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Identification of fish species using DNA barcode from Visakhapatnam, east coast of India

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Abstract
Tripletail (*Lobotes surinamensis*), a pelagic fish species found throughout tropical and subtropical regions of the world's oceans, support commercial and recreational fisheries throughout much of its geographic range. Applications of DNA barcoding tools are emerging in the fields of fish conservation, management aspects such as quota, by-catch monitoring and sustainable fisheries monitoring science. Therefore, in this study, we generated DNA barcodes for fish species found at Visakhapatnam fishing harbour, developed a DNA barcode reference library, and investigated the efficiency of the library for identifying specimens at the species-level and analyzing the presence of cryptic species. Furthermore, we investigated the possible taxonomic misidentification of Tripletail (*Lobotes surinamensis*).

Keywords: DNA barcoding, *Lobotes surinamensis*, COI, phylogenetic tree, Clusta lX, mega

Introduction
Tripletail (*Lobotes surinamensis*), a pelagic fish species found throughout tropical and subtropical regions of the world's oceans, support commercial and recreational fisheries throughout much of its geographic range. This was a cosmopolitan fish species found in marine and brackish tropical and subtropical waters, whereas in the Mediterranean Sea is considered a rare species (Akyol and Kara, 2012) [2]. It inhabits a variety of habitats, from estuarine to open ocean waters and is usually found in association with submerged or floating structures. Juvenile specimens are usually found swimming on their side at the surface, probably mimicking a floating leaf in order to avoid predators, but also attracting potential prey (Froese and Pauly, 2016) [19]. Although tripletails sporadically occur at certain locations in the southern Mediterranean, this species is still considered rather rare for the Mediterranean as a whole (Akyol and Kara, 2012) [2].

DNA barcoding differs from these earlier approaches as it proposed (Hebert *et al*., 2004) [24] that the sequence of a single gene region could be used as the basis of a global bio-identification system for animals. The availability of broad-range primers for the amplification of a 655 base pair (bp) fragment of cytochrome c oxidase subunit I (COI) from diverse phyla (Folmer *et al*., 1994) [18] established the 5' end of this mitochondrial gene as a particularly promising target for species identification. COI encodes part of the terminal enzyme of the respiratory chain of mitochondria. Barcoding has also been employed to validate the identity of animal cell lines (Lorenz *et al*., 2005; Cooper *et al*., 2007) [36, 13] and is a recommended characterization step for materials in biodiversity repositories (Hanner and Gregory, 2007) [13]. Interestingly, the same gene region of COI has also been shown to be effective for species identification in red macroalgae (Saunders, 2005) [47], in single celled protists Tetrahymena (Chantangsi *et al*., 2007) [10] and for some fungi (Seifert *et al*., 2007) [50]. Its power to discriminate closely related species is largely attributable to the abundance of synonymous nucleotide changes (Ward and Holmes, 2007) [54].

The need for comprehensive and reliable species identification tools combined with early barcoding success with fishes (Savolainen *et al*., 2005; Ward *et al*., 2005) [48, 8] provoked the formation of the the Fish Barcode of Life campaign (FISH-BOL) initiative (http://www.fishbol.org). Furthermore, the database is assisting the reconciliation of divergences in scientific, market and common names across nations. For ichthyologists, FISHBOL promises a powerful tool for extending understanding of the natural history and ecological interactions of fish species. Indeed, high-throughput barcoding is complementary to phylogenetic studies because it sheds light on divergent lineages for subsequent inclusion in

such analyses (Hajibabaei *et al*., 2007) [20].

