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DNA Barcoding Of Coral Reef Associated Fishes Collected From Cuddalore, Southeast Coast Of India

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Abstract

DNA barcoding is the significant species identification method, it helps in the construction of phylogenetic tree and avert mislabeling of species. DNA barcoding of some of the coral reef associated fishes from Indian southeast coast were very scarce. Hence, in the present study DNA barcoding of five species of coral reef associated fishes such as *Lutjanus russellii*, *Siganus canaliculatus*, *Siganus javus*, *Acanthurus tristis* and *Trachinocephalus myops* collected from Cuddalore, southeast coast of India were studied. The sequences were submitted to the GenBank and their accession numbers were obtained. The GC content in the sequence of COI genes were also calculated, the maximum GC content was found in *Trachinocephalus myops* (50.47%) and minimum was in *Acanthurus tristis* (45.33%). The average GC content was 47.64 ± 1.01%. Among the five species analyzed the sequence for *Acanthurus tristis* was not available in the NCBI database earlier, hence this sequence may the first molecular evidence for GenBank database.

Key words: Cuddalore, DNA barcoding, Phylogenetic tree, Reef fishes

Introduction

DNA-related approaches are extensively used throughout different disciplines of biology and medicine (Duran et al. 2009). For the traceability of commercial fishes and sustainable exploitation of fishing resources, species identification is very important (Rasmussen, Morrissey 2008). Mislabeling in several fish groups distinguished by DNA-based methodology has been evidenced the need of genetic tools for species authentication (Marko et al. 2004; Machado-Schiaffino et al. 2008). Estimation of stock size could also adversely affect by mislabeling, if it impacts the reporting of catch data which are used in fisheries management (Marko et al. 2004). DNA barcoding is a potential identification method, which encompasses amplification and sequencing of a short standard nucleotide sequence of (approximately) 651 bp of the mitochondrial cytochrome coxidase subunit I (COI) gene (Hebert et al. 2003; Sadurudeen et al. 2017). Mitochondrial cytochrome coxidase subunit I gene is held as DNA barcode since it encode the highly

conserved protein in animals (Walia and G.K. Dhillon). The term "DNA barcode" was anticipated to propose the characteristics of nucleotide sequences used to epitomize a species in the similar way as the 11-digit Universal Product Codes in labeling the retail products (Ekrem et al. 2007; Chandan Haldar, Suchismita Nath 2020). The characteristic of a standard sequence that resembles to a single homologous gene region which can be amplified by a PCR with "universal primers", and discriminates a species from similar ones across a varied range of taxa is the chief concept of this useful tool. This can be a prospective tool for identifying even the larval forms of an organism and for incomplete specimens on which a morphological identification cannot be performed (Ekrem et al. 2007).

The use of DNA barcodes for recognizing marine fishes has now become an accepted concept (Ward et al. 2005). Usually, DNA barcoding can be used in two ways such as identification of previously described species and discovery of new species (Hebert et al. 2003; Hebert et al. 2004a). This technique primarily depends on the genetic divergence among the species which is high when compared to within the species. The distance found between and within species was therefore called as 'DNA barcoding gap' (Meyer, Paulay 2005). For assessing the DNA barcode for species identification, various approaches have been followed. Each and every method has its specific merits and demerits (Casiraghi et al. 2010). Predominantly, the distance based tactics like Neighbour joining (NJ) algorithm with Kimura-2-parameter (K2P) correction have been applied in several studies (Hebert et al. 2004b; Ward et al. 2005; Wong, Hanner 2008). Despite many contest have deliberated on this method, Kimura-2-parameter correction was professed as the foremost DNA substitution model for low genetic distances (Nei, Kumar 2000) and similarity based methodology like BLAST (Basic Local Alignment Search Tool) Altschul et al. (1990) with clustering method like Maximum likelihood (ML), Bayesian inference (BI), Neighbour joining (NJ) have been employed in species identification and decided that BLAST and NJ performing remarkably faster (Elias et al. 2007).

