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Molecular phylogeny of Shrimps from west coast of Maharashtra using DNA barcoding

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ABSTRACT

Crustaceans represent metazoan group with enormous morphological and ecological diversity but are difficult to identify with traditional approaches and require help of expert taxonomists. DNA barcoding technique is the most reliable molecular approach of species identification and provides permanent species tags. In case of crustaceans, sequence of two mitochondria genes COI and 16S rRNA have been found useful for correlation between taxonomic ranks and molecular divergence. *Parapenaeopsis stylifera, Solenocera crassicornis* and *Fenneropenaeus indicus among penaeids* and *Nematopalaemon tenuipes, Exhippolysmata ensirostris* and *Acetes indicus* considered as non-penaeids representing 5 families were collected from Mumbai, the major fishing region in Maharashtra. DNA barcode and Accession numbers were generated for these species. Molecular phylogenetic evolutionary study was carried out to evaluate genetic divergence between species with respect to their families and based on protein coding COI and non-protein coding 16S genes.

Keywords: COI gene, DNA Barcoding, shrimp, 16S rRNA

INTRODUCTION

DNA barcoding is the technique of sequencing a short fragment of mitochondrial DNA generating a 'DNA barcode'. DNA barcode generated from a taxonomically unknown organism is then matched against a reference library of barcodes of known species source in order to identify the species. Since 2004 a universal consortium has been promoting DNA barcoding as a worldwide standard for well-ordered recognizable pieces of proof for taxonomist (Savolainen *et al.*, 2005). It has become a new research hotspot in recent years to obtain DNA barcode fragments which can be used to restructure the phylogenetic relationships of biological groups (Wu *et al.*, 2019). DNA barcoding guarantees quick, correct species identifications by focusing

analysis on a brief standardized section of the genome (Hebert *et al*., 2003). In addition, DNA barcoding also provides reliable method for the identification of larval forms, damaged specimens and processed food. The progress of molecular science introduced a replacement approach, that relies on nucleotide sequence variabilities among species. (Murray *et al*., 1995). Main advantage of DNA barcoding over conventional species distinguishing proof strategies is the capacity to effectively recognize creatures from any stage of life cycle. The honesty of DNA sequences through replication and creature development is essential to their role as the "blueprint of life". ("Righting the Wrongs," 2006).

Mitochondrial gene COI have been found useful for correlation between taxonomic ranks and molecular divergence in molecular studies. From several studies, a 650-bp fragment of the mitochondrial gene cytochrome c oxidase subunit I (COI) (Hebert *et al*., 2003) has been chosen as the standard barcoding marker. It provides strong species-level resolution, high interspecific variation, low intraspecific variation, and relatively universal primers (Hebert *et al*., 2003; Smith *et al*., 2006) for varied animal groups including birds (Hebert *et al*., 2004), fishes (Ward *et al*., 2005). It is also be used for detection of cryptic species (Ni *et al*., 2012) and for the identification of fish products (Daniel C. Carvalho *et al*., 2011; Daniel Cardoso Carvalho *et al*., 2015). The studies related to DNA barcoding of crustaceans have been carried out at various international levels. Drumm *et al*., (2013) and Bilgin *et al*., (2014) approached, barcoding to characterize the genetic diversity of shrimps of Alaska and Turkish waters respectively. Palumbi and Benzie, 1991 focused on penaeid shrimps using COI gene.

