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Research Article

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DNA Barcoding for the Identification and Authentication of fiddler crabs from Mumbai mangroves.

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Abstract

Molecular authentication using DNA barcoding techniques offers an efficient methodology that can identify existing species and discover unknown species with the help of analysis of sequence variation in a conserved domain region of DNA. These techniques can identify animal species and discriminating the authentic from the invasive species. Molecular characterisation using DNA Barcoding technique has been carried out to identify fiddler crabs *Uca annulipes* and *Uca urvillei* from the Mumbai mangroves using the standard DNA barcodes Cytochrome Oxidase I(COI), 16S rRNA, 18S rRNA and Internal Transcribed Spacer (ITS). The above genes were sequenced and the confirmation of *Uca annulipes* and *Uca urvillei* was confirmed.

Keywords: DNA Barcoding, Cytochrome Oxidase I, 16S rRNA , 18S rRNA, ITS, Phylogenetic tree

1. Introduction

Fiddler crabs belonging to the genus *Uca* comprises of 100 species of semi-terrestrial marine crabs found in the tropical and sub-tropical regions(Peer, Miranda, & Perissinotto, 2015). *Uca* are the members of the family Ocypodidae and are most closely related to the ghost crabs of the genus *Ocypode* (Rosenberg, 2001). Ocypode comprises of small crabs in which the largest crab is slightly over two inches long (Levinton, Judge, & Kurdziel, 1995). Fiddler crabs habitat is located along sea beaches and brackish inter-tidal mud flats, lagoons and swamps (Levinton et al., 1995). Fiddler crabs are known for their peculiar sexually dimorphic claws; the males have a larger major claw than the minor claw while the females have claws of the same size (Levinton et al., 1995). In the year 1992 ,the diversity of fiddler crabs in India was reported and as per the reports over 9 species of *Uca* are present in the east and west coast of India (Krishnan, 1992).

Mangle and Kulkarnihad reported the presence of *Uca annulipes* from the intertidal area of Mumbai region (Mangale & Kulkarni, 2013). However, *Uca annulipes* was identified on the basis of morphological characteristics (Mangale & Kulkarni, 2013). In this study we have identified the fiddler crabs *Uca annulipes* and *Uca urvillei* from Mumbai region using the mitochondrial genes Cytochrome Oxidase I (COI), 16S rRNA, 18S rRNA and Internal transcribed Spacer (ITS).

2. Materials and Methods

The fiddler crabs were collected from the Sewri18.9924° N, 72.8547° E mangrove regions of Mumbai and the live specimens were brought to the laboratory. Few fiddler crab specimens were sent to Central Marine Fisheries Research Institute (CMFRI),

Mumbai for morphological identification. The identification was performed by group of expert taxonomists who were involved in the validation of the identity of the voucher specimens.

DNA was extracted from the merus, carpus and propodus region of the cheliped using CTAB buffer method consisting of 2% CTAB (hexadecyltrimethylammonium bromide), 100mM Tris-Cl [pH 8.0], 20mM EDTA (Ethylenediaminetetraacetic acid) [pH 8.0], 1.7M NaCl and 0.3% β-mercaptoethanol. Mitochondrial gene Cytochrome Oxidase subunit I, 16S rRNA, 18S rRNA and Internal Transcribed Spacer genes were amplified using the universal primers for the respective genes. A 25 µL reaction was set up for the amplification of the genetic loci mentioned above containing 100 ng of DNA, 1X Taq Buffer, 2.5mM $MgCl_2$ (Magnesium Chloride) along with their respective primers using the Applied Biosystems thermocycler. The PCR amplified products were sent to Eurofins India, Bengaluru for DNA sequencing (Sanger's method). The results acquired with the help of DNA sequencing were observed and edited using Finch TV 1.4. Expasy tool was used to translate the COI sequence to locate the stop codons. The DNA sequences obtained for *Uca annulipes* and *Uca urvillei* found in Mumbai region were compared with the most similar sequences available in the NCBI GenBank database using BLAST (Basic Local Alignment Search Tool). Multiple sequence alignment was applied to study the variation between the DNA Sequences of *Uca annulipes* and *Uca urvillei* with the DNA sequences of the other Brachyuran crabs which were retrieved from NCBI using the software MultiAlign by Florence Corpet. Phylogenetic analysis using Clustal W software was achieved and the genetic diversity between *Uca annulipes* and *Uca urvillei* with other brachyuran crab species was studied using the Neighbour Joining method. The sequences were submitted to NCBI GenBank using NCBI Bankit and the sequences are now available. (KX951459.1,MK348671.1,MK346040.1,MK350357.1, K629663, MK348672.1, MK350359.1, MK350359.1)