Materials and methods

DNA isolation

The coral reef associated fishes such as, *Lutjanus russellii*, *Siganus canaliculatus*, *Siganus javus*, *Acanthurus tristis* and *Trachinocephalus myops* were collected from Cuddalore landing center and were preserved in 95% ethanol. For precise and quick isolation of DNA from the fish tissues, salting out protocol were followed (Miller et al. 1988). Universal CO1 gene primers FishF1-5′-TCAACCAACCACAAAGACATTG GCAC-3′ and FishR1-5′-TAGACTTCTGGGTGGCCAAA GAATCA-3′ (Ward et al. 2005; Ajmal Khan 2010) were used for the amplification of the COI genes.

Polymerase chain reaction

The COI fragment was amplified by the GeneAmp PCR system 9700. The PCR was carried out in 25 µl volume [2.5 µl of 10 x $MgCl_2$ buffer, 1 µl of primer mix, 1 µl of DNA template, 2 µl dNTPs (each 2.5 mM), 0.5 µl of Taq polymerase (3U/µl) and 18 µl of distilled water) Polymerase chain reactions were performed in the following temperature and timing condition programmed in TechGene™, thermal cycler which includes initial denaturation at 94ºC for 5 min, thirty five cycles of 94ºC for 30 sec, annealing at 54ºC for 30 sec, extension at 72ºC for 1 min and final the extension at 72ºC for 10 min, and hold at 4ºC indefinitely.

Sequencing

The purified products of PCR were sent to Macrogen, Inc. (Seoul, Korea) for bidirectional sequencing. The DNA sequence analyzer, 3730 x l DNA analyzer with BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA) was used for the sequence analysis.

Sequence editing

The DNA sequences obtained were edited by the electropherogram peak clarities. Sequences containing noisy peaks were omitted from the analysis. The sequences were further assessed to check the insertion or deletions and stop codons in MEGA 5.0 (Molecular Evolutionary Genetic Analysis) software.

Sequence characterization and GenBank submission

Multiple sequence alignment and pair wise sequence alignment of all the sequences were executed by using Clustal W program implemented in MEGA 5.0. Nucleotide differences had been carefully observed and the differences were detected and edited manually. Sequences were translated into amino acid sequences using vertebrate mitochondrial codon pattern in the MEGA 5.0 for checking the pseudogene status. All the

sequences were correctly translated into amino acid sequences with their respective starting primes without any internal stop codon.
Sequences were verified for integrity by MEGABLAST searches using the BLAST tool. All the sequences were submitted to the NCBI's GenBank through BankIt according to NCBI's procedure with required information. GC content of each species was calculated by BioEdit software (V.7.0.9) according to Hall (1999). For the sequence comparisons, pairwise genetic distances were calculated for the species based on the Kimura 2-parameter (K2P) model according to Kimura (1980) using MEGA 5.0 (Tamura et al. 2011).

Phylogenetic analysis
DNA sequences were aligned by using Clustal W software (Thompson 1994), which was suitably integrated with MEGA 5.0 (Tamura et al. 2011) and default options were used for the first step of alignment. Large gaps were deleted manually and the final alignments with decreased levels of penalties for both the pair-wise and multiple alignments were made. In the substitution models both coding (all positions) and the non-coding sections of the sequences were used. The phylogenetic tree of CO1 gene sequence was constructed along with similar sequences taken from database according to the neighbor joining method (Saitou and Nei 1987). The percentage of replicate trees in the bootstrap test (1000 replicates) were constructed (Felsenstein 1985). The evolutionary distances were computed by using the p-distance method using the software MEGA version 5.0 (Nei and Kumar 2000; Tamura et al. 2011).

