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Research Article

IDENTIFICATION OF BLUE SWIMMING CRAB *PORTUNUS PELAGICUS* USING MOLECULAR TAXONOMY

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ABSTRACT

The main aim of this study was to perform DNA barcoding of *Portunus pelagicus* from Mumbai region using DNA markers specifically used as DNA barcodes. In this study, we have performed molecular taxonomy of *Portunus pelagicus* based on gene loci namely Cytochrome Oxidase Subunit I, 18S rRNA and Histone H3. In the beginning, genomic DNA was isolated using CTAB method. Polymerase Chain Reaction of COI, Histone H3 and 18S rRNA genes were performed and sequenced. The DNA sequences of the respective genes were analysed using Finch TV version 1.4 and BLAST was performed to understand the sequence similarity. FASTA sequence of all the three barcodes of *Portunus pelagicus* were compared with the FASTA sequence of other related crab species using Multialin tool and Clustal Omega. With the help of Multialin tool, it was easier to study the evolutionary relationship between the sequences for the respective gene loci. Clustal Omega was used to evaluate the phylogenetic relationships between *Portunus pelagicus* and other crabs from the same family. Thus, in this study we propose that the three DNA barcodes viz; COI, Histone H3 and 18S rRNA can be used to discriminate between crab species by comparative evaluation using phylogenetic tree.

INTRODUCTION

Portunus pelagicus (Rathbun)[1], commonly known as the blue swimming from the family, Portunidae is one of the important commodities in the world's fisheries. The crabs are valuable seafood of great demand both in domestic markets and the export industry. The crabs live in a wide range of inshore and continental shelf areas, including sandy, muddy or algal and seagrass habitats, ranging from the intertidal zone to at least 50 m depth [2]. Increase in demand for crab species along the Indian coast has led to an increase in illegitimate labelling by seafood restaurants in this region. There is an increasing need for upgrading quality control with the help of proper labelling of seafood utilizing molecular tools. This problem can be solved by the taxonomic identification of species [3].

In recent times, there has been a debate over identification of species using morphological characteristics. According to some scientists, morphological identification is ineffective and often misleading[4]. Many times, due to improper handling the seafood is damaged. This may often result in forgery. To resolve such problems, DNA barcoding acts as a reliable tool. In DNA barcoding the mitochondrial cytochrome oxidase I (COI) gene which is around 648 base pairs is the chief identification tool that is used to single out species at a molecular level [5]. This identification system is steadfast and attainable and hence is becoming popular day by day.

In this paper, we have studied the molecular taxonomy of *Portunus pelagicus* from Mumbai region. DNA Barcoding was performed using the standard DNA barcode used for identification of animals Cytochrome Oxidase Subunit I , as well as other important markers like Histone H3, and 18S rRNA [6].The data generated with the help of these barcodes were then examined using a phylogenetic tree.

MATERIALS AND METHODS

Sample collection and authentication

The species *Portunus pelagicus* was collected from New Ferry Wharf (Bhaucha Dhakka) 18.956382°N 72.849261°E. The crab species were then sent to Central Marine Fisheries Research Institute, Mumbai for further authentication.

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DNA extraction, Polymerase Chain Reaction and DNA Sequencing

A small amount of tissue was obtained from the manus, carpus and merus region of the chelipeds. The tissue was then crushed in mortar and pestle using Liquid nitrogen. Lysis buffer consisting of 2% CTAB (Cetyl trimethyl ammonium bromide), 100mMTris (Tris Hydroxy Amino Methane)- pH 8.0,20mM EDTA(Ethylene diamine tetra acetic acid),1.7 M NaCl (Sodium Chloride) with 0.3% (v/v) β-mercaptoethanol was used for extraction of genomic DNA and the sample was kept for 1 hour incubation at 60^0C.Genomic DNA was isolated and analysed with the help of Agarose Gel Electrophoresis and quantitated with the help of Nanodrop.