3. Results and Discussion

According to the reports drafted by the expert taxonomists of CMFRI, the collected fiddler crabs were identified as *Uca annulipes*(figure 1a)and *Uca urvillei* (figure 1b)

Key Diagnostic characteristics of *Uca annulipes* (As per the reports of CMFRI, Mumbai)

1. Eyes round, terminal on slender eyestalks. Antennular flagella small, not hidden beneath front.

2. Front is wider and small chelipeds with merus are not posteriorly flattened. Row of tubercles absent on merus of small chelipeds. Oblique tuberculate ridge on palm present. Crest on major merus absent.

3. The fingers widely gaping, the movable finger being curved inward at the tip. Dactyl of major cheliped with convex upper margin throughout.

4. Dorsal edge of orbit narrow. Dactylus of major cheliped without large subdistal tooth. Manus of major cheliped with smooth outer surface. Outer manus of major cheliped without small round depression near base of pollex and carpal cavity with distal extension.

5. The enlarged pincer is usually pink, sometimes nearly white. Body varies from black to pale,with dark or blue or white stripes.

Key Diagnostic characteristics of *Uca urvillei* (As per the reports of CMFRI, Mumbai)

1. Eyestalks slender and equipped with round cornea. Major cheliped of male is prominently enlarged. Carapace is not covered with tubercles. Front narrow. Carpus pf major cheliped with flattened anterodrosal area. Ratio of right or left major chela approximately 50%

2. Major chela armed with forceps shaped fingers. Straight cutting margins of fingers <1/2 length fingers. Merus of minor cheliped with short and stiff setae.

3. Row of tubercles present on floor of orbit. Distal tube of male gonopod elongated.

Figure 1Dorsal view of Uca annulipes *Figure 2.bDorsal view of Uca urvillei*

The first step required before the amplification of the mitochondrial genes is the isolation of good quality genomic DNA which can be achieved using DNA extraction buffer. For this study we have used three different types of buffers namely SDS buffer, Triton X Buffer and CTAB Buffer. Triton X buffer and SDS buffer did not prove to be ideal extraction buffers for isolation of gDNA from the fiddler crabs as the concentration of the DNA isolated was not satisfactory. Nanodrop spectrophotometer quantification of the DNA isolated using the detergents Triton X and SDS showed a poor peak at 260 nm. However, the genomic DNA isolated with the help of CTAB buffer was of good quality and gave a good absorption peak at 260 nm.

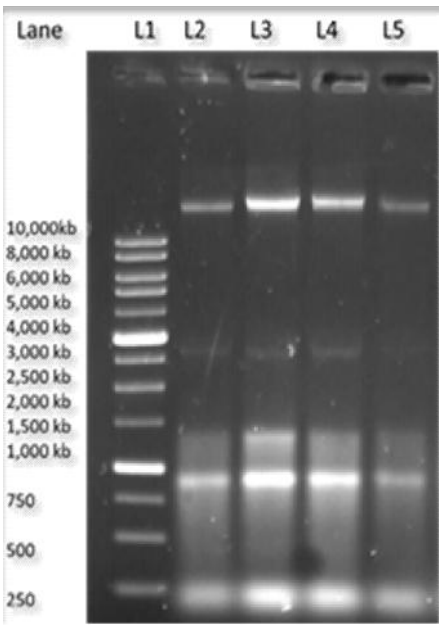

Figure 2– 0.8% gel stained with 0.1µg/ml of EtBr.Lane 1 – 1kb Standard DNA ladder, Lane 2 to Lane 3 – gDNA isolated from Uca annulipes in CTAB buffer.Lane 4 to 5- gDNA isolated from Uca urvillei in CTAB buffer

