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RESEARCH ARTICLE

MITOCHONDRIAL COI GENE BARCODING OF *PENAEUS SEMISULCATUS* COLLECTED FROM FOUR ACCESSIONS

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ABSTRACT

DNA Barcoding is a new taxonomic approach for identifying biological specimens and managing species diversity. Intraspecific variation in a sequence is in order of magnitude less than that observed inter-specifically and this provides the means by which species are differentiated. It is also being used as a research tool for refining the understanding of biological diversity, and as a system for assigning biological samples to their species of origin. The objective of this study was to investigate the applicability of DNA barcoding as a tool for species identification of the green tiger prawn, *Penaeus semisulcatus.* The present study on molecular identification based on DNA barcoding of MT-COI gene was also aimed to construct a possible molecular phylogenetic tree with selected shrimp species for understanding their evolutionary relationship/ significance. Penaeid shrimps are an important resource in crustacean fisheries, representing more than the half of the gross production of shrimp worldwide. The results showed that DNA barcodes present a high degree of interspecies variation making classification possible even at the species level and this technique is relatively cheap, fast, and useful in the identification of incorrectly labelled market products.

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INTRODUCTION

DNA barcoding is an efficient approach of species identification that uses information within a single gene region common across all taxa, and has become a powerful discipline since its inception (Hebert *et al.,* 2003a, b), aiming to assess and document biodiversity at a quicker pace than traditional methods. This approach is particularly valuable for species identity establishment of rare, fragile or small organisms, especially when morphological detection is problematic and errors are likely to crop up due to simple or evolutionary conserved body plans. One of the main advantages of this method is its ability to flag species that are potentially new to science (cryptic species). It also has advantages over morphological approaches when analyzing stomach contents and identifying larval forms and damaged specimens. The two main goals of DNA barcoding are (i) to assign unknown specimens to already described and classified species, and (ii) to enhance the discovery of new species and facilitate identification, particularly in cryptic, microscopic, and other organisms with complex or inaccessible morphology (Hebert *et al.,* 2003).

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DNA barcoding has become an important tool in numerous biological disciplines, e.g. modern biodiversity assessment studies (Barber *et al.,* 2006), conservation biology (Bucklin *et al.,* 2011; Witt *et al.,* 2007), or the authentication of sea food (Haye *et al*., 2012; Nicole *et al*., 2012). As consequence, many recently published species descriptions and taxonomic studies included barcode sequence data. Accurate species identification is critical for understanding their distribution and abundance and to inform ecosystem-based management. Within the Arthropoda, most DNA barcoding publications have focused on insects (Zhou *et al.,* 2011; Hausmann *et al.,* 2011; Woodcock *et al.,* 2013), whereas the number of comprehensive studies evaluating the utility of DNA barcodes for the identification and discrimination of crustaceans is still limited (Lefebure *et al*., 2006; Costa *et al.,* 2007). Nevertheless, crustaceans represent one of the most economically and ecologically important invertebrate groups (Brusca, 2003). Currently, more than 67,000 extant species have been described so far (Ahyong *et al.,* 2011), and probably five or ten times of that number are still waiting to be discovered in the marine realm. Morphological identification of crustaceans is very critical because, this group has different larval stages, sexual dimorphism, plasticity, trading etc. Species identification by morphological characteristics is sometime misleading and ineffective, because, larval stages of some species groups often

cannot be assigned to the correct species (Hebert *et al.,* 2003). Morphological identification becomes more complex when the species are damaged due to rough handling, and there may have chances for fish fraud (CSIRO, 2013). The unique color system in crustacean plays a significant role in aquaculture because their color affects the quality and market price (CSIRO, 2013). Prawns, like most other crustaceans are able to change color depending upon growth, time of day, and background coloration due to chromatophores (Montgomery. 2010). These problems can be overcome by molecular identification or DNA Barcoding. It is difficult to estimate the true species richness of freshwater shrimps, as every year new taxa continue to be described. In the present study, DNA barcoding technique based on MT-COI gene was adopted to identify the marine shrimp species inhabiting the Coromandel coastal region in the Bay of Bengal, Tamil Nadu, India. MT-COI (COX I) gene has been employed as a possible DNA marker for species identification. This gene has two important advantages, (i). Universal primers are very robust for this gene, enabling recovery of its 5 primer end from the representatives (Simmons *et al.,* 2001), and, (ii). COI likely possesses a greater range of phylogenetic signal than any other MT gene. In common with other protein-coding genes, its third position nucleotides show a high incidence of base substitutions. However, changes in its amino acid sequence occur more slowly than those in any other mitochondrial gene (Cox *et al.,* 2001). Therefore, this gene is conserved and less subjected to external forces.