Results and Discussion

In the present study, DNA was isolated from the coral reef associated fishes viz., *Lutjanus russellii, Siganus canaliculatus, Siganus javus, Acanthurus tristis* and *Trachinocephalus myops* and the barcoding region of COI gene were amplified by using Universal COI gene primers. DNA barcoding is an efficient method for the species level identifications by using a range of species specific molecular tags obtained from the 5' region of mitochondrial cytochrome oxidase I (COI) gene. The mitochondrial DNA analyses have appeared as a powerful methods to resolve questions regarding species identification, fish taxonomy and population genetics (Hsu et al. 2009; Nalugwa et al. 2010). Hence, in the present study standard barcode region of mitochondrial COI gene was used for the identification of fish species. The sequences of all the five species of coral reef associated fishes were successfully amplified using universal COI primer set followed by Ward et al. (2005). The gene sequences obtained from the fishes were submitted to the GenBank and their accession numbers were acquired (Table 1). Out of 650-655 bp (base pairs) of the basic taxonomic sequence length, in the present study it was able to get 648 bp for *Lutjanus russellii,* 642 bp for *Siganus canaliculatus* and *Acanthurus tristis,* 640 bp for *Siganus javus* and 644 bp for *Trachinocephalus myops.*

The GC content in the sequence of COI genes were calculated by BioEdit software. In COI region, maximum GC content was found in *Trachinocephalus myops* (50.47%) and minimum was in *Acanthurus tristis* (45.33%). The average GC content was 47.64 ± 1.01%. Similarly, Saccone et al. (1999) derived GC contents of 38.4% and 43.2% from the complete mitochondrial genomes data of three Chondrichthyes species and nine Osteichthyes respectively. Ward et al. (2005) also concluded the 655 bp mitochondrial cox1 region containing the GC content was on average higher in 143 of Osteichthyes species (47.1%) than in 61 of Chondrichthyes species (42.2%). These values correspond reasonably well to the present study, particularly with respect to the higher GC content in the studied fishes. The evolutionary of proteins were influenced by the GC content, because of its energy cost, besides the synthesis of both amino acids and bases are involved in this process (Du et al. 2018).

Table 1. The GenBank accession numbers and GC contents of barcoded coral reef associated fishes

Family	Species	Sample code	Accession number	GC content (%)
Lutjanidae	*Lutjanus russellii*	CRAF1	KJ679901	45.37
Siganidae	*Siganus canaliculatus*	CRAF2	KJ679902	49.07
Siganidae	*Siganus javus*	CRAF3	KJ679903	47.97
Acanthuridae	*Acanthurus tristis*	CRAF4	KJ679904	45.33
Synodontidae	*Trachinocephalus myops*	CRAF5	KJ679905	50.47

Genetic distance
Using Maximum Composite Likelihood method, the genetic distance of each species was analysed based on the pair-wise distance analysis in MEGA 5 software (Table 2). The nucleotide sequence of *Lutjanus russellii*

had highest genetic distance with *Siganus javus* (1.468) and lowest with *Acanthurus tristis* (1.421). *Siganus canaliculatus*, *Siganus javus* and *Acanthurus tristis* had highest genetic distance with *Trachinocephalus myops* (0.249, 0.265 and 0.274 respectively). *Siganus canaliculatus* had lowest genetic distance with *Siganus javus* (0.143) and *Siganus javus* had lowest with *Siganus canaliculatus* (0.143). *Acanthurus tristis* had lowest genetic distance with *Lutjanus russellii* (1.421). *Trachinocephalus myops* had highest genetic distance with *Acanthurus tristis* (0.274) and lowest with *Lutjanus russellii* (1.432). The number of base differences per sequence was averaging from the overall sequence pairs (overall mean distance) of COI is 0.7%.

Table 2. Pair-wise genetic distance (%) of COI sequence of coral reef associated fishes

	L. russellii	***S. canaliculatus***	***S. javus***	***A. tristis***	***T. myops***
L. russellii	0.00				
S. canaliculatus	1.444	0.00			
S. javus	1.468	0.143	0.00		
A. tristis	1.421	0.192	0.200	0.00	
T. myops	1.432	0.249	0.265	0.274	0.000

The attainment of barcoding approach relies on the distribution of genetic distances between heterospecific individuals and conspecific individuals (Meyer, Paulay 2005). The lineage diversifies more quickly within species than between species (Pons 2006). As consequence of that, mutation has determined the diversification within species at a rate higher than the speciation within the lineages. Hence, the branch length between species which leads to be much deeper than between the conspecific individuals which leading to a gap in the distribution of pairwise distance between the conspecific individuals and between species that has been mentioned to the barcoding gap (Meyer, Paulay 2005). In the present study, genetic distance of coral reef fishes ranged between 0.143-1.468, which is considered rather high compared to most studied fish species (Gao 2011; Tzeng, Chiu 2012). In terms of interspecific comparisons, a high K2P distance should signify a clear species separation.