For this study Cytochrome Oxidase, I, 16S rRNA, 18S rRNA and ITS genes were used as the genetic markers. The primers ordered for COI(Black, Hoeh, Lutz, & Vrijenhoek, 1994),16S rRNA (Garcia-pichel, Muyzer, & Nubel, 1997), 18S rRNA, ITS were universal. The final concentrations of all the primers were optimized. For each gene, the annealing temperatures were optimized. Once the annealing temperature was optimized the genes were readily amplified.

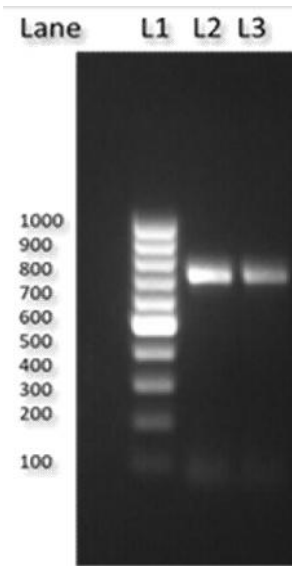

Figure 3a 1.5% agarose gel stained with 0.1µg/ml of EtBr.LANE 1 – 100 bp Standard DNA ladder LANE 2 and Lane 3 – COI amplification of Uca annulipes

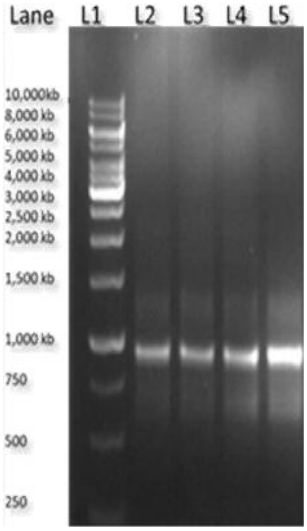

Figure 3b 1.5% gel stained with 0.1µg/ml of EtBr. LANE 1 – 1 kb Standard DNA ladder,LANE 2, LANE 3, LANE 4, LANE 5 – 16Sr RNA amplification of Uca annulipes

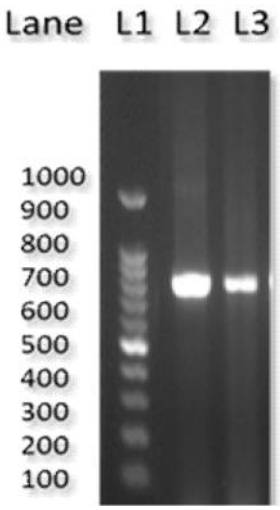

Figure 3c1.5% gel stained with 0.1µg/ml of EtBr. LANE 1 – 1 kb Standard DNA ladder LANE 2, LANE 3- 18Sr RNA amplification of Uca annulipes

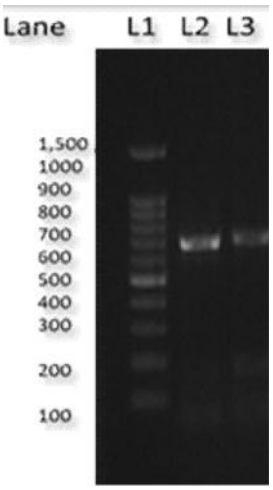

Figure 3d1.5% gel stained with 0.1µg/ml of EtBr. LANE 1 – 100 b Standard DNA ladder, LANE 2, LANE 3- ITS amplification of Uca annulipes

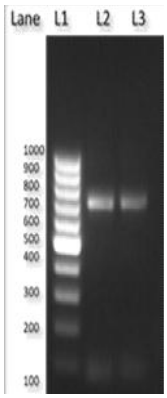

Figure 4a – 1.5% gel stained with 0.1µg/ml of EtBr.LANE 1 – 100 b Standard DNA ladder, LANE 2, LANE 3- COI amplification of Uca urvillei