MATERIALS AND METHODS

Species collection and identification: A total of four shrimp samples were collected from four different sites, Pondicherry (11°.05 N, 79°.05 E), Royapuram (13°.10 N, 80°.29 E), Pazhaverkadu (12°.98 N, 80°.18 E), and Thalankuppam (13°.22 N, 80°.32 E) situated along the Coromandel Coast of the Bay of Bengal, India. Fresh specimens were spot examined for specific morphological characters that define the green tiger prawns. Each catch sampled from different sites were investigated to ensure correct sampling and labeling. These species were identified by using taxonomic keys described by "Edible Penaeid Shrimps in India" in the Training Manual "GIS and Marine Biodiversity" edited by John Milton (2008). Finally, these species were confirmed by Dr. R. Venkitesan, Scientist, Zoological Survey of India, Chennai.

Molecular analysis: Shrimp genomic DNA was extracted in accordance with Andrew *et al*. (1996) who described a simple method for insect genomic DNA extraction. Isolated nucleic pellet was washed with 70% ethanol and re-suspended in TE buffer (10mM TrisHCl, 1mM EDTA, pH 7.4). 0.7% Agarose Gel Electrophoresis (GENEI, Bangalore) was performed to detect the genomic DNA using Gel documentation (Mediccare, India). DNA amplification of MT-COI gene was carried out in Eppendorf Thermo Cycler by using the forward (COI F: 5' - GGTCAACAAATCATAAAGATATTG-3') and reverses (COI R:5'-TAAACTTCAGGGTGACCAAAAAATCA- 3') primers. Amplification was performed in a total volume of 20 µl containing 1µl of DNA template, 0.3 µM of each primer, 0.2 mM of dNTP and 0.2µl of Taq DNA polymerase (Genedirex). Thermo cycler conditions were as follows: 5 min at 95°C for pre-running, then 35 cycles of 60 s at 95°C for denaturation, 60 s at 48°C for annealing, and 80 s at 72°C for extension followed by 7 min at 72°C for a final extension. The final product was stored at -20 °C for further usage. The amplified product was resolved with 1.5% AGE. Sequencing was done by using ABI 3500 XL Genetic Analyzer with manufacturer's protocol of Eurofins India Pvt. Ltd., Bangalore, India.

Sequence statistical analysis: The sequences were aligned pair wise by using EMBL-ABI. Stop codons were removed by using BLAST, and the reading frame shift was deducted by ORF finder. The trimmed sequence was authenticated with GenBank. The similarity between sequences was identified by BLAST. The multiple sequence alignment was done by using CLUSTAL W and the aligned sequence was highlighted with multiple align show (MAS). The evolutionary relationship in the form of Phylogenetic tree was constructed using Neighbor-Joining method based on Kimura-2-parameter (MEGA6). Phylogenetic tree is a graphical representation of the evolutionary relationship among three or more gene or organism. MEGA6.1: Mega6 performs tree inference using the Mega6 program and the input file should be '.meg' format. The file contains aligned DNA sequences in mega format. Using Mega6it is possible to estimate a NJ tree and perform the bootstrap test in an automated fashion. The program will display the tree in a new window and superimpose bootstrap support values along each branch of the tree.