Phylogenetic relationship

The phylogenetic relationship of the fish species in the present study was compared with the very most similar sequences present in NCBI database and the results were given in Table 3. The phenotypically distinguished gene sequences of *Lutjanus russelli* (DQ900716) from China (99% identity), *Siganus spinus* (JQ432158) from France (97%), *Siganus javus* (EU752210) from USA (98%), *Acanthurus triostegus* (KF929569) from USA and *Trachinocephalus myops* (KF930506) from USA (98%) in the NCBI's nucleotide database were closely related to *Lutjanus russellii*, *Siganus canaliculatus*, *Siganus javus*, *Acanthurus tristis* and *Trachinocephalus myops* respectively. It was the maximum identical percentage for the study species sequences with GenBank sequences.

Table 3. List of sequences of similar species used to construct the Phylogenetic tree

Species name	**Reference species from GenBank**	**Accession number**	**Similarity (%)**	**Country**
Lutjanus russellii KJ679901	*Lutjanus russelli*	DQ900716	99	China
	Lutjanus russelli	DQ900714	99	China
	Lutjanus russelli	DQ900715	99	China
	Lutjanus fulvus	DQ900709	95	China
	Lutjanus stellatus	DQ900702	93	China
Siganus canaliculatus KJ679902	*Siganus canaliculatus*	KJ872545	89	China
	Siganus spinus	JQ432158	97	France
	Siganus spinus	KC970420	97	Philippines
	Siganus luridus	JQ350366	91	France
	Siganus fuscescens	EF609464	89	Australia
Siganus javus KJ679903	*Siganus javus*	EU752210	98	USA
	Siganus javus	EU752209	98	USA
	Siganus javus	KC959885	98	Philippines
	Siganus stellatus	KF930444	93	USA
	Siganus virgatus	FJ584112	91	Canada
Acanthurus tristis KJ679904	*Acanthurus triostegus*	KF929569	97	USA
	Acanthurus triostegus	JQ349667	97	France

	Acanthurus sp.	FJ582691	91	Canada
	Acanthurus olivaceus	KC970448	89	Philippines
	Acanthurus pyroferus	HM034199	88	USA
Trachinocephalus myops KJ679905	*Trachinocephalus myops*	JX519395	98	USA
	Trachinocephalus myops	JQ841030	97	USA
	Trachinocephalus myops	JQ843092	96	USA
	Trachinocephalus myops	EU595323	86	Canada
	Scarus russelii	KF489744	81	Canada

Molecular phylogenetic evaluations of closely related species contribute insights into their evolutionary relationships letting us to authenticate their morphological taxonomic classification. Sometimes such studies specify that the formerly assumed classification is wrong or not sufficient (Persis 2009). For the identification of species, DNA barcoding is tremendously the most powerful tool than that of protein fingerprinting. Although barcode analysis prospects only to define species boundaries, there is noticeably some phylogenetic signal in CO1 sequence data. Even so methodologies for phylogeny reconstruction from molecular data endure somewhat controversial with a wide range of disparate approaches possible (Nei, Kumar 2000).

