



Genetic identification of marine eels through DNA barcoding from Parangipettai coastal waters



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ABSTRACT

Anguilliformes, also known as “true eels”, are an ecologically diverse group of predominantly marine origin whose members were easily recognized by their extremely elongated bodies with reduced cross-sectional areas and universal lack of pelvic fins. The Marine Eels were collected from landing centres of Parangipettai coastal waters and identified based on their morphometric and meristic characters. The newly recorded species were used for the barcoding analysis. Information on molecular taxonomy of marine eels was very meagre and hence, the present study was aimed to study the barcoding of marine eels which were present along the southeast coast of India. The cube of lateral muscle was exercised for DNA isolation followed by its amplification. Cluster IX 2.06 was used to align the nucleotide sequences (Thomson, 1997). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). The barcodes sequences were submitted in NCBI (National centre for Biotechnological Information). The species within genera of Muraenidae, Muraenesocidae and Ophichthidae family were clustered in a same clade with high bootstrap value. The evolutionary relationships of six species were analyzed using neighbor joining method. This results of phylogenetic tree showed maximum genetic relatedness with the sequenced results which were submitted in gene bank.

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1. Introduction

Genetics analysis has many advantages on fisheries management especially by facilitating the tools that enables unequivocal specimen identification and assessment of stock structure [1]. Genetic data have become increasingly important in assessing the gene flow between populations, which is crucially important for the maintenance of genetic diversity. Genetic technologies have been recognized for their usefulness in species identification [2], monitoring fisheries [3] and aquaculture [4]. Interpretation of genetic data will serve as a key platform in setting up conservation priorities [5].

In many situations, genetics may be the best way to decide whether the species is worthy of protection under endangered category. Genetic uniformity of highly threatened species will pave the way for restocking of the same through translocation [6] by employing planned program based on the awareness of quantitative genetics, life history and DNA variation [7]. An individual possessing high genetic variability seems to exhibit increased growth rates, stability, developmental variability, fecundity and resistance to environmental stress [8].

DNA bar-coding is a concept in which a small fragment of nucleotide sequence of mitochondrial genome acts as a DNA barcode for the identification of organism at its species level. DNA bar-coding is based on the fact that the intra-species variations are lesser than inter-species variations. Single-gene assays can help to identify an individual animal to its species level or disclose the inconsistency between its molecular variation and existing view of species boundaries. It shouldn't be confused along with the efforts to determine the “tree of life” rather it should resolve phylogeny at scales from species to major eukaryotic clades that necessitate a importance of gene selection. Indeed, the unique character that makes the COI gene as a candidate for high-through put DNA barcoding is its high constrained amino acid sequence and thus enables wide applicability of primers [9] and also restricts its information content over in-depth phylogenetic level [10,11].

Anguilliformes, the “true eels”, forms an ecologically diverse group, predominantly of marine origin whose members were easily recognized by their extremely elongated bodies with reduced cross-sectional areas and universal lack of pelvic fins. Despite a conserved body plan, some Anguilliformes exhibit high diversity in cranial morphology and prey capture mode [12,13]. Earlier phylogenetic studies of Anguilliformes relationships based on morphological data were unable to resolve the relationships among the three Anguilliformes suborders [14,15] while mitochondrial analyses have revealed some of the largest

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Anguilliformes groups to be paraphyletic [16,17]. Information on molecular taxonomy of marine eels from South East Coast of India was very meagre and hence, the present study was aimed to study the barcoding of marine eels from Parangipettai, Tamil Nadu.

2. Materials and methods

The eels were collected from Annan Kovil and Mudasalodai landing centres and are subjected for molecular identification. Species sampled were *Gymnothorax undulatus*, *G. punctatus*, *G. pictus*, *Muraenesox cinereus*, *Myrophis microchir* and *Brachysomophis cirrocheilos*. A cube of lateral muscle from right side of each fish samples were exercised for DNA isolation.

