- *Halichoeres timorensis:* Randall, 1980. The two new Indo-Pacific Labrid Fishes of the Genus *Halichoeres*, with Notes on the Other Species of the Genus. Pacific Science, Vol. 34, No.4.

Phylogenetic analysis

The 656 bp fragment of COI gene of *Halichoeres timorensis* were analyzed to examine the phylogenetic relationship of eight closely related species. The Maximum Likelihood tree of COI was estimated using the MEGA v5 clearly showed a significantly long branch length leading to the clade of *H. timorensis* (Fig. 1). In contrast, the COI was highly conserved that forming an unresolved dichotomy with a short branch length in the Maximum Likelihood tree. In phylogenetic analysis, there are two major clades are resolved in the COI phylogeny corresponding to clearly distinct. The base of the tree forms a dichotomy leading to the following clusters: clade I and clades II. The results also indicated that there was a closer genetic relationship between *H. bicolor*, *H. marginatus*, *H. argus*. (Fig.1). Clade I consists of five *Halichoeres* genus note that the other *H. timorensis* sequence are obtained falls into clade II, whereas *H. timorensis* sequences are obtained with the similar *Halichoeres* sequences. The branch length leading to the Genus *Halichoeres* clade (0.006) was 15 times longer than those of other branches leading to the major clades (0.101).

Genomic analysis

Genetic distances of *H. timorensis* with the eight closely related Labridae species were calculated based on Kimura's 2-parameter method (Table 1). The pairwise genetic distance was calculated it ranged from (0.095 to 0.280) that showed the smaller genetic distances indicate a close genetic relationship whereas large genetic distances indicate a more distant genetic relationship. Genetic variation within populations can be lost through genetic drift or a bottleneck in the population (You *et al.*, 2001; Kim *et al.*, 2010). DNA Barcoding has the power to provide valuable insight into patterns of genetic divergence affected by species-level or ecological variation. The average genetic distance among species does not exceed the average genetic distance between sisters' species (Hebert *et al.*, 2003). The genetic distance is a short term computational and mathematical exact prediction has been the most widely used for genetic diversity measure between same and different species (Chakraborty, 2010). It should help us to identify closely related species and have a recent common ancestor (Nei *et al.*, 1987). Genetic distance can be used to compare the genetic similarity between different species and within a species genetic distance can be used to measure the divergence between different sub-species. One crucial barcoding criteria is that the identification of fish belonging to the same species should be higher than the fish that belongs to the same genus (Hubert *et al.*, 2008).

As expected, species from the same genera were clustered into a two different clades with well supported bootstrap proportion (Steinke *et al.*, 2005). The *H. timorensis* mtDNA COI sequences contained three open reading frames (ORFs) are shown in Fig. 2.

Table 1. Genetic distances of *H. timorensis* sequences from the related species. The low genetic distances are highlighted and the high genetic distances are marked as bold

