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Research Article

DNA BARCODING OF ROCK LOBSTER *P. HOMARUS* FROM THE MUMBAI COAST

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ABSTRACT

The aim of this study was to identify the rock lobster *P.homarus* with the help of DNA Barcoding. Molecular taxonomy of *P.homarus* was performed using Cytochrome Oxidase I, Histone H3 and 18S rRNA gene loci. Isolation of genomic DNA was performed with the help of CTAB buffer and later with the help of Polymerase Chain Reaction COI, Histone H3 and 18S rRNA genes were amplified. Sanger's method of DNA sequencing was used, and sequence chromatogram was obtained for all the three gene loci. The DNA sequences of the respective genes of *P.homarus* were analyzed using Finch TV 1.4 and the similarity of sequences was understood using BLAST (Basic Local Alignment Search Tool). FASTA sequences of *P.homarus* were compared with the FASTA sequences of other related lobster species using Multalin tool and Clustal Omega. The evolutionary relationship between the *P.homarus* and other lobster species was studied with the help of Multialin tool. Evaluation of Phylogenetic relationships between *P.homarus* and other lobsters from same family was performed using Clustal Omega. Therefore, in this research study we conclude that COI, Histone H3 and 18S rRNA can be used to identify various lobster species.

INTRODUCTION

Explicit and detailed species determination is condemnatory to ascertain the evolution and taxonomy of various marine organisms since it helps us to identify organisms that are recently diverged or under divergence using the available genetic data.

Classical DNA barcoding technique was used for molecular analysis of *Panulirus homarus* - an economically important and significant lobster that is spread wide across the Indo-West Pacific region, with an erratic taxonomic status(Lavery *et al*., 2014). Commercial exploitation of Palinurid lobsters is on a high rise due to its interest and lucrative price for live lobsters in international markets(Thangaraja & Radhakrishnan, 2012). Primarily, 3 subspecies of *P. homarus* have been identified based on their pattern of sculpturing on the abdomen and colorations(Lavery *et al*., 2014). Recent reports contour a decline in the fisheries status of this species throughout its distribution which makes it critical to clarify the ambiguity about the regional variation, maintenance, and evolution of *P. homarus*.

Hence, an authentic method like the DNA barcoding analysis and molecular taxonomy studies renders a comprehensive look at its biological divergence and phylogeny. DNA barcoding is the latest technique which ultimately helps for identification, discrimination and taxonomic classification of various organisms by establishing a community resource of DNA sequences(Bandyopadhyaya, Ramakrishnan, Kulkarni, & Rajanna, 2013). It employs short genetic markers to identify and distinguish organisms from their belonging species and provides promising results and reproducibility (Bandyopadhyaya *et al*., 2013).

Hence, this technique extensively assists in resolving taxonomic ambiguities of indistinguishable species based on their morphological variations(Al-Senaidi R, 2015). Mitochondrial DNA is a powerful tool to identify species, understand their relationship with other organisms and gives us a better idea of the divergent haplotypes worthy of conservation attention. In order to validate and obtain a detailed taxonomy, we analysied the Mitochondrial Cytochrome Oxidase (COI) gene, Histone genes and 18S gene.

The results of this study are intended to give us a closer approach towards the divergence and evolution of *P. homarus* and related species.

MATERIALS AND METHODS

Collection of P.homarus samples

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P.homarus samples were collected from landing centres in Mumbai. The rock lobster specimens were then brought to the lab in boxes containing ice for the proper maintenance of the lobster specimens. *P.homarus* specimens were taken out and were measured as well as photographs were taken and recorded. One specimen of *P.homarus* was sent to Central Marine Fisheries Reasearch Institute for morphological identification. A small tissue from the tail region of *P.homarus* was extracted and used for genomic DNA isolation.

Genomic DNA isolation, Polymerase chain reaction and DNA sequencing

Approximately 1 gm of *P.homarus* tissue was weighed and homogenised using mortal and pestle. For the homogenisation of tissue, CTAB lysis buffer consisting of 2% Cetyl trimethyl ammonium bromide,100mM Tris(Tris Hydroxy Amino Methane) pH 8.0, 20 mM EDTA(Ethylene Diamine Tetracetic Acid), 1.7 M NaCl(Sodium Chloride) with 0.3%(v/v) β-mercaptoethanol was used. Once the homogenisation of tissue was completed, the homogenised samples were kept for 1 hour incubation at 60^{o}C. 0.8% Agarose Gel was used to analyse the genomic DNA isolated from *P.homarus* and the concentration of the isolated genomic DNA was found out with the help of Nanodrop (Thermoscientific).

Table 1 Forward and Reverse sequences for COI,18S and Histone gene primers.