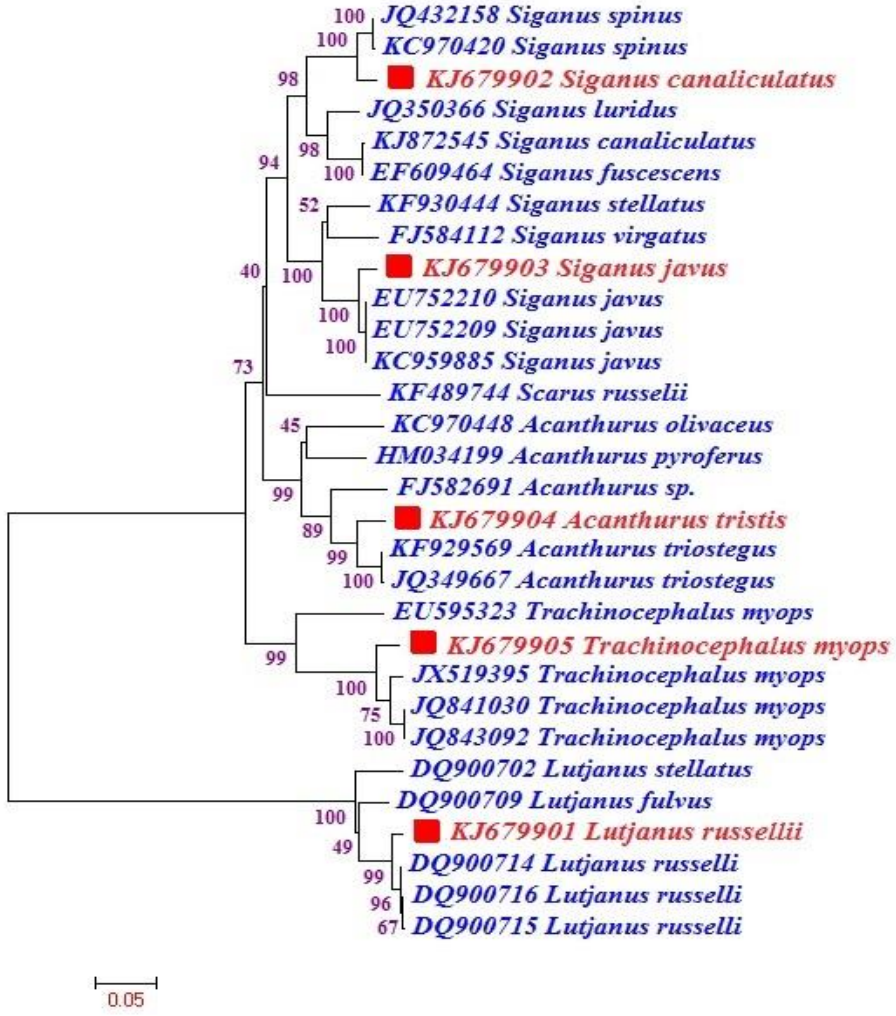

Figure 1. Phylogenetic relationships of sequences of the coral reef associated fishes along with the similar species

The evolutionary history of the fishes was determined using Neighbor-Joining technique. The most favourable tree with the sum of branch length = 1.58973958 was shown. The percentages of linked taxa of the replicate trees which clustered together in the bootstrap test (1000 replicates) were exposed next to the branches. The tree drawn with the same scale of branch lengths units used to conclude the phylogenetic tree

by the evolutionary distances. The evolutionary distances were calculated using the p-distance methods and were in the units of number of base differences per site. Thirty nucleotide sequences were involved in the analysis. 1st+2nd+3rd+Noncoding codon positions were included. All the positions containing missing data and gaps were eliminated. In the final dataset a total of 375 positions were there.
Figure 1, shows the neighbour-joining tree of the coral reef associated fishes barcoded. The sequences of currently barcoded species with those of barcoded earlier were analysed through construction of phylogram. As barcodes of the same species invariably get clustered in same clad it is clear that across geography, barcodes of the same species do not contain much variations, all the related specimens of formed cohesive units were separated from each other in the Neibour joining. In the present study the Neibour joining tree of coral reef associated fishes revealed identical phylogenetic relationship among the species. All the species were found genetically distinct from each other, similar result was reported for *channa* species by Lakra et al (2010).

Conclusion

The present results reveal that DNA barcoding of species will permit the definite identification of majority of fish species. With the increasing uses of DNA barcoding, numerous previously unrecognized fish species will be discovered through discovery of the deep divergence of CO1 sequences within the currently recognized species. Among the five species studied, the sequence for *Acanthurus tristis* was not available in the NCBI database earlier, hence this studied sequence may the first molecular evidence for GenBank database. The generated sequence data will act as a benchmark and reference data for identifying the respective species around the world.

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