The fish DNA barcoding observation seems to be reflected in species identification results 98% of investigated in marine species (Ward *et al*., 2009) [55]. The current DNA barcoding methodology it is possible to separate the species. Further, DNA barcoding COI gene sequences produced regional genetic differentiation and shared haplotypes genetic differences due to the different habitants (Hubert *et al*., 2008; Ward *et al*., 2009) [27, 55]. The unambiguous identification of taxa is the main requirement to prevent/control this type of activity, and forensic molecular approaches using the cytochrome oxidase subunit I (COI) gene have successfully addressed this issue (Filonzi *et al*., 2010; Cawthorn *et al*., 2012; Carvalho *et al*., 2017) [17, 9, 8]. Such an assessment is crucial to alert the relevant authorities regarding the need for effective measures of organization, standardization, and surveillance of the fishery sector for combating replacement practices and safeguarding consumer rights.

In this study, we generated DNA barcodes for fish species found at Visakhapatnam fishing harbour, developed a DNA barcode reference library, and investigated the efficiency of the library for identifying specimens at the species-level and analyzing the presence of cryptic species. Furthermore, we investigated the possible taxonomic misidentification of Tripletail (*Lobotes surinamensis*). The literature about Tripletail (*Lobotes surinamensis*) of DNA barcoding is scanty. Our main objective was to generate a reference library of DNA barcodes that can be applicable in our study area and adjacent similar habitats and used in future monitoring programs for improved environmental assessments.

Material and methods

Sampling

To consider possible intra-species sequence variations, fishes were collected from fishing boats operating from north-east coast of Visakhapatnam, Andhra Pradesh. Species identification was based on descriptions of Roper *et al* (1984). Biometric measurements such as dorsal mantle length (DML; in cm) and weight (g) for individual species were recorded and a total of 50 individuals were used for this study. After initial rinsing with seawater, the samples were preserved in polyethylene bags and kept frozen at -20°C until further analyses. One muscle tissue sample was collected from fillet piece, and the sample was stored in micro-tubes containing 90% alcohol, received a registration code and stored at -20°C.

Extraction of genetic material

0.1 – 0.2 g of tissue preserved in ethanol was kept in a fresh 1.5 ml vial (sterile) and add 180 µl of Lysis Buffer I. mechanically grind the tissue, using the tissue grinder provided. 20 µl for Proteinase k was added and mixed thoroughly by vortexing and were incubated at 55℃ until complete lysis is observed. Added 200 µl of lysis buffer II and mixed gentle by vortexing and incubated at 70℃ for 15 – 20 minutes to neutralize and were centrifuged for 10 minutes at 10000 rpm to remove debris. Transferred the supernatant to a fresh 1.5 ml vial and added Add 4 ml of RNase A and mixed gentle by vortexing and incubate at room temperature for 5 – 10 minutes. 200 µl of absolute ethanol was added in a collection tube of GeneiPure column and centrifuged for 1 minute at 11000 rpm, then discard the GeneiPure column collection tube and replace the GeneiPure column into a new collection tube. Added 500µl of wash buffer I and II – Ethanol Mixture and centrifuged at 11000 rpm for 1 minute (wash buffer I) and 2 minutes (wash buffer II) and discard the collection tube with flow through and replaced the GeneiPure column in a new collection tube.

Elution Buffer has taken in a sterile 1.5 ml vial and warm for 5 minutes at 70℃ in a dry bath. 100µl of pre-warmed Elution Buffer (70℃) transferred into the center of GeneiPure column, incubated for 5 minutes at room temperature also were centrifuged to elute the DNA for 1 – 2 minutes and stored at -20℃. After that, the samples were exposed under ultraviolet light to assess the quality of the extracted DNA. Mitochondrial DNA was screened as potential markers for species identification in this study were identified was COI.