A new tool was developed recently to be complementary marker for cytochrome c oxidase subunit I (COI) DNA barcoding. 16S rRNA has become one of the popular genes for reconstructing animal phylogenies because of the combination of variable and conserved regions within the same gene. The COI is mitochondrial protein coding gene, while the 16S rRNA is structural and non-coding gene. The 16S ribosomal RNA, mitochondrial DNA (mt DNA) gene has proven useful for studying evolutionary phylogenetic relationships at both the inter and intra-specific level in a number of major crustacean groups (Crandall *et al*., 1999; Munasinghe *et al*., 2003; Silberman *et al*., 1998). The 16S rRNA gene can provide useful information across a broad taxonomic spectrum from the population to the family level because of the presences of both fast and slow evolving regions. Some studies have established evolutionary relationships at different taxonomic levels based on 16S rRNA gene among shrimp (Lavery *et al*., 2004; Machado *et al*., 1993; Palumbi and Benzie, 1991; Voloch *et al*., 2005). Molecular data and phylogenetic hypothesis testing have already made known to be effective for explaining many aspects of *Macrobrachium* systematics based on 16S rRNA and should allow confident insights to be made into the patterns and processes influencing the evolution worldwide (Murphy and Austin, 2002;and 2003; and2004). Superfamily Penaeoidea was studied in detailed by Vazquez-Bader *et al*., 2004 using 16S gene and Quan *et al*., 2004 using both COI and 16S gene.

A great diversity of organisms is seen in marine, freshwater and semiterrestrial ecology from order Decapoda especially from crustaceans which represent metazoan group. Crustaceans have enormous morphological and ecological diversity so are difficult to identify with traditional approaches and require help of expert taxonomists. Crustaceans includes crabs, shrimps, lobsters, and stomatopods are ecologically and economically very important group of invertebrates majorly in fishing. Shrimps are important economically as it is exported more than some other fish with high value rate. Penaeids shrimps are typically exported because of their size and different taste whereas little size shrimps or non-penaeid shrimps are devoured by local people. Non-penaeid shrimp have incredible incentive in nearby market as they are consumed fresh and furthermore in dried form. Some piece of this non-penaeid shrimp landing goes to food industry for shrimp paste which is widely used in preparing different food items. Non-penaeids get contribution in economy as well as ecologically as it carries on vital factor in marine food chain.

Among marine shrimp, *Parapenaeopsis stylifera, Fenneropenaeus indicus, Solenocera crassicornis, Nematopalaemon tenuipes, Exhippolysmata ensirostris and Acetes indicus* were targeted. *Fenneropenaeus indicus* and *Parapenaeopsis stylifera* from family Penaeidae, *Solenocera crassicornis* from family Solenoceridae, *Exhippolysmata ensirostris* from family Lysmata (De Grave *et al*., 2014), *Nematopalaemon tenuipes* from Palaemanidae and *Acetes indicus* from

Sergestidae family were selected as they are available in western coast of Mumbai and Maharashtra. From these *Nematopalaemon tenuipes*, *Exhippolysmata ensirostris* and *Acetes indicus* are considered to be non-penaeids because of their small size and rest are called penaeids (Deshmukh, 1994, 2004).

This research incorporates the phylogenetic investigation using barcoding technology involving two different molecular markers; one protein coded gene COI and other nonprotein coding gene 16S rRNA. The study will help in developing our understanding of evolutionary biology of shrimps.

MATERIAL AND METHOD

Sample Collection

Fresh samples of six species of shrimps viz. *Parapenaeopsis stylifera*, *Solenocera crassicornis* and *Fenneropenaeus indicus* (penaeids) *Nematopalaemon tenuipes*, *Exhippolysmata ensirostris* and *Acetes indicus* (non-penaeid) were collected from New ferry wharf, Sassoon dock and Versova – the major fish landing centers of Mumbai. The samples were identified morphologically with the help of field identification key (Fischer and Bianchi, 1984) and authenticated by Central Marine Fisheries Research Institute, Regional Centre, Mumbai.