Polymerase Chain Reaction (PCR) was performed for the amplification of COI (Cytochrome Oxidase I), 18S rRNA and Histone H3 gene (Table 1). The total volume of the PCR reaction was 25 µl which contained 100ng DNA, 1X Taq Buffer, 2.5mM $MgCl_2$ (Magnesium Chloride). The final primer concentration for COI (Folmer *et al*.1994), 18S rRNA (Kobori *et al*., unpublished.) and Histone H3[7] gene was 0.5 µm. Amplification was carried out in thermal cycler (Applied Biosystems) with an initial denaturation at 96°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, followed by the annealing temperature of the respective primers used which was different for the for 30 seconds and later extension at 72°C for 30 seconds. The program concluded with one final extension at 72°C for 10 minutes. The amplified products were later observed by electrophoresis on 1.5% Agarose Gel.

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

Name of Primers	Forward Sequence	Reverse sequence
Cytochrome Oxidase 1 gene	5 - GGTCAACAAATCATA AAGATATTGG-3	5 - TAAACTTCAGGGTGAC CAAAAAATCA-3
18S rRNA	5 - CTGGTTGATYCTGCC AGT-3	5 - CTTCGAACCTCTGACTT TCG-3
Histone	5- ATGGCTCGTACCAAG CAGACVGC-3	5- ATATCCTTRGGCATRAT RGTGAC-3

Data Analysis

The chromatogram sequence obtained from Xcelris Genomics for COI, Histone H3 and 18S rRNA gene for both forward and reverse primers were analysed using Finch TV version 1.4. BLAST (Basic Local Alignment Search Tool) tool was used to identify the homology of DNA sequences amplified for COI, Histone and 18S rRNA gene. Multiple Sequence Alignment for the gene sequences based on Cytochrome Oxidase subunit I, Histone H3 and 18S rRNA data of *Portunus pelagicus* was implemented using the Multi Align (Florence Corpet) software. With the help of Clustal Omega software, we could construct a phylogenetic tree for COI, Histone H3 and 18S rRNA gene.

RESULTS AND DISCUSSION

Morphological identification of Portunus pelagicus

The blue crab was submitted to CMFRI, Mumbai (Central Marine Fisheries Institute) and was identified based on morphological characters. The species identification report provided thereby confirmed the identity of the crab *Portunus pelagicus*. (Figure 1 and 2)

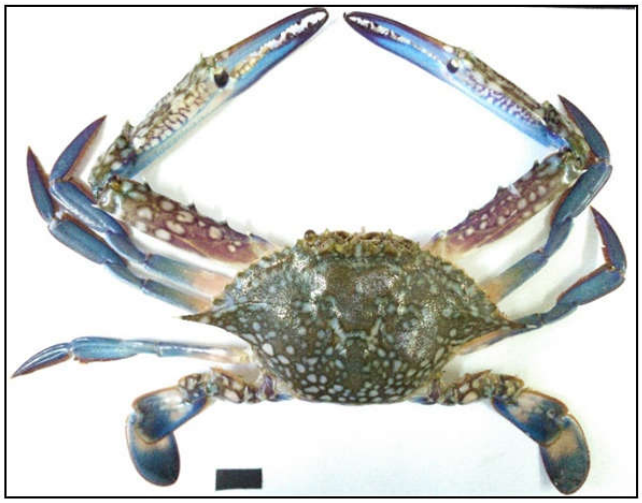

Figure 1 Dorsal Side of *Portunus pelagicus*

Figure 2 Ventral Side of *Portunus pelagicus*

For any genotyping analysis, a pure intact double stranded DNA of a decent concentration is a prerequisite. In this study, we have used Lysis buffer along with a quaternary ammonium surfactant called Cetyltrimethylammonium bromide. Due to its nature C-TAB forms micelles in aqueous solution which in turn helps in the lysis of cell membrane as well as there is a separation of polysaccharides from nucleic acids[8]. Before treating the *P.pelagicus* tissue with the lysis buffer it was treated with liquid nitrogen followed by the grinding of the frozen tissue with the help of mortar and pestle. The purity of genomic DNA was assessed on Nanodrop spectrophotometer. The ratio of absorbance at 260 nm and 280 nm was approximately 1.8.