Amplification and sequencing of genetic material

Polymerase chain reaction (PCR) with a total volume of 15 ml contained 1.5 ml 106 reaction buffer, 1.5 ml dNTPs (10 mM), 0.05 ml of each primer (100 pmol/ml), 5 ml DNA-extract, 0.3 ml Teg polymerase (3U/ml; comparable with Taq polymerase; Prokaria, Reykjavik, Iceland), and 6.6 ml deionised ultra-pure water. Thermal profile began at 94µC for 4 min, followed by 35 cycles of 94µC (30 s), 52µC (30 s), and 72µC (90 s), with a final step of 7 min at 72µC. In order to amplify a fragment of COI, degenerated primers were designed on the basis of the universal COI primers for fish published by Ward *et al*. (2005) [55]: COI-Fish-F (5'-TTC TCA ACT AAC CAY AAA GAY ATY GG-3') and COI-Fish-R (5'- TAG ACT TCT GGG TGG CCR AAR AAY CA-3'. The volume of the PCRs was 15 ml and contained 1.5 ml 106reaction buffer, 1.5 ml dNTPs (10 mM), 0.05 ml of each primer (100 pmol/ ml), 3 ml DNA-extract, 0.2 ml Teg polymerase (3 U/ml; Prokaria, Reykjavik, Iceland), and 9.7 ml deionised water. Thermal profile started with 94µC for 4 min, followed by 30 cycles of 94µC (50 s), 59µC (50 s), and 72µC (90 s), finalized at 72µC for 7 min. PCR products were purified by using the ExoSAP-IT for PCR clean-up (GE Healthcare, Uppsala, Sweden). The COI product was sequenced with the PCR primers shown above. The Big Dye Terminator Cycle Sequencing Kit (ver. 3.1, PE Biosystems, Foster City, USA) and an ABI Prism 3730 automated DNA Analyser (Applied Bio systems, Foster City, USA) were used according to the manufacturer's instructions.

Results

DNA barcoding uses small regions of mitochondrial DNA that work as a barcode to amplify a gene. DNA sequencing and matching of unidentified sequence with the closely related individual in BOLD or NCBI libraries can be conducted within hours, so the response time depends greatly on available infrastructures, such as reference sequence or voucher specimen in NCBI and BOLD libraries. DNA barcoding is now well established; leads typically to accurate results and the DNA sequencing costs are low and constantly dropping. A major benefit of DNA-based analytical procedures is that they can be applied throughout the food supply chain, from whole specimens to trace samples (scales and fins), to highly processed and cooked fish products In addition, DNA analysis is use readily on not only fresh fish samples but also preserved historical material (bones and/or scales from museums). The positive PCRs were sequenced using the dideoxy-terminal method (Sanger *et al*., 1977) [45], with the Big Dye Kit (ABI Prism-TM Dye Terminator Cycle Sequencing Reading Reaction), and employing an ABI 3500 XL automated capillary sequencer (Life Technologies). A chromatogram was generated in a specific file format called

ABI format. The chromatogram is visualized by a locally installed tool, *viz*. Finch TV Version 1.4.0 (Geospiza.com, 2015) [26]. The chromatogram was converted to a sequence, in FASTA format using Finch TV which was represented in the (figure 1).