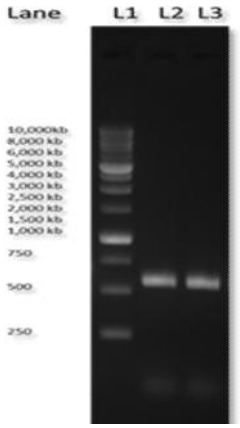

Figure 4b – 1.5% gel stained with 0.1µg/ml of EtBr. LANE 1 – 1Kb Standard DNA ladder , LANE 2, LANE 3- 16 S r RNA amplification of Uca urvillei

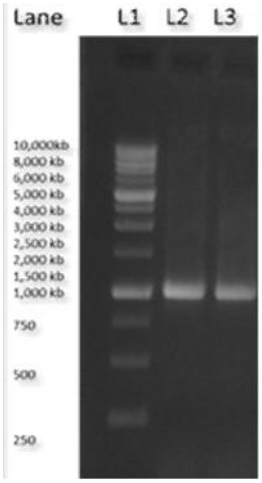

Figure 4c – 1.5% gel stained with 0.1µg/ml of EtBr. LANE 1 – 1Kb Standard DNA ladder, LANE 2, LANE 3- 18 S r RNA amplification of Uca urvillei

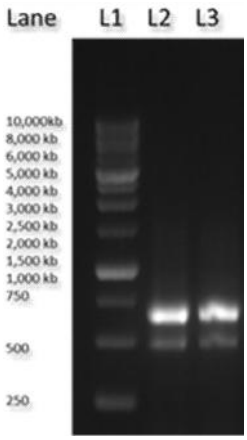

Figure 4d– 1.5% gel stained with 0.1µg/ml of EtBr. LANE 1 – 1Kb Standard DNA ladder, LANE 2, LANE 3- ITS amplification of Uca urvillei

Final aim was to have a comparative analysis of the four genes in various brachyuran crabs. Analysis of how evolution has taken place in *Uca annulipes* with the help of comparison of its genes with that of other crabs was needed to be done. Hence, we generated phylogenetic trees using the nucleotide sequences of COI, 16S rRNA, 18S rRNA and ITS. Before generating a phylogenetic tree, multiple alignment of the sequences was performed to find out the nucleotide substitutions. This was done with the help of MultAlign software. Comparison of COI data of *Uca annulipes* and *Uca urvillei* from Mumbai region with the COI data of other brachyuran crabs present in NCBI database namely *Scylla serrata*, *Portunus pelagicus*, *Charybdis feriata*, *Carcinus maenus*, and *Ocypode quadrata* was done. With this the nucleotide substitutions were known, the missing of a single nucleotide that made *Uca annulipes* and *Uca urvillei* different from the other crabs. This tells us how evolved *Uca* species is from that of the other crabs. Similar multiple sequence alignment was performed for 16S, 18S and ITS as well.