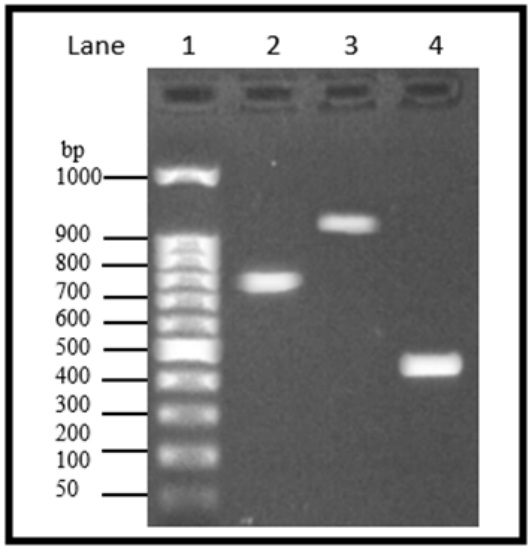

Figure 3 1.5% gel stained with 0.1µg/ml of ETBR.

LANE 1 – 100 bp ladder **LANE 2** – COI amplification **LANE 3** – 18S amplification **LANE 4** – Histone amplification

Figure 3 1.5% agarose gel showing COI, 18S rRna, and Histone gene amplification

For the study, genetic markers namely Cytochrome oxidase I,18S rRNA and Histone were considered. Cytochrome oxidase subunit I is a protein-coding animal mitochondrial gene. Due to slow evolution rate, it has been used as a unit to measure the rate of evolution in animals . 18S rRNA is a non-protein coding gene of the mitochondria where 18S gene is responsible for the synthesis of 18S rRNA. Another DNA marker that we used was that of Histone H3 gene. The gene that codes for the Histone proteins play an important role in the formation of chromatin. They are one of the highly-conserved proteins in eukaryotes[9]. Polymerase Chain Reaction(PCR) of COI, Histone and 18S genes was performed with primers specific for the respective genes (figure 3). Before performing PCR, it is necessary to check the concentration of DNA. The concentration of DNA was examined on Nanodrop spectrophotometer. The genomic DNA obtained gave a satisfactory 260/280 ratio determining its quality. The genomic DNA was appropriately diluted and then PCR amplification was performed (Figure 3). On amplification, the samples were eluted from the Agarose gel with the help of Qiagen Gel Elution Kit. On completion, the samples were sent for DNA sequencing.

The DNA sequencing data was obtained in the form of a chromatogram. The chromatogram data was further analysed using the application Finch TV 1.4. Then BLAST was used to identify the origin of the DNA sequence that was amplified with the *Portunus pelagicus* sequences already present in the databases and the results showed 95%, 98% and 94% similarity of COI, 18S and Histone respectively. On processing the complete data of the nucleotide gene sequences of *Portunus pelagicus* for COI, histone and 18S the sequences were submitted to NCBI having the following accession ID s KY865316, KY865317, KY865318.

The final aim of the study was to have a comparative analysis of the three genes in various brachyuran crabs, therefore, it was important to analyse how evolution had taken place in *Portunus pelagicus* with the help of comparison of its genes with that of other crabs. Hence, the phylogenetic tree was generated for the nucleotide sequences of COI, Histone and 18S. Before generating a phylogenetic tree, multiple alignments of the sequences were performed to find out the nucleotide substitutions. This was done with the help of MultAlign software. The COI data of *Portunus pelagicus* from Mumbai region was compared with the COI data of other crab present in NCBI database namely, *Portunus sanguinolentus*, *Charybdis lucifera*, *Charybdis natator*, *Portunus sanguinolentus*, *Portunus pelagicus* and *Scylla serrata*. (Table 2)

Table 2 COI sequence variation among the Brachyuran infra-order

	Aligned position									
	196	197	201	202	203	204	205	206	210	214
Portunus pelagicus	.	G	.	G	.	.	G	.	.	A
Portunus sanguinolentus	.	.	.	A	.	.	C	.	.	T
Charybdis lucifera	.	.	.	A	.	.	T	.	.	A
Charybdis natator	.	.	.	T	.	.	C	C	.	A
Charybdis feriatus	C	.	C	A	T	A	C	.	C	T
Scylla serrata	T	.	C	T	T	A	T	.	C	G

Dots (·) indicate nucleotides identical to those of *Portunus pelagicus*.