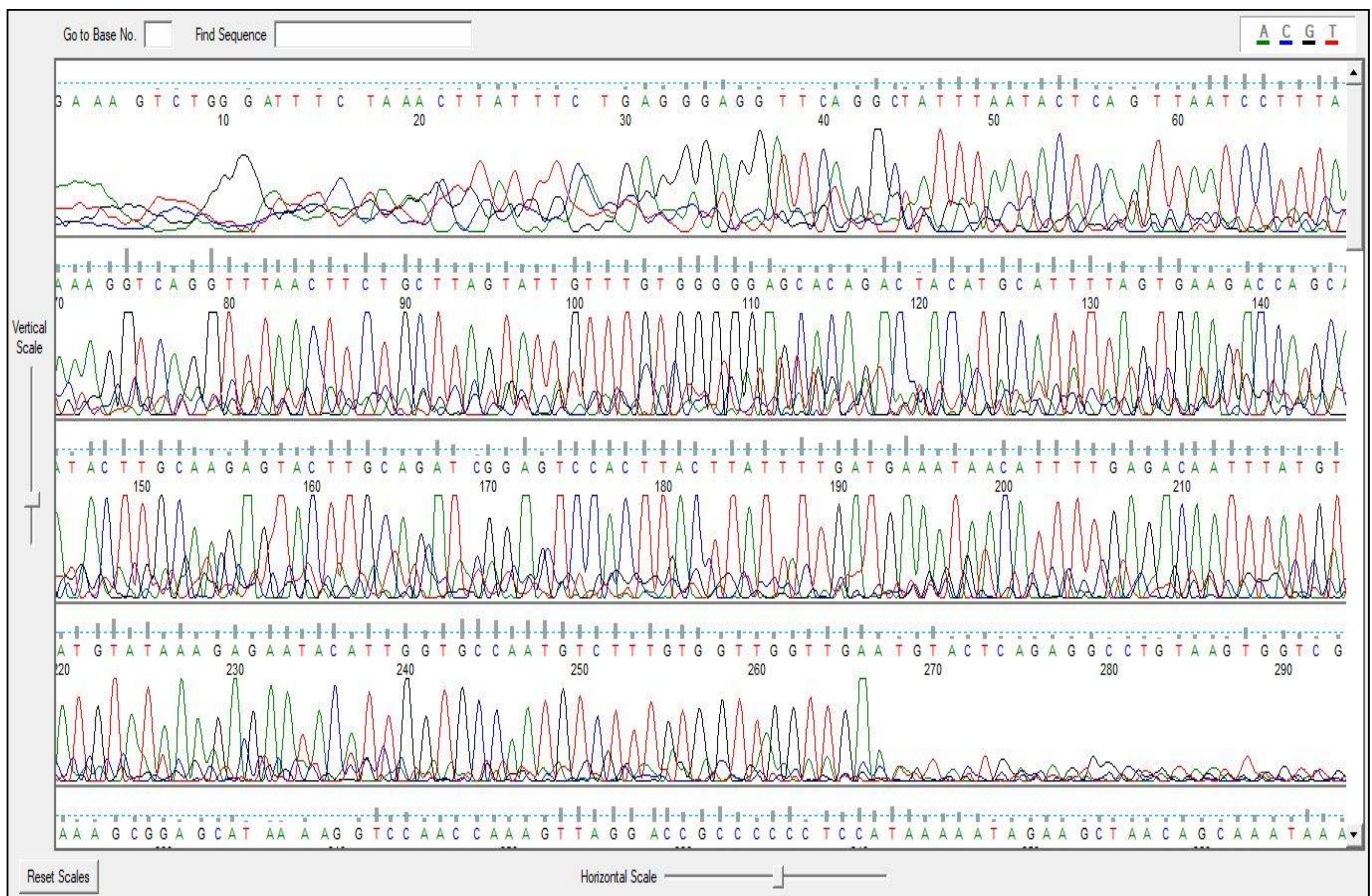

Fig 1: Chromatogram showing the nucleic acid residues with quality at that position

Sequence analysis

ORF Finder

The raw DNA sequence was analyzed, using ORF finder tool (https://www.ncbi.nlm.nih.gov/orffinder/), was used to search for the open reading frames in the gene sequence. All the Parameters used were default, except the ‘Genetic Code’ parameter was set to be ‘Vertebrate Mitochondrial’. The second frame was downloaded and saved in FASTA format for further analysis. The ORF finder tool also predicted the protein.

Performing Blast search

The open reading frame deciphered in the previous step was subjected to Basic Local Alignment Search Tool (BLAST, Altschul *et al*., 1990) [3] using default parameters, except BLAST algorithm parameter ‘Max target sequences’ was set to 5000 sequences. Specific COI sequences were selected for further analysis The Blast results showed diverse homologous sequences all of them with significant e-value. Only good scoring COI sequences of various organisms those having query coverage as well as sequence Identity more than 70% were selected. Total number of 70 sequences was found.

Data analysis

The Multiple Sequence Alignment (MSA) depicted the less conserved regions at its ends due to this editing operation was performed using Bio Edit (Hall TA, 1999) [21]. The edited file was stored in Mega v7.0.26 supported formats. After creating a database of fillet sequences, some corrections were performed manually in sequence positions with errors or doubts regarding the nucleotide present.

