

MtDNA Barcode Identification of Finfish Larvae from Vellar Estuary, Tamilnadu, India

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Abstract

Planktonic larvae were captured in the shallow Vellar estuary, Tamilnadu, India, during January to June 2013 using zooplankton net. Larvae were identified to the species level by comparison with Genbank data base using MtDNA sequence data. Further analysis showed that there is no sequence variation within-species, whereas between-species variation was more than 25%, supporting the suitability for species identification. The highest genetic distance was observed between *A. nigropunctatus* and *M. cephalus* (0.434) and the lowest genetic distance was observed between *T. jarbua* and *S. commersoni* (0.256). Given the current worldwide interest in DNA barcoding and species identification using MtDNA gene marker (CO1), it was confirmed the efficacy of the Fish-F1 and -R1 primer set, which gave the possibility for identification of finfish larvae at species level.

Keywords: cytochrome oxidase I, genetic distance, ichthyoplankton, mitochondria

Introduction

In the early life history of fishes, many species share the same morphology and their morphometric measurements are often duplicated (Victor *et al.*, 2009). Traditionally, the larval fish has always been identified by morphological characters, such as the body shape, pigmentation, meristic count and measurements. However, these characters are not enough to identify each species, especially those are rare and cryptic (Matarese *et al.*, 2011). The morphology of the same species can change quickly and significantly during its development from preflexion larvae to postflexion and to the pre-juvenile stage, when the fish settles down. Thus, the same species at different developmental stages may be identified as several different species when using just morphological characters (Ko *et al.*, 2013). The basic characters normally employed for the identification of finfish larvae belonging to various families are the shape of the body, shape of the gut, position of anal opening, type of alimentary canal, arrangement of muscle fibres, number of myotomes, origin and position of paired and unpaired fins besides changing pattern of pigmentation etc. (Sundaramanickam, 2004).

Marine fish larval identification would be a very difficult task among the huge species diversity of teleost fishes if the research is merely based on limited morphological observation of field sampling larval fish species (Yamaguchi *et al.*, 2000). Shao *et al.* (2002) has pointed out that only molecular identification can give guarantee identification of the fish eggs to the species level and this conclusion can be applied to larval fish identification too. The application of DNA barcoding to the larval fish identification has become

so popular in recent years (Pegg *et al.*, 2006). 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. DNA barcoding works under the principle that inter species variations are greater than the intra species variations, allowing one to distinguish the species using nucleotide sequences. Cytochrome c oxidase subunit I gene (CO1) have been accepted as a universal barcode to delineate animal life of this planet. DNA barcode technique could be used as a rapid tool to survey many uncertain species, species composition and cryptic species of adults in a study area (Spies, 2006) as well as morphologically similar species (Ward *et al.*, 2008). DNA barcoding can speed the discovery of new species, as large sequence differences in animal MtDNA generally give species status (Krishna *et al.*, 2012).

Vellar estuary is in the southeast coast of India (11°30'N; 79°46'E) and harbours many euryhaline fishes, many of them commercially important. It joins to Bay of Bengal at Parangipettai and is said to be a 'true estuary' as there is no complete closure of the mouth. This estuary has been demarcated into marine, gradient, tidal and freshwater zones, based on salinity characteristics. It is subjected to semi-diurnal tides with maximum tidal amplitude of about 1 m. The average depth ranges between 2.3 and 5.0 m. Influence of the neritic water with the estuarine environment promotes a perfect exchange of both biotic and abiotic components and the tidal influence extends over a distance of 16 km upstream of the estuary. The estuary extends over an area of 262 ha and 600 meters wide at its junction with the sea. Mangroves have

been developed over an area of 10 ha along the northern bank of the estuary in the estuarine zone. A recent study reported that there are 95 species of finfishes available in this estuary (Murugan *et al.*, 2014). Never the less, the larval biodiversity data is sparse in this estuary. The objective of this study is to identify some dominant fish larvae in Vellar estuary upto species level using CO1 gene and find the phylogenetic relationship among them.

Materials and methods

Larvae collection and identification

Fish larvae were collected under the mangrove areas of Vellar estuary during tide periods early in the morning and in the evening, with the help of zooplankton net with a mesh size of 158 μ m. The collected larvae were divided into four groups as per the initial morphometric characters, such as body shape, length and width and transferred into 2 ml eppendorf tubes containing 95% ethanol. Photographs were taken by using a microscope mounted with digital camera. Morphometric identification was done by following the report of Ramaiyan *et al.* (2005). The basic characters used for the identification were: size of the body, shape of the larvae, nature of muscle fibers, number of myotomes, alimentary canal, position of the vent, origin and location of paired and unpaired fins and changing pattern of pigmentation.