	Forward Sequence	Reverse sequence
Cytochrome Oxidase 1 gene	5 -GGTCAACAAATCATAAAGATATTGG-3	5 -TAAACTTCAGGGTGACCAAAAAATCA-3
18S rRNA	5 -CTGGTTGATYCTGCCAGT-3	5 -CTTCGAACCTCTGACTTTCG-3
Histone	5-ATGGCTCGTACCAAGCAGACVGC-3	5-ATATCCTTRGGCATRATRGTGAC-3

COI(Cytochrome Oxidase subunit I), 18S rRNA, and Histone H3 gene amplification was achieved using Polymerase Chain Reaction. The primers for the respective genes were ordered from Eurofins Table no. 1 shows the forward and reverse sequences of the respective gene loci used. A total volume of 25 µl was kept for each gene PCR which contained about 100ng DNA, 1X Taq Buffer, 2.5mM $MgCl_2$ (Magnesium Chloride). The concentration of primers for COI, 18SrRNA and Histone H3 for the reaction was kept 0.5 µM. PCR amplification was accomplished using the Applied Biosystems thermal cycler with an initial denaturation temperature 96^{o}C for 5 minutes followed by 35 cycles of denaturation for 95^{o}C for 30 seconds, followed by the annealing temperatures 52^{o}C(COI,18S), 49.2^{o}C(Histone H3) and later followed by extension at 72^{o}C for 30 seconds respectively. The PCR was completed with a final extension at 72^{o}C for 10 minutes and later the amplified products were observed on a 1.5 % Agarose gel.

Data Analysis

Finch TV version 1.4 was used to analyse the chromatogram sequence for COI, 18S and Histone H3 obtained from Xcelris Genomics. Homology of the amplified *P.homarus* COI, 18S and Histone H3 sequences was determined using Basic Local Alignment Search Tool(BLAST) as it has the ability to detect sequence homology which allows us to identify putative genes in a novel sequence. Multiple sequence alignments are the prerequisite for phylogenetic analysis thus with the help of Multialin tool(Florence Carpet) sequence alignment was implemented for the COI, 18S and Histone gene sequences of *P.homarus*. The software Multalin creates a multiple sequence alignment from a group of related sequences using progressive pairwise alignments. With the assistance of ClustalW we constructed a phylogenetic tree for the respective gene sequences as a phylogenetic tree shows the relatedness of the sequences.

RESULTS AND DISCUSSION

Panulirus homarus (Linnaeus, 1758) is commonly known as the rock lobster, belonging to the infraorder *Acheleta*. It is the member of the family Palinuridae and order Decapoda, Class Malacostraca and Phylum Arthropoda. *Panulirus homarus* is a marine lobster and is widely distributed in the Indo-West Pacific region(Thangaraja & Radhakrishnan, 2012). It is consumed across large scale in Mumbai region. It is found in a very wide-range in all the crustacean and fish landing centers in Mumbai. Even though the rock lobster has been morphologically identified (Kizhakudan & Patel, 2010), there are rare reports on its molecular taxonomy. Hence in our study we have tried to analyze the rock lobster at a molecular level using the technique - DNA Barcoding.

Figure 1a Dorsal side of *P.homarus*

Figure 1b Ventral side of *P.homarus*

Rock lobster *P.homarus* were collected from the fish landing centre in Mumbai (figure 1a and 1b). Once the lobsters were brought, our next challenge was to isolate genomic DNA. We wanted a good quality genomic DNA hence we used different methods of DNA isolation. The tissue was taken from the carapace region of *P.homarus*. We used different types of buffers for the Genomic DNA isolation namely, Triton-X Buffer, Sodium Dodecyl Sulfate buffer and C-TAB buffer. An exclusive quality of genomic DNA is very much important for PCR amplification and therefore, the DNA isolation must be appropriate. The DNA isolated with the help of Triton-X buffer and SDS Buffer were of poor quality. When the DNA was quantified using Nanodrop spectrophotometer it did not give a good peak at 260nm. However, the genomic DNA isolated with the help of C-TAB buffer was of a good quality and gave optimum absorption peak at 260 nm. For the isolation of genomic DNA, we have also used Liquid nitrogen. The DNA isolated with the help of Liquid nitrogen(Wang, Wang, Zhang, & Dong, 2011) and C-TAB were of very high quality. Hence, it was noted that use of Liquid nitrogen was beneficial for isolating genomic DNA along with C-TAB buffer. Liquid nitrogen allowed proper crushing of the rock lobster tissue.