RESULTS AND DISCUSSION

Our analyses, based on the commonly used mitochondrial genes cytochrome coxidase I (the standard DNA barcode for animal species) was capable of discriminating prawn species with high accuracy. A total of 4 COI barcodes of 658bp were thus obtained for the species of Penaeid. Well defined peaks and the absence of stop codons indicated that co- amplification of nuclear pseudo-genes did not occur (Zhang and Hewitt, 1996).The isolated genomic DNA was measured greater than 10 kb nucleotides (Fig. 1) in each sample, and the PCR amplified products showed ~658bpamplicon for each of the sample. The sequences generated in this study were submitted to BLAST and authenticated by GenBank and the accession numbers are generated. GenBank analyses indicated that all sequenced samples have been correctly identified morphologically based on low e-values and high sequence similarity scores to sequences from the same species already submitted to GenBank as indicated in Table. All the samples was morphologically identified as *Penaeus semisculatus* and the GenBank results for the all bi-directional primer sequences indicated, E-value 0,00 and average similarity score of 98%, high likelihood that this Prawn specimen was rather more likely to belong to the species *Penaeus semisculatus.* The analyzed sequences were submitted in the genetic sequence database at the National Center for Biotechnology Information (NCBI) (GenBank ID: KY069063- Thalankuppam sample; GenBank ID: KY069064- Royapuram Sample; GenBank ID: KY069065- Palavanthangal Sample; GenBank ID: KY069066- Pondicherry Sample) (Fig. 2). It is worth noting that the BLAST search function of NCBI GenBank accomplishes the task of associating an 'unknown' sequence with its closest 'known' cognate in the GenBankdatabase efficiently, quickly, and without proprietary complications. Deposition of sequences in this public database will facilitate the professed goal of achieving comprehensive availability of comparative data across a broad taxonomic range more readily.Four obtained sequences viz. Accession No. JQ812738, JQ812739, JQ812740, JQ812741, JQ812742, JQ812743 and the additional 24 COI sequences downloaded from the GenBank were aligned

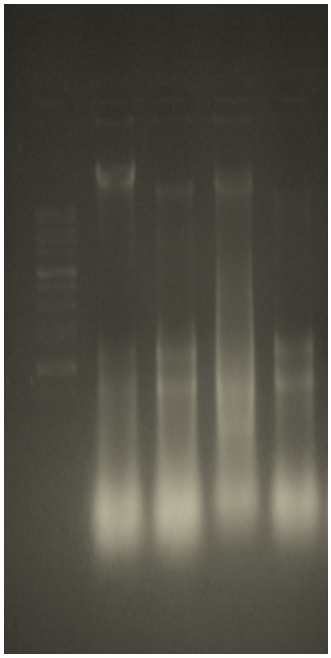

Fig. 1. : Agarose gel (0.8 %) for genomic DNA of prawn (a): Lane 2 to 5 representing prawn collected from Pondicherry, Palavanthangal, Royapuram and Thalankuppam respectively. Lane 1: DNA ladder (Fermentas, 1 kb)

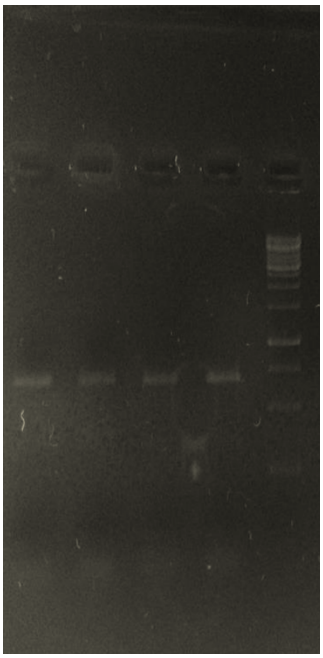

Fig. 2. PCR amplification of *CO1* regions: Lane 1-4 showed PCR results of *prawn species*from Pondicherry, Palavanthangal, Royapuram and Thalankuppam respectively Lane 4: DNA ladder (Fermentas, 1 kb)

to yield an equal length of 650bp with no gaps and no indels. Multiple alignments of sequences from various animals were performed with the Clustal W program (Higgins *et al*., 1994). Based on the results of multiple alignments, a phylogenetic tree was constructed using MEGA 6.1 software (Tamura *et al*., 2013) by the neighbor-joining method. The robustness of topology nodes was tested by the bootstrap method with 500 replications. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. Initial tree (s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 28 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 416 positions in the final dataset. The constructed phylogram clearly distinguished the out-group with high bootstrap values at the internal nods indicating the reliability of tree topology (Fig. 3). All the sequences of the present study formed a monophyletic relation with the *P. semisulcatus*obtained from NCBI. But the presence of *P. merguiensis*in the monophyletic clade with *Fenneropenaeus* subgenera weakened the support slightly.