2.1. DNA extraction and PCR amplification

The tissue sample was placed in 1.5 mL Eppendorf tube and 500 μ L of solution I (50 mM Tris – HCL - pH 8, 20 mM EDTA - pH 8 and 2% SDS) was added. The tissue was homogenized with sterile homogenizer and 5 μ L of proteinase K (20 mg mL⁻¹) was added and vortexed. The sample was incubated at 55 °C in water bath for 2 h with occasional mixing. Then the sample was placed on ice for 10 min and 250 μ L of 6 M NaCl solution was added and inverted to ensure thorough mixing. The tube was kept over ice for 5 min and centrifuged (8000 rpm for 15 min). About 500 μ L of supernatant was carefully collected in to new labelled 1.5 mL Eppendorf tube and twice the volume (1 mL) of 100% ethanol was added so as to precipitate the DNA. The precipitate was pelleted down at 8000 rpm for 5 min and the supernatant was removed without touching the pellet. The DNA pellet was rinsed with 500 μ L of ice cold ethanol and centrifuged at 1100 rpm for 5 min. The supernatant was carefully removed and the excess liquid was drained using pipette. The pellet was partially dried with lid open at 55 °C. The pellet was re-suspended with 50–200 μ L of fresh sterile distilled H₂O depending on size of pellet (100 μ L average) by gently pipetting the sample with wide bore filter tip until dissolved. This dissolved DNA acted as template for Polymerase Chain Reaction (PCR).

The fragment of COI was amplified by Gene Amp PCR system 9700. PCR was carried out in 25 μ L volumes [2.5 μ L of 10 \times PCR buffer, 1.5 μ L of MgCl₂ (2 mM/ μ L), 1 μ L of DNA template, 1 μ L of each primer (10 pmoles μ L⁻¹), 2 dNTPs (1 mM/ μ L), 10 U of 1 μ L Taq polymerase (BioServe Biotechnologies Pvt., Ltd., Hyderabad, India) and 15 μ L of sterile Milli-Q water].

Fish F1 [5'-TCAACCAACCACAAAGACATTGGCAC-3'] and Fish R1 [5'-TAGA CTTCTGGTGGCCAAGAATCA-3'] primers were used for COI amplification [18]. The thermocyclic conditions for PCR included the initial denaturation at 94 °C for 1 min, five cycles of 94 °C for 34 s, annealing at 45 °C for 40 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min, followed by indefinite hold at 4 °C.

Following PCR, about 10 μ L of PCR product with 2 μ L of bromothymol blue was added to 2% agarose gel, prepared with 2.5 μ L of 1% Ethidium bromide and electrophorized at 90 V until the dye moved for 6 cm in the gel. The gel was transferred to gel doc system provided with UV transilluminator. Sequencing of PCR product was carried out using Dye terminator mix v3.1 and quantified in Euro BioServe Biotechnologies, Pvt., Ltd., Hyderabad, India.

2.2. Sequence data analysis

The electropherogram generated by automated DNA sequencer was read by ChromasPro v1.42 and the sequences were carefully checked for mis-calls and base spacing. Cluster IX 2.06 was used to align the nucleotide sequences [19]. The evolutionary history was inferred using the Neighbor-Joining method [20]. The optimal tree with the sum of branch length = 1.06326244 is shown. The percentage of replicate trees by which the related taxa were clustered together among the bootstrap test (1000 replicates) is shown near to the branches. The tree is drawn

to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Phylogenetic analysis was conducted in MEGA4 [21].

3. Results

3.1. Phylogenetic analysis

Blast search for *Gymnothorax punctatus*, *Gymnothorax undulatus*, *Gymnothorax pictus*, *Muraenesox cinereus*, *Brachysomophis cirrocheilos* and *Myrophis microchir* showed the genetic relatedness at the genus level and were positioned in the same clade. Two phylogenetic trees were constructed to verify the following hypothesis. The phylogenetic tree was constructed to test the efficacy of COI in delineating the members of Anguillidae fishes to its species level. Barcode sequence of six species of eels viz., *G. undulatus*, *G. pictus*, *G. punctatus*, *M. microchir*, *M. cinereus* and *B. cirrocheilos* from Parangipettai coastal waters were used as an out-group and this has been clearly distinguished as an out-group in the phylogenetic tree. *Gymnothorax undulatus* revealed more genetic relatedness to other species of the genus *Gymnothorax* and they were placed among the clusters of *Gymnothorax* species. *Muraenesox cinereus* was placed among the Clade with members of *Muraenesox bagio*. *Brachysomophis cirrocheilos* exhibited much genetic relatedness to *B. henshawi*. *Myrophis microchir* exhibits more genetic relatedness with members of *M. platyrhynchus*. *Gymnothorax pictus* displayed more genetic relatedness to *G. chilospilus*.