Molecular Identification

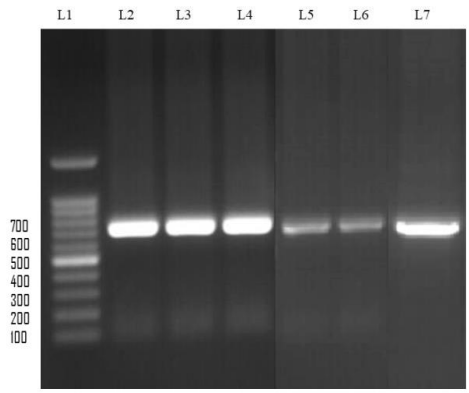
The collected shrimp samples were carried to the laboratory in ice-box to maintain its freshness. DNA was extracted from the fresh muscle tissues. DNA extraction was carried out by standardized modified CTAB method (Doyle and Doyle). Agarose Gel Electrophoresis (AGE) technique was used to check purity of DNA and NANO DROP was used to quantify pure genomic DNA. The selected gene were amplified by polymerase chain reaction (PCR) technique using primer LCO1490 (5′-GGTCAACAAATCATAAAGA TATT GG-3′) HCO2198(5′-TAAACTTCAGGGTGACCAAAAAA TCA-3′) (Vrijenhoek, 1994) for COI gene and 16SAR (CGCCTGTTTATCAAAAACAT) 16SBR (CCGGTTTGAA CTCAGATCATG) (Palumbi and Benzie, 1991) for 16S gene. The GeneAmp 9700 Applied Biosystem thermal cycler was used to carry out PCR reaction. The reaction volume of 25 µL includes 2.5 µl of 10X buffer, 2 µl of 10 mM dNTP, 1µl of Taq Polymerase, 10 p.mol of each primer and 100 ng concentration of DNA. Cycling parameters were optimized to the following condition: 5 min at 96^{O}C for initial denaturation, followed by 35 cycles of 30 sec at 95^{O}C for denaturation, 30 sec at 50^{O}C and 60°C for annealing of COI and 16S gene respectively and 30 sec at 72^{O}C for extension, and followed by 10min at 72^{O}C for final extension. Sanger's Sequencing Method was used for DNA Sequencing and were outsourced from Eurofins, Bangalore, India.

Sequence Data Analysis

The sequences were edited by Chromas 2.6.4 version software and Multi Align (Multalin interface page, n.d.) software online tool to see the similarities between the sequences. The nucleotide and protein sequences from GenBank were compared by the algorithm Basic Local Alignment Search Tool (BLAST) and BLASTx search. The corrected sequences were submitted to the National Center of Biotechnology Information (NCBI). Nucleotide sequences of listed species with respect to COI and 16S rRNA were submitted successfully.

RESULTS and DISCUSSION

The pure genomic DNA isolated from shrimp samples were run on 0.8% agarose gel and the PCR products were made to run on 1.5% agarose gel with Ethidium bromide as staining dye. Amplified COI and 16S gene in the form of bands were observed on 1.5% agarose gel. The bands for COI gene were observed around 700 bp and around 600bp for 16S rRNA gene. In amplification of COI and 16S gene gel images (Figure 1 and 2); lane 1 represents 1 KB DNA ladder and lane 2 to 7 representing species *Fenneropenaeus indicus*, *Parapenaeopsis stylifera*, *Solenocera crassicornis*, *Exhippolysmata ensirostris*, *Nematopalaemon tenuipes* and *Acetes indicus* respectively. Raw sequences obtained from sequencing amplified selected genes of listed species were analyzed, trimmed, edited using bioinformatic tools and submitted to the National Center of Biotechnology Information (NCBI) and DNA Barcode was generated (Table 1). Accession No. MH724294, MK503360, MK331952, MK341171, MK488093 and MK537304 for COI gene sequence whereas MH045067, MH156198, MK245778, MH161413, MH352449, MH045066 for 16S rRNA gene were allotted to *Parapenaeopsis stylifera, Solenocera crassicornis, Acetes indicus, Exhippolysmata ensirostris, Fenneropenaeus indicus* and *Nematopalaemon tenuipes* respectively.