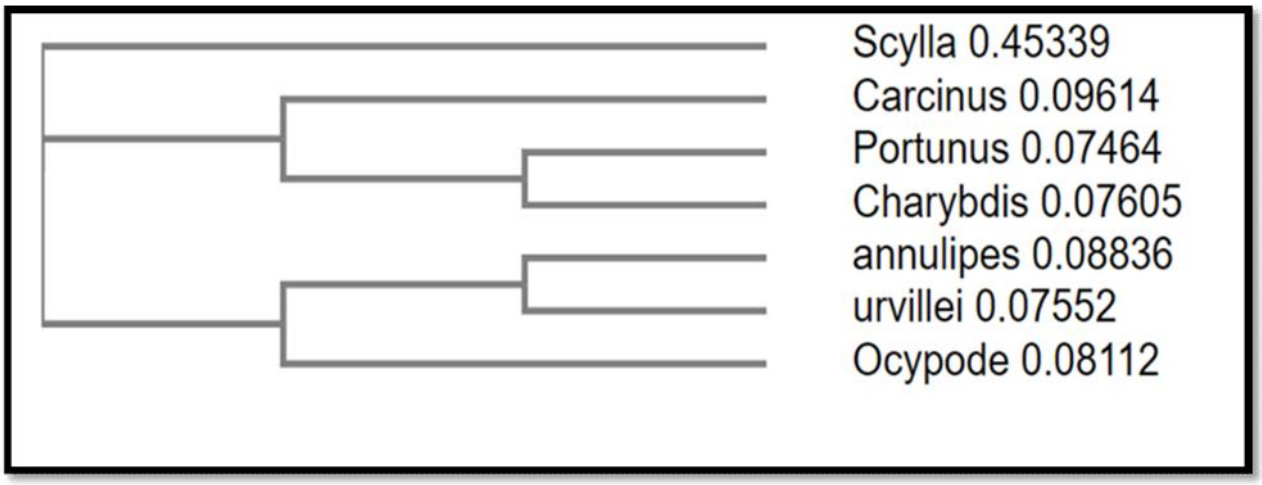

Figure 5 Phylogenetic tree of Uca annulipes and Uca urvillei and other brachyuran crabs for the COI sequence

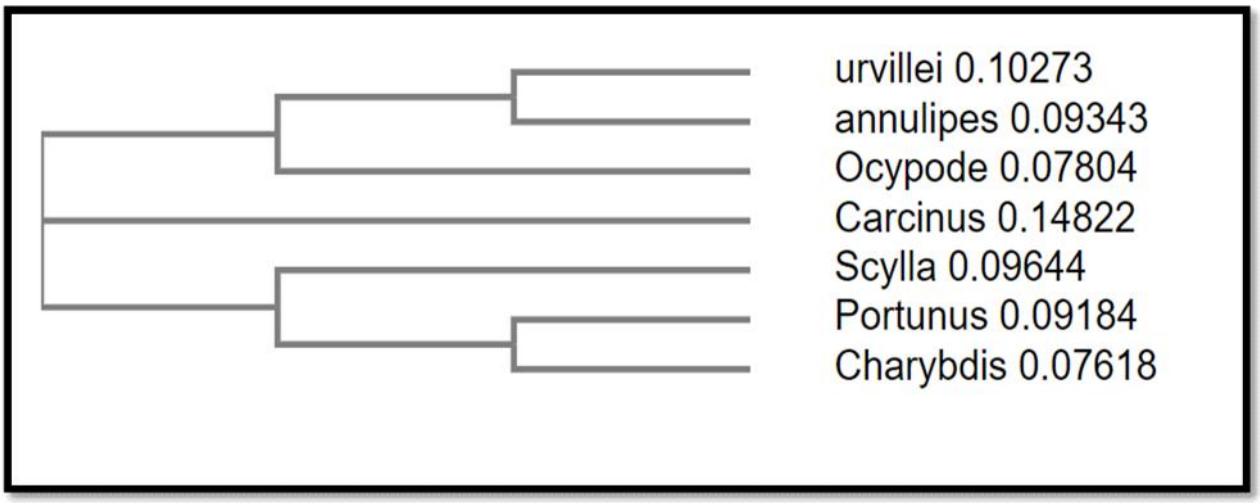

Figure 6 Phylogenetic tree of Uca annulipes and Uca urvillei and other brachyuran crabs for 16S rRNA sequence