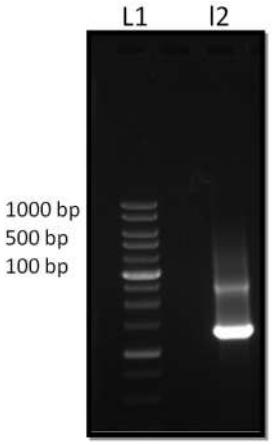

Figure 3- 1.5% gel stained with 0.1µg/ml of EtBr
Lane 1- 100 bp standard DNA ladder
Lane 2- COI Amplification

Figure 2 Amplification of COI gene in *P.homarus*

Once the Genomic DNA isolation was done our next step was to amplify the genomic DNA using different DNA markers. Cytochrome oxidase I is the standard DNA Barcode gene in animals (Hebert *et.al*). For our study we have used Cytochrome oxidase I gene as well as other DNA markers like 18S rRNA and Histone. Cytochrome oxidase I gene codes for the protein Cytochrome Oxidase, which is one of the enzymes in the respiratory chains. Due to slow evolution rate it has been used as a unit to measure the rate of evolution in animals (Hebert *et.al*). 18S rRNA is a non-protein coding gene of the mitochondria. 18S gene is responsible for the synthesis of 18S rRNA. Another DNA marker that we used was that of Histone gene. The gene codes for the Histone proteins that play an important role in the formation of chromatin. They are one of the highly-conserved proteins in eukaryotes (Derycke, Vanaverbeke, Rigaux, Backeljau, & Moens, 2010). We performed Polymerase Chain Reaction (PCR) of COI (figure 2), Histone(figure 3) and 18S(figure 4) genes with primers specific for the respective genes. Before performing PCR, it is important to check the concentration of DNA. The concentration of DNA was examined on Nanodrop spectrophotometer. For the PCR, a specific concentration of the template DNA is required. The genomic DNA that we isolated had a very high concentration. Therefore, the genomic DNA was diluted and then used for PCR. The other important thing we had to check before jumping to PCR were the primers required. The primers ordered were universal. The final concentrations of all the primers were optimized. For each gene, we had to optimize the annealing temperatures. Once the annealing temperature was optimized the genes were readily amplified. Once the genes were amplified we had to elute the amplified samples from the Agarose gels with the help of Gel Elution kit. Once that was done, we sent the samples for DNA sequencing.

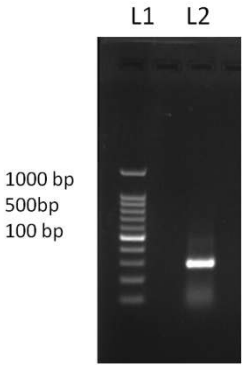

Figure 4: 1.5% gel stained with 0.1µg/ml of EtBr
Lane 1- 100 bp standard DNA ladder
Lane 2- Amplification of Histone gene

Figure 3 Amplification of Histone gene in *P.homarus*

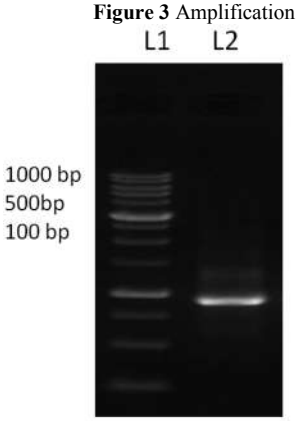

Figure 5: 1.5% gel stained with 0.1µg/ml of EtBr
Lane 1- 100 bp standard DNA ladder
Lane 2- 18 S amplification

Figure 4 Amplification of 18S gene in *P.homarus*

The DNA sequencing data is in the form of a chromatogram. Firstly, we had to edit and trim the sequence of DNA for all the three DNA markers namely COI, Histone and 18SrRNA with the help quality data from the chromatogram. We analyzed the chromatogram data using the application Finch TV 1.4. Secondly, we translated the edited sequence to check for the stop codons. Then we used NCBI'S BLAST tool to identify the origin of the DNA sequence that we have amplified with the *Panulirus homarus* sequences already present in the databases. We found 95%, 98% and 94% similarity of COI, 18S and Histone respectively. This was done for all the three markers. Once this was done, we had a proper data of the nucleotide gene sequences of *Panulirus homarus* for COI, histone and 18S. We submitted the sequences to NCBI and soon our sequences of the rock lobster will be uploaded on NCBI.

Table 2 COI sequence variation among *Panulirus*.

	Aligned position									
	656	662	665	669	671	674	675	677	680	683
Panulirus ornatus	A	C	T	C	T	T	C	A	C	G
Panulirus homarus	A	T	T	C	C	C	T	A	A	A
Panulirus penicillatus	C	T	C	T	A	T	T	G	T	A
Panulirus polyphagus	-	-	-	-	-	-	-	-	-	-

Table 3 Histone sequence variation among *Panulirus*

	Aligned position									
	27	33	78	81	84	99	114	117	120	132
Panulirus polyphagus	C	C	C	T	C	C	A	T	G	A
Panulirus penicillatus	C	T	T	C	C	T	G	C	T	G
Panulirus ornatus	T	C	C	T	T	T	A	T	G	A
Panulirus homarus	C	C	C	T	T	C	A	T	G	A

Table 4 18S sequence variation among *Panulirus*.