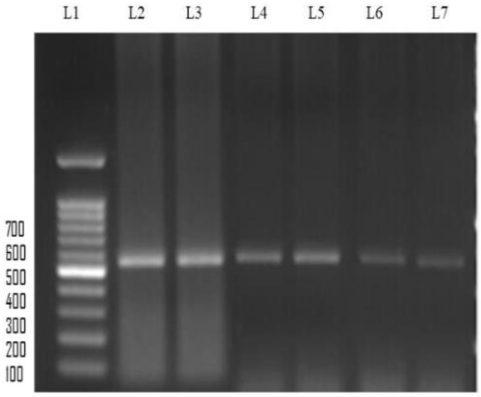


Fig.1: **Fig.2:**

Fig 1: Gel image representing an amplified COI gene at 700 bp. Lane 1 represents 100 bp DNA Ladder. L2 to L4 represents species *Fenneropenaeus indicus, Parapenaeopsis stylifera, Solenocera crassicornis, Exhippolysmata ensirostris, Nematopalaemon tenuipes* and *Acetes indicus*

Fig 2: Gel image representing Amplified 16S gene at 600 bp. Lane 1 represents 100 bp DNA Ladder. L2 to L4 represents species *Fenneropenaeus indicus, Parapenaeopsis stylifera, Solenocera crassicornis, Exhippolysmata ensirostris, Nematopalaemon tenuipes* and *Acetes indicus.*

SR. NO.	SAMPLES	GENE	ACCESSION NO.	BARCODE
1	*Fenneropenaeus indicus*	COI	**MK488093**	
		16S	**MH352449**	
2	*Parapenaeopsis stylifera*	COI	**MH724294**	
		16S	**MH045067**	
3	*Solenocera crassicornis*	COI	**MK503360**	
		16S	**MH156198**	
4	*Nematopalaemon tenuipes*	COI	**MK537304**	
		16S	**MH045066**	
5	*Exhippolysmata ensirostris*	COI	**MK341171**	
		16S	**MH161413**	
6	*Acetes indicus*	COI	**MK331952**	
		16S	**MK245778**	

Table 1: Accession Number and barcode of *Fenneropenaeus indicus, Parapenaeopsis stylifera, Solenocera crassicornis, Nematopalaemon tenuipes, Exhippolysmata ensirostris* and *Acetes indicus.*

Molecular Phylogeny

Molecular Phylogenetic study is nothing but to represent genetic evolutionary history of organisms using tree like diagrams called as cladograms which signify pedigrees of the organisms with the help of molecular data i.e, DNA or protein sequences (Xiong, 2006). A gene phylogeny is the study which expresses the evolution of that particular gene. The evolutionary cladogram drawn from the gene/protein sequenced can be unlike from species evolutionary history. Henceforth, the evolutionary approach of species not certainly link with the gene evolution. Therefore, to find true species phylogenetic evolution, various gene phylogeny trees need to constructed and complied evaluation study to be carried out.

In this study, five major families of Order Decapoda were studied with respect to COI and 16S molecular markers. The ecologically and economically important species from Fishing Area 51 were selected to represent the families. *Fenneropenaeus indicus* and *Parapenaeopsis stylifera* belong to the family Penaeidae, *Solenocera crassicornis* belong to the family Solenoceridae, *Exhippolysmata ensirostris* found in family Lysmata (De Grave *et al*., 2014), *Nematopalaemon tenuipes* belongs to Palaemanidae and *Acetes indicus* from Sergestidae family.

Evolutionary analyses were conducted in bioinformatic software MEGA version 7.0.26 1993-2020 (Kumar *et al*., 2016). COI gene is protein coding gene so the cladogram constructed was from both nucleotide and protein sequences. The evolutionary history was inferred by using the Maximum Likelihood (ML) method based on the Kimura 2-parameter model (Kimura, 1980). When the COI sequences of all 6 species were compared (Fig.3), it was noted that *P. stylifera*, *S. crassicornis* and *E. ensirostris* with respect to their families had recent ancestor. Whereas *A. indicus*, *F. indicus* and *N. tenuipes* have a lesser recent common ancestor. *P. stylifera* and *S. crassicornis* are monophelytic group which has common ancestor with *E. ensirostris*. *N. tenuipes* and *F. indicus* are sister taxa having common ancestor *A. indicus*.