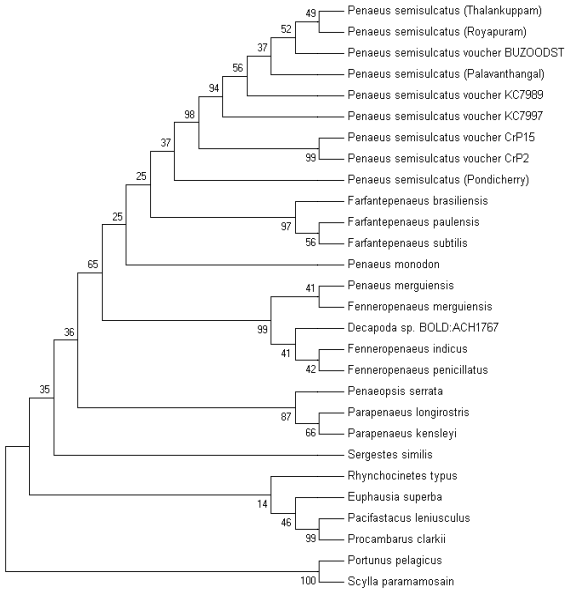

Fig. 3. Phylogenetic tree for the populations of *P. semisulcatus* based on Neighbor-joining tree analysis of COI data sets

The sequences of the present study and the *P. semisulcatus* obtained from Repository were most closely related. *Farfantepenaeus spp* then joined this subclade with *P. monodon* as the most outlying sister taxon. The two species, *P. merguiensis* and *F. merguiensis*, were placed in the same clade as sisters in the present analyses with relatively weak bootstrap support. *Penaeopsisserrata* were closely related *Parapenaeus spp* as the sister taxon. The out-group Sergestessimilis belonging to a closely related family Sergestidae formed a distinct clade. Four species used as out-group, Euphausiasuperba, one species of Euphausia, a krill genus; *Rhynchocinetestypus*, a rock shrimp; Pacifastacus leniusculus**,** a crayfish; Procambarusclarkii**,** a freshwater crayfish species , all that belongs to the same super order (Eucarida) that the Penaeid shrimps. All out-group lies within decapods and thus is more closely related to the in-groups.Two other out groups belonging to the same subfamily Portuninae, *Portunuspelagicus* and Scylla paramamosain were clustered together and formed a distinct clade.

The first important result of the phylogenetic tree is that the genus Penaeus is associated with *Farfantepenaeus, Fenneropenaeus, Penaeopsis, and Parapenaeus*. This is an important result because it is in line with the monophyletic status of the Penaeidae family, since these entire genuses belong to the same family.The group formed by *Farfantepenaeus, Fenneropenaeus, Penaeopsis, Parapenaeus*, and *Penaeus*, also known as the "old Penaeus genus," are clustered with high Bootstrap support values. This result is very

interesting and may eventually be used to justify a separate intermediate taxonomic level for the classification of this group. The genus *Penaeus* appears to be paraphyletic with Farfantepenaeusspp, clustering between the two Penaeus lineages: *P. semisulcatus* and *P. monodon.* The results of Carolina *et al*. (2005) that the genus Penaeus appears to be paraphyletic with *Fenneropenaeus spp*, clustering between the two Penaeus lineages: *P. esculentus* + *P. semisulcatus*and *P. monodon* are not in accordance with those in our study. But, the result of Carolina *et al*. (2005) requires verification because the support values were mostly low. The decapods phylogenetic relationships are as contentious as ever. In spite of the limited number of taxa tested in this study, studies with further taxa involving the complete mitochondrial genomes of this family are necessary to know the phylogeny of the Penaeidae. In summary, molecular phylogeny of this study has clarified the relationships within the genus Penaeidae family to a certain extent.

Conclusion

DNA barcoding has become a promising tool for rapid and accurate identification of various taxa and it has been used to reveal unrecognized species in several animal groups. In summary, this study has provided the COI barcodes for prawns collected the Coromandel Coast of Tamil Nadu, India and has established their effectiveness in discriminating species recognized through earlier taxonomic work contributing to the growing library of DNA barcodes of crustacean species of the world. This study validates that the partial cytochrome c oxidase I gene aidsthe accurate species identification where adequate reference sequence data exists. Also phylogenetic work on these species, using other genetic markers, will expose which of these highly divergent and geographically detached populations should be treated as fitting to the same species or sister species. It can provide some basic evidence for phylogenetic analyses among the species studied giving some suggestion for evolutionary relationship.

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