The following sequences were submitted in NCBI and the accession numbers were assigned viz., *Gymnothorax undulatus* PS 1 (KF297588), *Gymnothorax punctatus* PS 2 (KF297589), *Gymnothorax pictus* PS 3 (KF297590), *Myrophis microchir* PS 4 (KF297591), *Muraenesox cinereus* PS 5 (KF2975892) and *Brachysomophis cirrocheilos* PS 6 (KF297593).

4. Discussion

The limitations of morphology-based identification systems and the diminishing group of taxonomists lead to the implementation of molecular approach for species identification. The efficiency of species identification by molecular methods is judged by the levels of intraspecific homogeneity and interspecific heterogeneity displayed by the intended method. Mitochondrial COI gene acts as an attractive “species barcode” as it is highly efficient in species identification. Hebert et al. [22] stated that DNA barcoding is to identify species and to uncover biological diversity. For many animal taxa, sequence divergences within the 5' region of the mitochondrial cytochrome oxidase subunit I (COI) gene were much useful between species than within them, and it suggests that this is greatly applicable throughout the phylogenetically distant groups. Till date, very few publications were concerned regarding the COI barcodes from Indian waters that efficiently distinguish the dissimilar species. In this study, mitochondrial cytochrome oxidase subunit I (COI) genes was used for molecular identification of eels.

Members of Anguilliformes comprise the most species within Elopomorpha, with about 937 species. Based on the fused frontal bones, Robins (1989) treated Congroidei as a monophyletic taxon within Anguilliformes [23], but it was in contradiction with several molecular databases [16]. The true eels (Anguilliformes) are the most species-rich elopomorph group and are characterized by long cylindrical bodies with several absent characters (e.g., no pelvic fins and, in some species, lack of pectoral fins). Most Anguilliformes are marine, inhabiting the range from shallow coastal waters to deep sea. The order Anguilliformes contains around 937 species currently assigned to 15 or 16 families in three suborders, Anguilloidei, Muraenoidei, and Congroidei [23,24]. They are distinct from other elopomorphs in having a very elongated body (eel-like), lacking pelvic fins and their supporting skeletal structures and having dorsal and anal fins confluent with a caudal fin that is reduced or sometimes completely lost. The three most species-rich families (Muraenidae [201 species], Ophichthidae [336 species] and