DNA isolation, PCR and CO1 gene sequencing

DNA was isolated from the individual fish larvae by the standardized salting-out procedure (Sambrook *et al.*, 1989). Quantity and quality of the extracted DNA was checked in UV spectrophotometer, 0.8% agarose gel electrophoresis respectively. The CO1 gene was amplified in a 50 μ L of PCR mix containing 5 μ L of 10X Taq polymerase buffer, 2 μ L of $MgCl_2$ (50 mM), 0.25 μ L of each dNTP (0.05 mM), 0.5 μ L of each primer (0.01 mM), 0.6 U of Taq polymerase and 2 μ L of genomic DNA. The universal primers for CO1 gene, FishF1-5' TCAACCAACCACAAAGACATTGGCAC-3' and FishR1-5' TAGACTTCTGGGTGGCCAAA GAATCA-3' (Ward *et al.*, 2005) were used. The thermal regime consisted of an initial denaturation step for 3 min at 95 $^{\circ}$ C followed by 35 cycles of 30 S at 94 $^{\circ}$ C, 30 S at 45 $^{\circ}$ C and 1 min at 72 $^{\circ}$ C, followed by final extension for 10 min at 72 $^{\circ}$ C. The PCR products were visualized on 1.5% agarose gels and the most intense products were cleaned up and sequenced by a commercial sequencing facility.

Sequence analysis

The CO1 gene partial sequences of the selected individuals were unambiguously edited, aligned using CLUSTAL-W in BioEdit and checked manually. All the sequences were confirmed by similarity index by the NCBI's BLAST program. Based on the higher percentage similarity and query coverage against the reference species, the species were confirmed. All the sequences were submitted to the NCBI's Genbank. Genetic distance within species and between species were determined by Kimura 2-Parameter method (Kimura, 1980) using the software program MEGA 3.1 (Kumar *et al.*, 2004). The neighbour-joining (NJ) tree for CO1 was constructed and to verify the robustness of the internal nodes, bootstrap analysis was carried out using 1,000 pseudo-replications.

Results and discussions

Molecular identification

Totally, 456 larval samples were collected from the intertidal region of Vellar estuary. Among these 456 larvae, four morphotypes were segregated based on the morphological characters and proceeded for molecular identification to confirm to species level. The four morphotype larval CO1 gene was successfully amplified and sequenced. PCR amplified CO1 gene showed 650 bp in length and partial at both ends after editing. All the sequences showed first frame and no internal stop codons were observed. In BLAST, all the sequences showed >99% similarity with CO1 gene of specific species in the NCBI nucleotide database. Based on the BLAST results, the morphotype 1 was identified as *Mugil cephalus*, morphotype 2 was identified as *Scomberomorus commersoni*, morphotype 3 was identified as *Arothron nigropunctatus* and morphotype 4 was identified as *Terapon jarbua*. The average percentage of base composition of the CO1 gene fragment in four fish species was as follows: *M. cephalus* A: 24.5, T: 31.0, G: 17.5, C: 27.0%; *S. commersoni* A: 23.0, T: 29.5, G: 18.0, C: 29.5% and *A. nigropunctatus* A: 23.5, T: 26.5, G: 19.0, C: 31.0%; *T. jarbua* A: 22.5, T: 28.0, G: 18.0, C: 31.5%. The high AT content (55.05%) was observed in *Mugil cephalus* (Fig. 1).

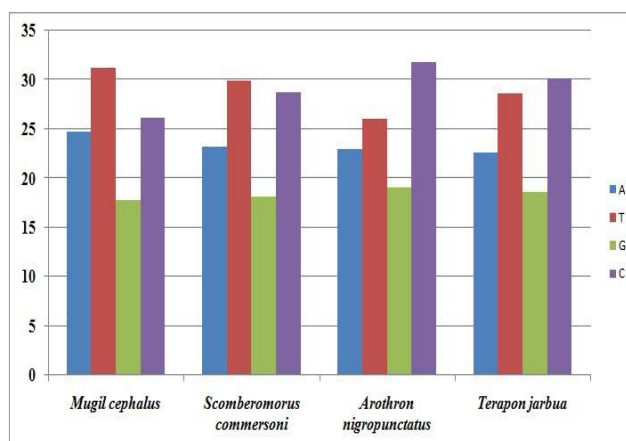


Fig. 1. Nucleotide percentage compositions of CO1 gene in the four fish species

Genetic distance

The genetic distances between the four species of fish larvae are given in Table 1. The highest genetic distance was observed between *A. nigropunctatus* and *M. cephalus* (0.434) and the lowest genetic distance was observed between *T. jarbua* and *S. commersoni* (0.256). The overall mean genetic distance was 0.306 based on the CO1 gene sequence.