	Aligned position									
	262	275	277	288	289	294	295	309	310	313
Panulirus penicillatus	A	C	C	G	G	C	C	C	A	T
Panulirus homarus	A	C	C	C	G	A	C	A	G	T
Panulirus polyphagus	C	T	T	A	A	A	T	A	A	C
Panulirus ornatus	C	T	T	A	A	A	C	A	A	C

Now our final aim was to have a comparative analysis of the three genes in various *Acheleta* lobsters. We had to analyze how evolution has taken place in *Panulirus homarus* with the help of comparison of its genes with that of other lobsters. Hence, we generated phylogenetic trees using the nucleotide sequences of COI, Histone and 18S. Before generating a phylogenetic tree, we performed multiple alignment of the sequences to find out the nucleotide substitutions. This was done with the help of Multalin software. We compared *Panulirus homarus* from Mumbai region's COI data with the COI data of other lobsters present in NCBI database namely *Panulirus homarus, Panulirus ornatus, Panulirus polyphagus and Panulirus penicilatus* (table 2). With this we got to know the nucleotide substitutions, the missing of a single nucleotide that made *Panulirus homarus* different from the other lobsters. This tells us how evolved *Panulirus homarus* is different from that of the other lobsters. Similar multiple sequence alignment was performed for Histone (table 3) as well as for 18S (table 4) respectively and thus evolution was seen in those two genes as well.

With the help of Clustal Omega Software we were successful in making phylogenetic tree. We selected the FASTA file of our lobster *Panulirus homarus* and compared to the FASTA sequences of *Panulirus homarus, Panulirus ornatus, Panulirus polyphagus and Panulirus penicilatus* already present in NCBI database. We got the data of phylogenetic analysis for COI, 18S and Histone. Hence with help of phylogenetic tree we could find out the evolutionary relationships between *Panulirus homarus* and other lobsters. When we compared the COI sequences of *Panulirus homarus* (figure 5) to other lobsters we saw that *P.homarus and P.polyphagus* have a less recent ancestor whereas *P.ornatus and p.penicilatus* have a recent common ancestor. When we compared the 18S rRNA data of *P.homarus* (figure 6) with other lobsters we found out that *P.homarus* and *P.penicilatus* are sister species. This means that *P.homarus* shared most recent common ancestor with *P.penicilatus*. Finally we compared the Histone sequences (figure 7) and we found out that *P.homarus* has a less recent ancestor than the other genus.

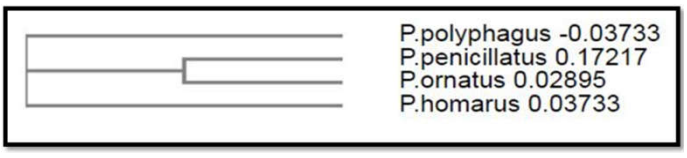

Figure 5 Phylogenetic tree of *P.homarus* and related species of Panuliris on the basis of COI gene sequence

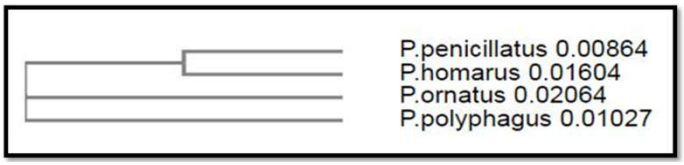

Figure 6 Phylogenetic tree of *P.homarus* and related species of Panuliris on the basis of 18S gene sequence

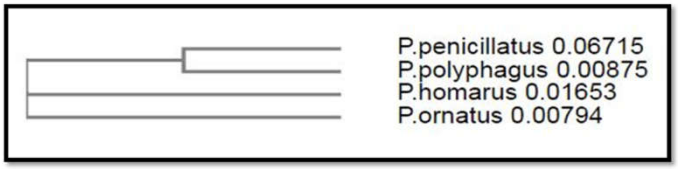

Figure 7 Phylogenetic tree of *P.homarus* and related species of Panuliris on the basis of Histone gene sequence

CONCLUSION

Hence with the help of the above data we could find out the relatedness of each *Panulirus homarus* with different lobster species at the genetic levels using the three different markers. *Panulirus homarus, Panulirus ornatus, Panulirus polyphagus and Panulirus penicilatus* are all commercially important species. Their meat has high value in the food industry. Even though they had been morphologically identified, it is not enough. Hence a proper authentication is required. DNA barcoding serves as the most valid system for authentication of the species. In our study, we thus authenticate that the sample of rock lobster that we collected from the landing centre in Mumbai was found to be *Panulirus homarus*. This shows that how DNA barcoding helped in identification.

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