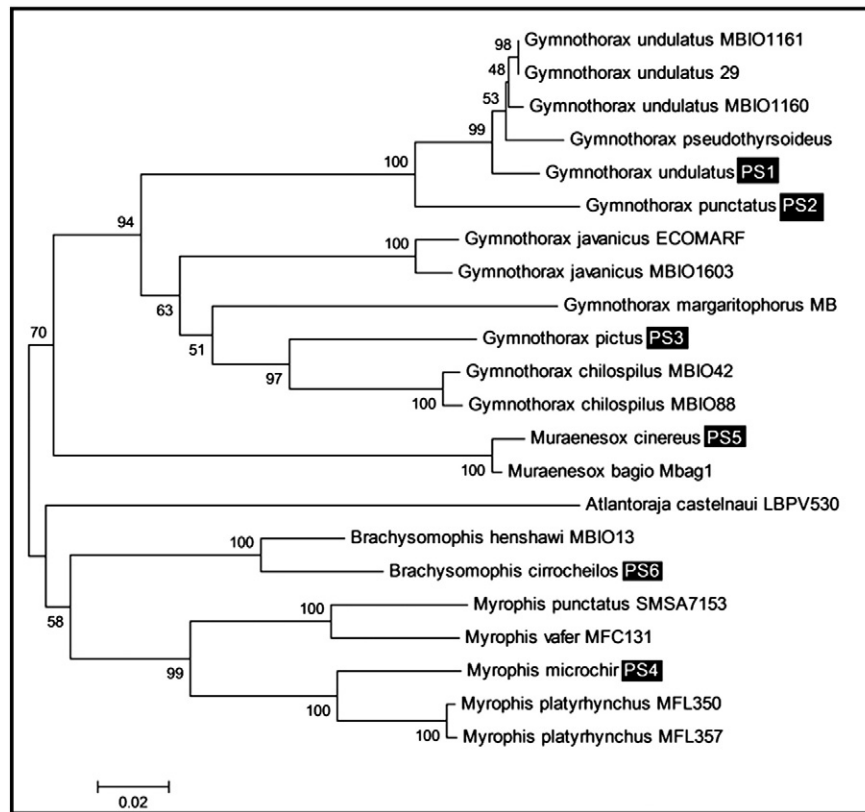


Fig. 1. Evolutionary relationships using neighbor joining method.

Congridae [200 species]) comprises about 80% of the total number of Anguilliformes species.

Mitochondrial and nuclear gene sequences have been used to test Anguilliformes intra-relationships [25,26]. None of these five previous studies found support for the monophyly of the three Anguilliformes suborders viz., Anguilloidei, Muraenoidei, and Congroidei as defined by Robins [23]. Wang et al. (2003) was first to examine the phylogenetic relationships of the Anguilliformes using partial sequences of the mitochondrial 12S rRNA gene (about 1000 positions) [25]. Moray eels are poorly studied group of organisms with respect to their evolutionary history and patterns of gene flow, yet they are one of the most widespread and common groups of reef fishes. Moray eels appear to have accumulated high species diversity and worldwide distributions through a long history of lineage accumulation, especially within the last 25 million years [27].

Despite the potential difficulties in identifying and classifying moray species [28], our results support the deepest morphology-based taxonomy within Muraenidae and Muraenesocidae. These families are distinguished by a suite of diagnostic characteristics, including vertical fins developed, dorsal fin originating on head and anal fin immediately beyond anus to both fins restricted to tail tip. In the present study, the phylogenetic relationships of the three species of *Gymnothorax* were similar to the earlier results [29,30].

Jimenez et al. had already noted that *Gymnothorax* was genetically closer to species of *M. robusta* [31]. In the present study, *G. undulatus* seems to be closer to *G. pseudothyrsoides* than to other species of *Gymnothorax*. Thus, there is a serious possibility that genera of *Gymnothorax* are monophyletic (Fig. 1). Only a broader phylogenetic analysis with an adequate coverage of the morays of different oceans will identify the existing clades and which taxa are to be included in a revised taxonomy of this family.

Furthermore, the significantly high bootstrap values indicated that freshwater eels (Anguillidae), moray eels (Muraenidae), and cutthroat eels (Synphobranchidae) are separate monophyletic groups, except

for the conger, snake and pike eels. Congridae and Ophichthidae cannot be supported as monophyletic or sister groups, but each of the subfamilies within the above-mentioned individual families remained so with a high bootstrap value. Muraenesocidae is regarded as a polyphyletic because some components such as *Gavialiceps taeniola*, *Muraenesox cinereus*, and *Oxyconger leptognathus* are not grouped together but separately as sister group of Ophichthidae. In the present study, the phylogenetic relationship for *Muraenesox cinereus* resembles maximum genetic relatedness with the report of Wang [29]. The *Myrophis microchir* exhibited genetic relatedness with the result of Santini and Tyler [26]. Wang et al. studied the phylogenetic relationship for the genus *Pisodonophis*, *Opichthus* which belongs to the family Opichthidae [28]. Several authors reported on the study of molecular taxonomy and phylogenetic relationship of Moray, Conger and Snake eel belonging to different genus [30,32,33]. In the present study, the phylogenetic tree showed maximum genetic relatedness with the sequenced results which were submitted in gene bank.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.gdata.2016.12.002>.

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