Table 1. K2P genetic distance within species (bold) and between species among the four fish larvae based on CO1 sequence data

Species	<i>Mugil cephalus</i>	<i>Scomberomorus commersoni</i>	<i>Arothron nigropunctatus</i>	<i>Terapon jarbua</i>
<i>Mugil cephalus</i>	0.000			
<i>Scomberomorus commersoni</i>	0.346	0.000		
<i>Arothron nigropunctatus</i>	0.434	0.340	0.000	
<i>Terapon jarbua</i>	0.411	0.256	0.343	0.000
Overall mean	0.306			

Phylogenetic analysis

In the dendrogram analysis, individuals of the four species clustered into two major clades (Fig. 2). The first major clade consists of *Arothron nigropunctatus*, *Terapon jarbua* and *Scomberomorus commersoni*. From the dendrogram it is ascertain that the fish genus is genetically homogenous. The second clade consists of *T. jarbua*. This phylogenetic construction using neighbour joining method shows the two clear branches without any change. All the species possess better node stability with good bootstrap values (100) for branch support.

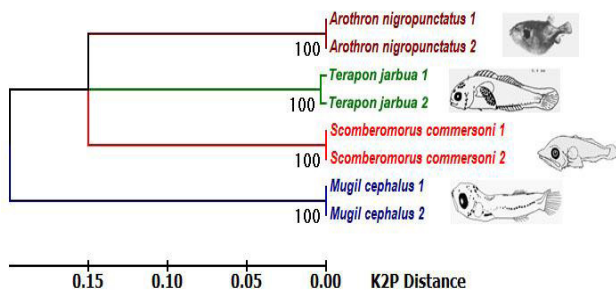


Fig. 2. Phylogenetic relationship of *Mugil cephalus*, *Scomberomorus commersoni*, *Arothron nigropunctatus*, *Terapon jarbua* from Vellar estuary

India is rich in diversity, not withstanding fish diversity and all the species occurring in the Indian waters need to be barcoded. Even so, only few efforts have been made for the protection of brackish water fishes, so in this view to extending this effort to Indian biodiversity, the present study was undertaken to barcode fish larvae in mangrove swamp of Vellar estuarine region. Recently, Lakra *et al.* (2011) were barcoded 115 species (belonging to 37 families and 79 genera) of marine finfishes that occur in Indian waters. CO1 sequences of *Terapon jarbua*, *Scomberomorus commersoni*, *Arothron nigropunctatus* and *Mugil cephalus* larvae of this study showed close relationship with those of the respective species collected by Lakra *et al.* (2011) from the Indian waters. The molecular phylogenetic clustering (Fig. 2) also reveal the same topology for clustering closely related species as per Lakra *et al.* (2011). The average percentage of base composition of the CO1 gene fragment in fish larvae is also in agreements with the earlier study (Lakra *et al.*, 2011). Where the average nucleotide composition of Scombrids was A: 23.20; T: 29.30; G: 18.90; C: 28.60%, whereas in this study, for *S. commersoni* A: 23.0, T: 29.5, G: 18.0, C: 29.5% were noted. However, Jamandre *et al.* (2014) recently showed that in *M. cephalus* the control region was AT rich and poor in G content. This result also supports the present study results for *M. cephalus* A: 24.5, T: 31.0, G: 17.5, C: 27.0%. For *A. nigropunctatus* the following data were scored: A: 23.5; T: 26.5; G: 19.0, C: 31.0%, whereas similar observations were made by Badhe and Nandanpawar (2013). In *T. jarbua* studied, A: 22.5, T: 28.0, G: 18.0, C: 31.5% were recorded. Khan *et al.* (2010) has shown the same result in the average nucleotide frequencies of *T. jarbua*.

The usage of K2P genetic distance revealed that within the species, genetic variation is low compared to the one among the species. The highest genetic distance was observed between *A. nigropunctatus* and *M. cephalus* (0.434), followed

by *T. jarbua* and *M. cephalus* (0.411) and the lowest genetic distance was observed between *T. jarbua* and *S. commersoni* (0.256). Similar observations were observed in various studies, which show that the genetic distance within the genus is lesser than between the genus (Blaxter and Floyd 2003; Borda and Siddall, 2004; Hebert *et al.*, 2003). Thus, the genetic distances keep increasing when the sequence of fishes belongs to the different genera or families and this proves once again the stringency of barcode region of CO1 gene in species identification.

Conclusions

It is clearly revealed that larval fish identification using traditional morphological characters should be more conservative and able to identify only to the family or genus level, and not to the species level. CO1 barcodes are essential to verify the species identification of fish larvae, especially of morphologically similar species. This study of molecular identification discriminate fish larvae from Vellar estuary as *Terapon jarbua*, *Scomberomorus commersoni*, *Arothron nigropunctatus* and *Mugil cephalus*. Although barcoding remains one of the best methods to confirm species identification, molecular identification of larval fishes still has some deficiencies and bottlenecks. This can be overcome by use additional markers of mitochondrial and nuclear origin which should be more conserved in nature.

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