Performing multiple sequence alignment

Sequences were aligned using ClustalX version 2.1 (Larkin *et al*., 2007) [34] installed locally. The results of Multiple Sequence Alignment (MSA) depicted gaps towards the ends of the MSA resulting in the decrease in sequence conservation in those positions. This was also supported by the Conservation Score Plot. Therefore, the Multiple Sequence Alignment (MSA) was edited using Bio Edit Sequence Alignment Editor (Hall TA, 1999) [21]. The Edited sequences were stored in Mega v7.0.26 supported formats. A conservation score plot was regenerated after editing the multiple sequence alignment.

Phylogenetic tree construction

The Phylogenetic tree was constructed by using Mega v7.0.26. The parameters used were Test of Phylogeny: The Number of Bootstrap replicates were set to 2000. It is saved in Newick Format and depicts the phylogenetic tree obtained from Mega v7.0.26. It indicates the diverse species related to each other in the tree and our sequence falls in one of the cluster as highlighted. The sequence obtained from *Lobotes surinamensis* species, shown close relation to many species like segmented worms and proteobacter.

Tree Visualization

The tree obtained in Newick format is opened in Interactive Tree of Life (iTOL, https://itol.embl.de/) (Letunic and Bork, 2016) [35]. Edited and presented figure 2. In order to have a more interactive picture of the phylogenetic tree, the Newick tree format was uploaded into iTOL (https://itol.embl.de/) browser to get a detailed picture. The corresponding trees developed in circular, un-rooted and rectangular format which were colored according to their classes to predict the relation of the given sequence in between those classes. Such interactive division of the phylogenetic tree depicts the given species *Lobotes surinamensis* shows a close relation to the segmented worms & gamma proteobacter.

Discussion

Despite the innovative applications of DNA barcoding, it has been controversial in some scientific circles (Ebach and Holdredge, 2005; Will and Rubinoff, 2004) [15. 56]. Interestingly recent results illustrated some straightforward benefits from the use of standardized species-specific molecular tags derived from COI gene for species-level identifications (Hebert *et al*., 2003; Ward *et al*., 2005; Lakra *et al*., 2011) [23, 33, 53]. DNA barcoding aims to provide an efficient method for species-level identifications using an array of species-specific molecular tags derived from COI gene (Pradhan *et al*., 2015) [42]. DNA barcoding clearly discriminated freshwater fish species from Canada (Hubert *et al*., 2008) [27] and Mexico and Guatemala (Valdez-Moreno *et al*., 2009) [52].

The validity of COI gene

Mitochondrial Cytochrome c Oxidase subunit I (COI) is a mitochondrial DNA gene that codes a protein, which helps in cellular respiration. Although COI gene is considered as a universal barcode in animals, its potency is challenging in some protists, fungi, and plants (Schoch *et al*. 2012) [49]. In fungi, Internal Transcribed Spacer (ITS) gene is more successful than COI gene to discriminate closely related taxa (Dentinger *et al*. 2010) [14]. Only a few published data are available on the successful of COI gene as a barcode in algae (Clarkston and Saunders 2010; Macaya and Zuccarello 2010) [12, 37]. It has been reported that a universal plastid amplicon (UPA) works well as a barcode in algae instead of COI (Sherwood, 2007) [51]. Furthermore, Saunder and Kucera (2010) reported that major advantage of UPA is its universality because this primer pair can reliably recover sequences from many groups of algae including green, red and brown marine macroalgae, diatoms, and also cyanobacteria (Sherwood and Presting, 2007) [51].