Other than protein coding COI gene, non-coding 16S gene sequences of all 6 species were also studied (Fig.4). *S. crassicornis* and *F. indicus* are sister taxa closely related to *A. indicus* and have common recent ancestor with *P. stylifera*. *N. tenuipes* and *E. ensirostris* are monophyletic group and shows comparative less recent ancestors.

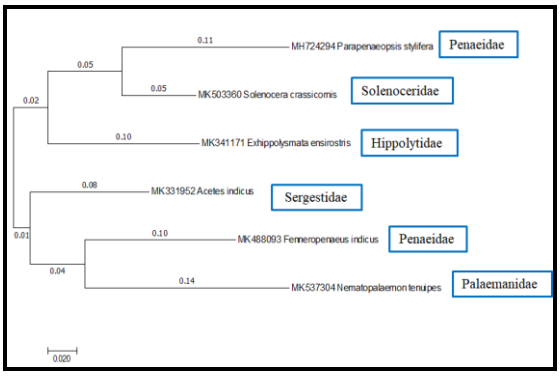


Fig 3: Phylogenetic tree of Fenneropenaeus indicus, Parapenaeopsis stylifera, Solenocera crassicornis, Exhippolysmata ensirostris, Nematopalaemon tenuipes and Acetes indicus representing Families Penaeidae, Solenoceridae, Hippolytidae, Palaemanidae and Sergestidae using COI gene.

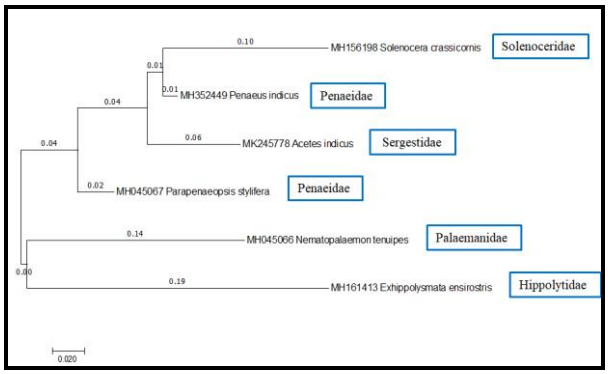


Fig 4: Phylogenetic tree of *Fenneropenaeus indicus, Parapenaeopsis stylifera, Solenocera crassicornis, Exhippolysmata ensirostris, Nematopalaemon tenuipes* and *Acetes indicus* representing Families Penaeidae, Solenoceridae, Hippolytidae, Palaemanidae and Sergestidae using 16S gene.

Statistical Analyses

The statistical analyses involved six protein coding COI gene sequences of six species representing their respective families to evaluate genetic data statistically. Evolutionary statistical analyses were conducted using MEGA7. The analyses are carried out with Maximum Composite Likelihood method. The composition of nucleotides with respect to species are *F. indicus* T36.0% C19.0% A25.7% G19.3, *P. stylifera* T30.8% C23.1% A27.9% G18.2%, *S. crassicornis* T31.5% C23.2% A28.6% G16.6%, *E. ensirostris* T33.2% C21.6% A26.9% G18.3%, *N. tenuipes* T30.4% C23.2%

A27.2% G19.2%, *A. indicus* T36.2% C16.6% A29.6% G17.6%. The transition/transversion rate of purine is 1.78 and pyrimidines is 3.35 ratio. The pairwise distance matrix (Table No. 1) shows the number of nucleotide base substitution per site from between sequences. The analyses were conducted by ML method using Kimura 2-parameter model which the construction of tree. *S. crassicornis* and *P. stylifera* are closely related with minimum distance 0.161 whereas maximum distance 0.265 between *N. tenuipes* and *P. stylifera* which are extremely separated from each other. The pairwise statistical analysis of non-protein coding 16S gene (Table No. 2) among species shows overall mean distance 0.195 and *P. stylifera* is closely related to *F. indicus* as it displays minimum distance 0.076 because they belong to same family Penaeidae.