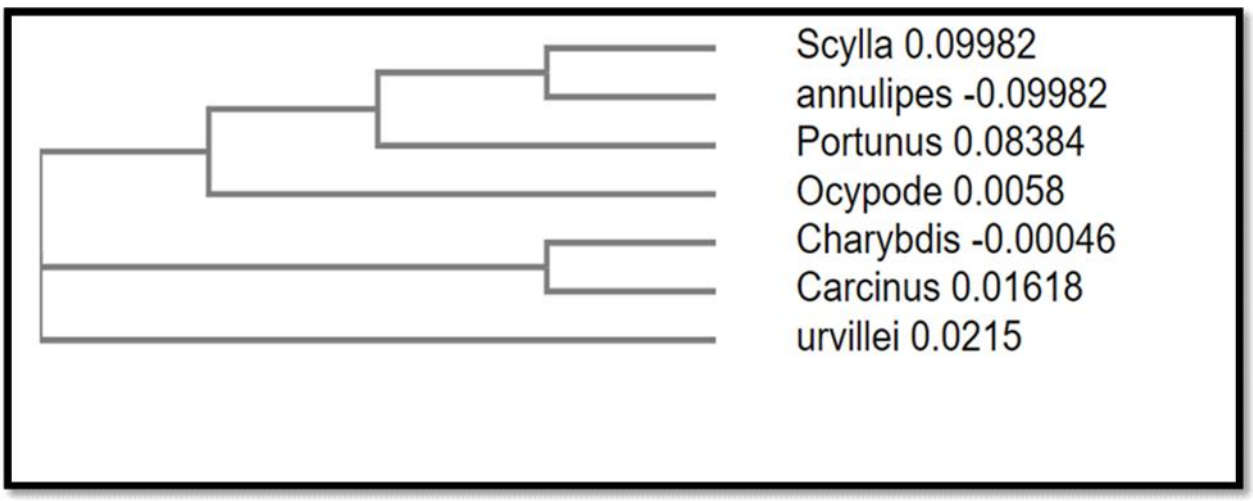

Figure 7 Phylogenetic tree of Uca annulipes and Uca urvillei and other brachyuran crabs for 18S rRNA sequence

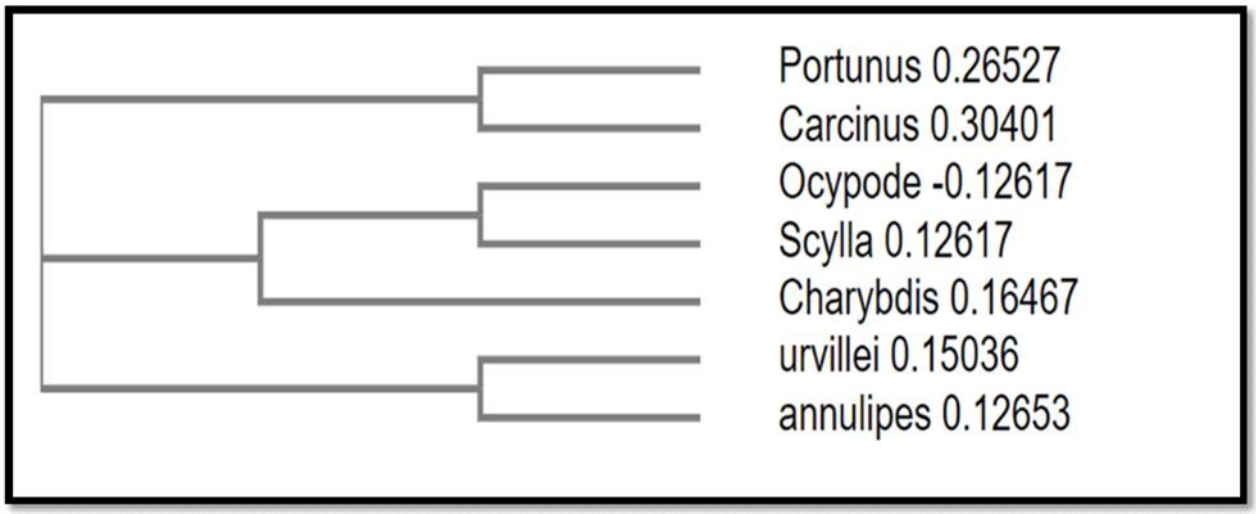

Figure 8 Phylogenetic tree of Uca annulipes and Uca urvillei and other brachyuran crabs for ITS sequence