Table 3 18 SRNA sequence variation among the Brachyuran infra-order

	Aligned position									
	79	80	82	84	162	233	241	256	390	391
Portunus pelagicus	T	G	G	T	A	T	G	A	.	G
Portunus sanguinolentus	.	.	.		A	T	G	A	.	.
Charybdis lucifera	.	.	.	.	.	.	.	C	.	.
Charybdis natator	.	.	.	.	.	.	.	C	.	.
Charybdis feriatus	.	.	.	.	A	T	.	C	.	.
Scylla serrata	.	.	.	.	.	.	.	G	A	.

Dots (·) indicate nucleotides identical to those of *Portunus pelagicus*.

Table 4 Histone sequence variation among the Brachyuran infra-order

	Aligned position									
	48	**49**	**52**	**57**	**76**	**79**	**82**	**94**	**109**	**112**
Portunus pelagicus	T		G	A	T	T	.	.	A	.
Portunus sanguinolentus	.	.	A		T	.	.	.	A	.
Charybdis lucifera	.	.	A	.	C	.	.	.	G	.
Charybdis natator	.	.	T	.	G	.	A	T	T	T
Charybdis feriatus	.	A	T	.	G	.	A	T	T	T

Dots (·) indicate nucleotides identical to those of *Portunus pelagicus*.

The missing of a single nucleotide made *Portunus pelagicus* different from the other crabs. Through This tells how *Portunus pelagicus* has evolved from other crabs. Similar multiple sequence alignment was performed for 18S and Histone H3 and thus evolution was seen in those two genes as well (Table 2 and table 3)

The Clustal Omega Software was used to create the phylogenetic tree. The FASTA sequence of Portunus *pelagicus* was selected and compared to the FASTA sequences of *Portunus sanguinolentus*, *Charybdis lucifera*, *Charybdis natator*, *Portunus sanguinolentus*, *Portunus pelagicus* and *Scylla serrata*. Hence with the help of phylogenetic tree, the evolutionary relationships between *Portunus pelagicus* and

other crabs could be studied. When the COI sequences of *P.pelagicus* was compared to other crabs it was noted that *P.pelagicus*, *C.feriata* and *S.serrata* had a recent ancestor (figure 4). While *P.pelagicus*, *C.lucifera*, *C.natator* have a less recent common ancestor. *P.pelagicus* and *P.sanguinolentus* also shared a common ancestor but *P.pelagicus* is much more evolved than *P.sanguinolentus* when compared with COI gene (figure 4) . The 18S rRNA data of *P.pelagicus*, when compared with other crabs, revealed that *P.pelagicus* and *P.sanguinolentus* are sister species (figure 5). This means that *P.pelagicus* shared the most recent common ancestor with *P.sanguninolentus*. Finally, we compared the Histone sequences (figure 6) and we found out that *P.pelagicus* shared the most recent common ancestor with *P.triberculatis*. Hence with the help of the above data, we could find out the relatedness of each *P.pelagicus* with different crab species at the genetic levels using the three different markers. *Portunus pelagicus*, *Portunus sanguinolentus*, *Charybdis feriata*, *Charybdis lucifera*, *Scylla serrata*, *P.triberculatis*, *C.natator* 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 crab that we collected from New Ferry Wharf was indeed *Portunus pelagicus*.