No	Organisms	1	2	3	4	5	6	7	8	9
1	*Halichoeres argus* KUT 4729									
2	*Halichoeres marginatus* ranglabhm90	0.157								
3	*Halichoeres bicolour* aqhb	0.170	0.160							
4	*Halichoeres cosmetus* ADC11_220.28#6	0.243	0.223	0.226						
5	*Halichoeres trispilus* ADC10_220.35A#2	0.209	0.198	0.206	0.095					
6	*Halichoeres hortulanus* ARO 83	0.209	0.205	0.217	0.184	0.180				
7	*Halichoeres zeylonicus* NBFGR:1333B	0.240	0.217	0.239	0.217	0.215	0.193			
8	*Halichoeres nigrescens* NBE0236	0.220	0.226	0.213	0.236	0.220	0.209	0.225		
9	*Halichoeres timorensis* DMSBDU 108	0.205	0.183	0.176	0.248	0.213	0.236	0.280	0.234	
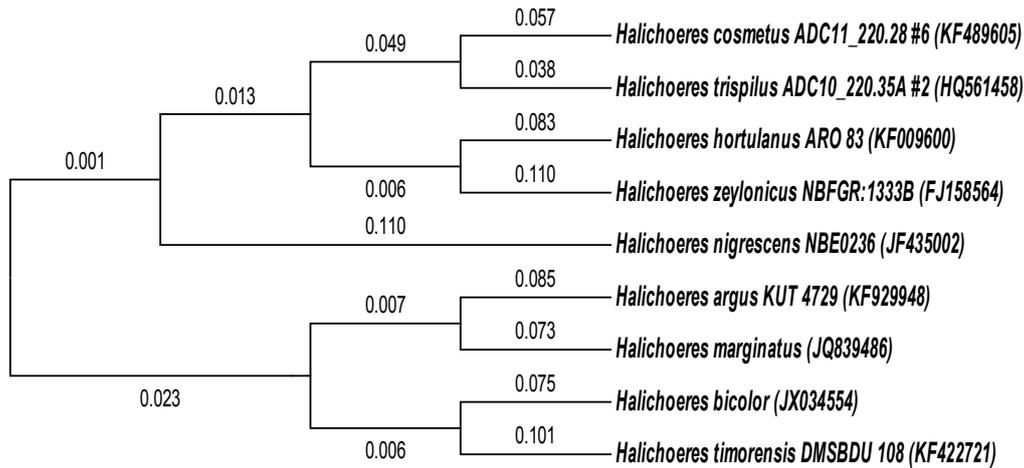
**Fig.1. Phylogenetic analysis of *H. timorensis* with closely related species by Neighbour Joining method**
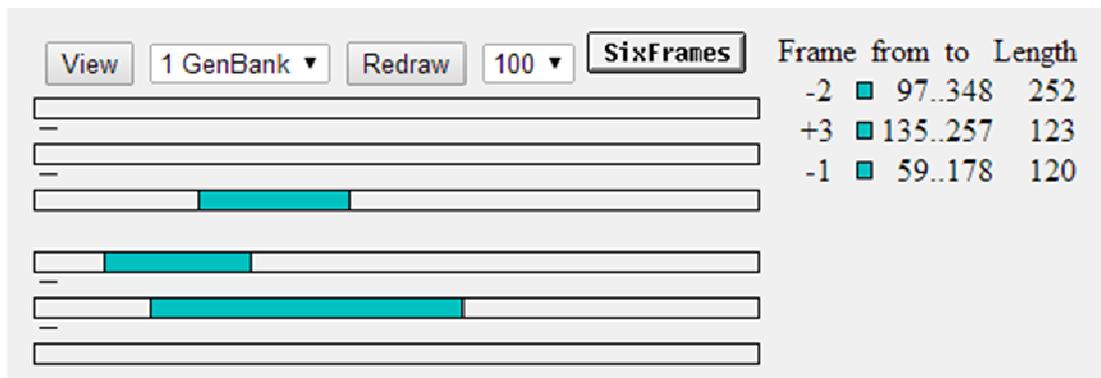
Fig.2. The Open reading frame for COI gene of *H. timorensis* contains three ORFs

Fig. 3. The mitochondrial COI sequence of *H. timorensis* about 656 bp. The highest open reading frames are marked from the start to end and the sequence were represent in bold is active start codon. The poly A signals was highlighted.

A simple gene prediction algorithm might look for a start codon followed by one open reading frame that is long enough to encode a typical protein, were the codon usage of the region that matches the frequency characteristic for the given organism's coding regions (Linsheng *et al*., 2001).

The polyadenylation signals from the *H. timorensis* are predicted that showed only one potential poly A signal at the position of 237 (Fig.3). It play major role in gene expression. The accurate predictions of polyadenylation sites play an important role in transcriptome diversity and regulating gene expression for defining the termination of genes and understanding its regulation mechanisms (Wu *et al*., 2012). When a DNA transcription region is start from the 5`-3` carboxyl group based on the start codon on which the DNA bound to methionine which denotes ATG and AUG for DNA and RNA respectively.

In conclusion, DNA barcoding is emerging as a valuable tool to identification of new species regulatory agencies and fisheries managers for species authentication, food safety, conservation management as well as consumer health and support (Costa *et al*., 2007). The results indicated that the COI gene is very conservative in *H. timorensis*, which is similar to the results obtained by Quan *et al*. (2001). This suggests that the COI gene could not be used to distinguish *H. timorensis* populations genetically, but it is useful for analysis of phylogenetic relationships among fish species. We conclude that *H. timorensis* is present in the Gulf of Mannar Region (South east Coast of India) and, to the best of our knowledge, this is the first record of *H. timorensis* in the Indian waters and also this species was sequenced and bar-coded at first time with identified as 656 bp region of the mitochondrial cytochrome oxidase subunit I gene, which strongly validated the efficacy of barcodes for identifying the ornamental fish species.

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