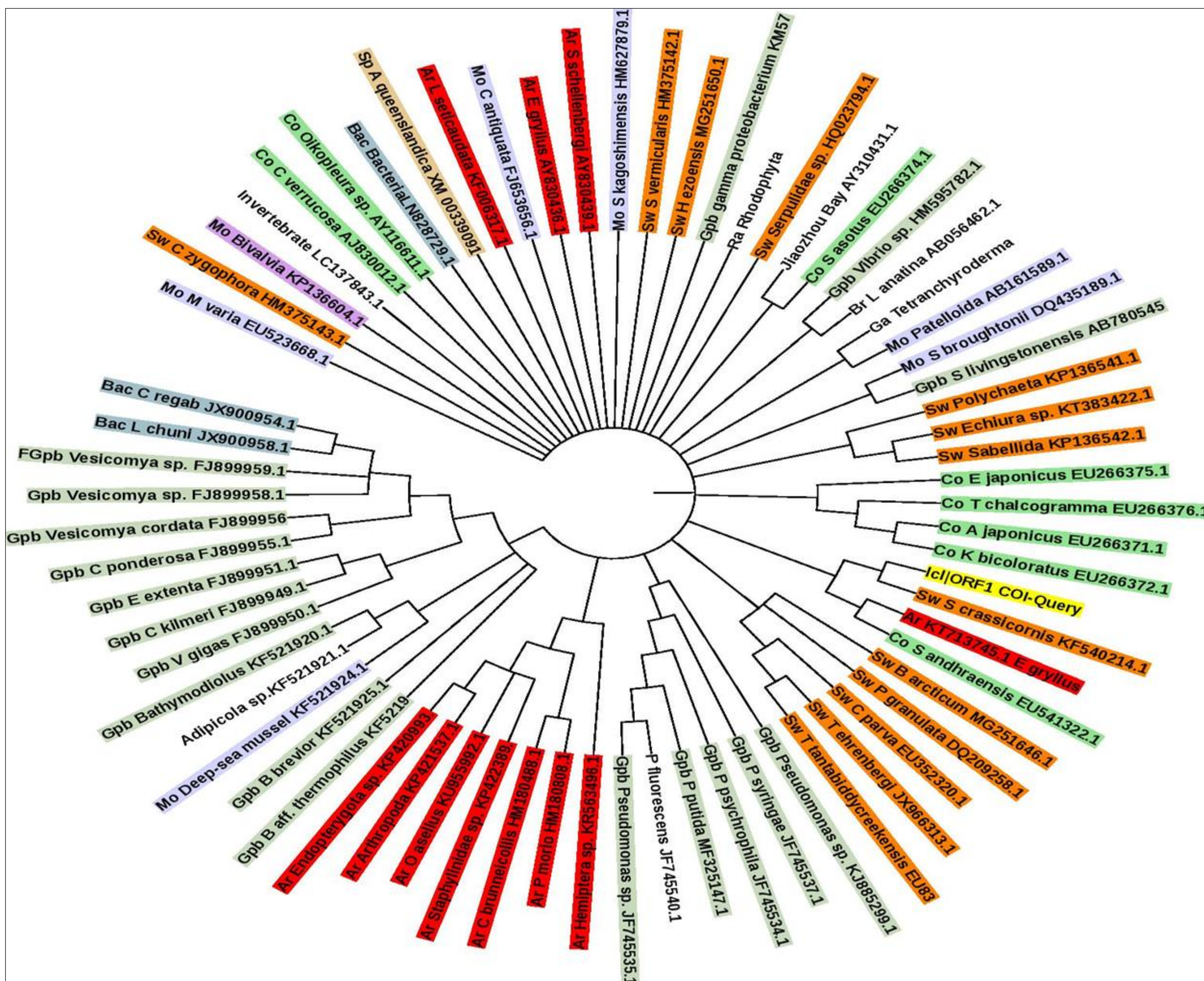

Fig 2: Phylogenetic tree of diverse groups of organisms in Circular tree from iTOL

DNA barcoding and species identification

Genetic identification of biodiversity is the necessity of time due to the presence of phenotypic similarities among neighboring species. Some organisms especially fish shows phenotypic plasticity with a change in its environment (Hutchings *et al*. 2007) [28]. DNA barcoding has the capability to identify not only in adult organisms but also at their early developmental stages. For example, Ko *et al*. (2013) [31] used DNA barcoding technique to successfully identified 100 specimens of fish larvae with a success rate of > 65 percent at the species level. However, with an increase in taxonomic level the identity rate also increased up to > 85 Percent. Likewise, Naim *et al*. (2012) [41] used COI gene to successfully identified approximately 60 individuals of mud crab into four species. Most recently, COI gene has been used to identify Ivory shell (Chiu *et al*. 2015) [11], and yellowfin tuna (Higashi *et al*. 2016).