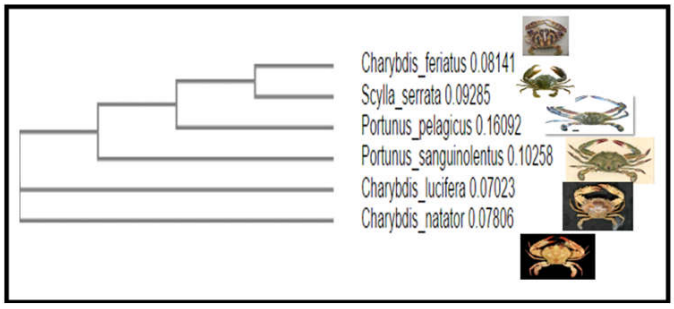

Figure 4 Phylogenetic tree of *Portunus pelagicus* and related species of COI sequence

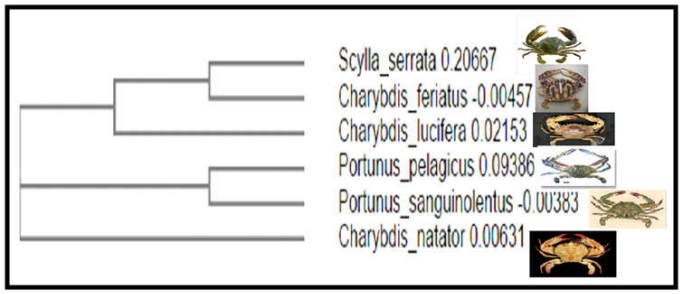

Figure 5 Phylogenetic tree of Portunus pelagicus and related species of 18S sequence

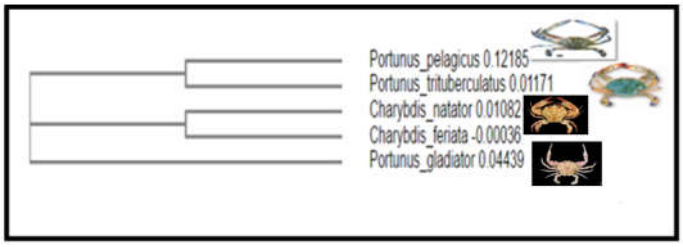

Figure 6 Phylogenetic tree of Portunus pelagicus and related species of Histone H3 sequence

CONCLUSION

Portunus pelagicus are members of crustacea. They belong to the order Decapoda and are placed under the infra-order Brachyura indicating that *Portunus pelagicus* is a true crab according to Guinot classification (1977, 1978, 1979). *Portunus pelagicus* is a freshwater crab which has a high diversity and conservation value. It is characterized on morphological basis but morphological data are sometimes inadequate and misleading when trying to determine the species at the larval stages. Therefore, molecular taxonomy has helped us to establish a genetic relationship between the members of different taxonomic categories and its identification. *Portunus pelagicus* was characterized on a morphological level by CMFRI showing 95% of confidence in the species name. *Portunus pelagicus* was then characterized on the molecular basis for taxonomical classification and to establish a genetic relationship between the members of Brachyuran infra-order of Portunidae family. (table 2`, 3, and 4).

Clustal Omega was used to evaluate the phylogenetic relationships among the crabs of same family and phylogenetic trees were constructed by applying the neighbour joining method to the entire sequences of mt COI, 18S rRNA, and Histone H3(figure 4-6). These results suggest that the traditional DNA barcodes, mt COI, 18S rRNA, and Histone H3 were able to discriminate the original crab species between *Portunus*, *Charybdis*, and *Scylla* species showing the rate of evolution. As shown in the results of the phylogenetic tree, when all the three DNA barcode sequences were employed, *Portunus pelagicus* was genetically closer to *Scylla serrata* and *Charybdis feriatus* and distant from *Portunus sanguinolentus* and more distant to *Charybdis lucifera* and *Charybdis natator* based on the COI sequence (figure 4). In regards 18S rRNA sequence, *Portunus pelagicus* was genetically closer to *Portunus sanguinolentus* and distant from *Charybdis* and *Scylla* species (figure 5). With respect to histone sequence, *Portunus pelagicus* was closer to *Portunus* species and distant from *Charybdis* species (figure 6). Based on these findings, we propose that the three DNA barcode regions mt COI, 18S rRNA, and Histone H3, can be used to discriminate between the crab species by comparative evaluation using phylogenetic tree. Furthermore, species-specific marker nucleotides identified in this study will help to accurately authenticate each species and standardize the diversity between the infra order Brachyura.

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