Table 1: Pairwise distance distribution table constructed in MEGA7 by ML method using Kimura 2-parameter model. Below diagonal matrix shows the protein coding COI gene divergence.

		1	2	3	4	5	6
1	MH724294_Parapenaeopsis_stylifera	0.000					
2	MK503360_Solenocera_crassicornis	0.161					
3	MK331952_Acetes_indicus	0.243	0.200				
4	MK341171_Exhippolysmata_ensirostris	0.244	0.183	0.202			
5	MK488093_Fenneropenaeus_indicus	0.246	0.208	0.208	0.250		
6	MK537304_Nematopalaemon_tenuipes	0.265	0.258	0.230	0.252	0.236	0.000

Table 2: Pairwise distance distribution table constructed in MEGA7 by ML method using Kimura 2-parameter model. Above diagonal matrix shows the nonprotein coding gene 16S gene matrix.

		1	2	3	4	5	6
1	MH045067_Parapenaeopsis_stylifera	0.000					
2	MH156198_Solenocera_crassicornis	0.172					
3	MK245778_Acetes_indicus	0.122	0.164				
4	MH161413_Exhippolysmata_ensirostris	0.238	0.271	0.240			
5	MH852449_Fenneropenaeus_indicus	0.076	0.113	0.076	0.252		
6	MH045066_Nematopalaemon_tenuipes	0.178	0.234	0.279	0.305	0.233	0.000

The present work shows *S. crassicornis* and *P. stylifera* share common clade whereas *F. indicus* form different clade constructed from Neighbor-Joining tree of Mt-COI gene sequence, which is similar to the results of Rajkumar *et al*. 2015.

The phylogenetic tree constructed by Samadi *et al*., 2016 using COI gene shows, *P. stylifera* and *Metapenaeopsis stridulans* make monophyletic group and showing close relation to Penaeus, *Fenneropenaeus merguiensis* and *Penaeus semisulcatus*. Lavery *et al*. 2004; Voloch *et al*. 2005; Samadi *et al*., 2016; Hurzaid *et al*. 2020; Katneni *et al*. 2021 constructed phylogenetic tree using both 16S rRNA and COI gene point that, *F. indicus* shows close relationship to sister group of *F. penicill* and *F. silasi*, *F. merguiensis* join as subclade and *F. chinensis* make outlying sister taxon.

Family Palaemonidae and Lysmatidae belongs to the infra order Caridea, the only group which shows distinct remarkable character, the second abdominal segment overlaps the first and third segment. The taxonomy is categorized on morphological basis but morphological data are insufficient to conclude phylogenetic relationship.

CONCLUSION:

The phylogenetic tree constructed from Maximum Likelihood method suggested the evolution of protein coding COI gene and non-coding 16S gene among the different families from order Decapoda. The distance estimated from both COI gene and 16S gene shows that *S. crassicornis*, *P. stylifera* and *F. indicus* are closely related as they belong to same family. Likewise, relation between individual species can be estimated with respect to the particular gene. The phylogeny trees constructed help to study molecular taxonomy as it also describes the relation between families. The family Penaeidae and Sergestidae are monophyletic group or sister taxa which satisfies the theory that both these families belong to suborder Dendrobranchiata, infraorder Penaidea and superfamily Penaeoidea. The phylogeny tree drawn from 16S gene derives that Family Palaemonidae and Lysmatidae are monophyletic group as they belong to infraorder Caridea.

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