DNA barcoding and population diversity

Mitochondrial DNA markers are not diploid because they are inherited from a single parent (maternal inheritance) so they are equally good for targeting population level studies. Although the cytochrome oxidase I (COI) gene information from DNA barcoding is not sufficient to investigate population-level questions (Bazin *et al*. 2006) [6], yet still it can be used to partially interpret the distribution of genomic diversity within taxa. Barcodes can give the status of species to an unknown specimen because they also consist information of genetic models (coalescent-model) based on population genetics (Abdo and Golding, 2007) [1]. The COI gene provides information about genetic variation within a population of a single species and this information can help to deduce the phenomenon of migration as well as genetic drift in fish populations (Mohammed Geba *et al*. 2016) [40]. To demonstrate, Boonkusol *et al*. (2016) [7] used mitochondrial COI gene sequences to access the genetic variability in snakehead fish of Thailand and deduced that genetic variation in the central river basin is due to fish dispersal by the flood. In the same fashion, the use of a combination of mitochondrial (COI) and nuclear genes (recombination activating gene-I (Rag I) and from nuclear alpha tropomyosin ‘intron V') can fully address the question of population structure as done by Eytan and Helburg (2010) [16] in Caribbean reef fish.

DNA barcoding and phylogenetic reconstruction

In barcoding, the information from an assemblage of species is in the form of genetic sequences, which is uploaded in a barcode library. However, the gene lengths from barcoding data are not sufficient to construct a deeper phylogenetic tree in resolving evolutionary relationship of organisms. Although the barcoding sequences have been used to construct Neighbour-Joining (NJ) tree, this barcode-based tree cannot be alternates of the phylogenetic tree. We strongly emphasize on the statement that DNA barcoding data can provide partial information about the phylogeny of species and can draw an outline for phylogeny that should be deeply analyzed by nuclear genes data. Lakra *et al*. (2011) [33] successfully identified 115 marine fish species that clustered into 79 genera when NJ tree was constructed to see the phylogenetic relationship among collected samples. Similarly, Krieger and Fuerst (2002) [32] showed that mutation rates are consistently lower for nuclear and mitochondrial genes in Acipenceri forms (sturgeons and paddlefish). The 5′ region of the COI gene was selected as the basis for a DNA barcoding system, in part, because of the availability of primers aiding its recovery from a broad range of taxa (Hebert *et al*., 2003) [23]. In the event that taxon specific primer mismatches are detected. Decisions on the nucleotide composition of new primers will be aided by the very large number of complete mitochondrial genomes available for fishes (Inoue *et al*., 2001; Miya *et al*., 2001; Miya *et al*., 2003) [29, 38, 39]. Further checked the sequences for nucleotide composition bias and there were no significant differences among them.

Conclusion

The reports from our data were similar with the taxonomic divisions of the finfish in the current study, based on the morphological characters as reported in FAO identification sheets. DNA barcoding focuses neither to build a tree-of-life nor to perform DNA taxonomy, however, moderately to produce a universal molecular identification key depends on the substantial taxonomic knowledge in the barcode reference library. The incontrovertible accomplishment of the DNA barcoding project is primarily due to the fact that DNA barcoding standards considerably enhance current practices in the molecular identification field and standardization offers virtually endless applications for different users. The present study has shown the efficacy of COI gene in identifying the *Lobotes surinamensis* fish species with designated barcodes as all the marine water fish species investigated corresponded to unique sequences that are distinct from each other. Our study strongly supports the potential utility of COI gene in barcoding the fish fauna.

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