Once our multiple sequence alignment was performed, the final aim was to generate a phylogenetic tree. With the help of Clustal Omega Software phylogenetic tree was made. FASTA file of our crab *Uca annulipes* and *Uca urvillei* was compared to the FASTA sequences of *Scylla serrata*, *Portunus pelagicus*, *Charybdis feriata*, *Carcinus maenus*, and *Ocypode quadrata*. With the help of the phylogenetic analysis data for COI, 16S, 18S and ITS, the evolutionary relationships between *Uca* species and other crabs were understood. When COI sequences of *U.annulipes* and *U urvillei* were compared to other crabs it was observed that *Uca annulipes* and *Uca urvillei* are sister species and share a very recent ancestor. It was also understood that *Uca annulipes* and *Uca urvillei* shared a recent common ancestor with *Ocypode quadrata* .It was also concluded that *Uca annulipes* and *Uca urvillei* shared a less recent ancestor with *Scylla serrata*, *Carcinus maenus*, *Portunus pelagicus* and *Charybdis feriata*. The 16 S phylogenetic tree data again proved that *Uca urvillei* and *Uca annulipes* are sister species .It also concluded that *U.urvillei* and *U.annulipes* shared a recent common ancestor with *Ocypode quadrata*. In the case of 18S rRNA sequences the phylogenetic data generated was not satisfactory .The phylogenetic tree concluded that *Scylla serrata* and *Uca annulipes* are sister species, which is not true. Also, it did not prove *Uca annulipes* as a sister species of *Uca urvillei* which is false. The phylogenetic tree generated for ITS sequence proved *Uca annulipes* and *Uca urvillei* as sister species but did not identify *Ocypode* as a recent common ancestor of the *Uca* spp. Hence with the help of the above data it was easy to find out the relatedness of each *Uca annulipes* and *Uca urvillei* with different crab species at the genetic levels using the four different markers.

4. Conclusion

Uca annulipes and *Uca urvillei* are true crabs and are placed under the infra order Brachyura (Ernesto & Bezerra, 2014). The *Uca* spp. or fiddler crabs as they are known were earlier characterised based on their morphology but sometimes the morphological features could be misleading (Rosenberg, 2002). The morphological identification is more complicated when the species are damaged. Molecular taxonomy has helped us to establish genetic relationship between the members of different taxonomic categories and its identification. *Uca annulipes* and *Uca urvillei* were characterized on morphological level by CMFRI showing 95% of confidence in the species name. *Uca* spp were then characterized on molecular basis for taxonomical classification and to establish genetic relationship between the members of Brachyuran infra order such as *Scylla serrata*, *Portunus pelagicus*, *Charybdis feraiata*, *Carcinus maenas* and *Ocypode quadrata.*

Extraction of DNA is a crucial step for DNA for DNA Barcoding. In case of the fiddler crab removal of tissue was a challenging task along with that selection of buffer for extraction. In this case CTAB Buffer based extraction was efficient in yielding quality DNA which was quantified using spectrophotometer. Selection of a gene for developing a DNA Barcode is a controversial topic and the best barcode differs from case to case. In the project undertaken four genetic loci namely COI,16S rRNA,18S rRNA, ITS were compared in order to understand the best combination of genetic loci which will act as potential DNA Barcode for *Uca* species. The universal primer was capable of binding and giving amplification for each loci but required degree of optimisation for annealing temperature. The sequence data obtained by sequencing the amplified genetic loci shows higher degree of polymorphism amongst the sequences. The sequence data for COI and 16S rRNA gave a satisfactory result while understanding the genetic diversity of the the fiddler crabs. The phylogenetic analysis for these two barcodes namely COI and 16S conclude that *Uca annulipes*, *Uca urvillei*, *Ocypode quadrata*, *Carcinus maenas*, *Scylla serrata* and *Portunus pelagicus* have evolved from the same ancestor. The observation that the fiddler crabs and *Ocypode quadrata* belong to same cluster in a phylogenetic tree confirms that they are a sister taxon belonging to the same family i.e Ocypodidae at the molecular level. This is also supported by the evident morphological and habitual similarities portrayed by these two crab varieties. The width of a fiddler crab is like that of the ghost crab i.e about 50mm(Fiddler & Uca, 1984). The male ghost crabs have unequal chelipeds (claws) and a similar morphology is evident in fiddler crabs where the males have one larger cheliped (Salmon, 2016). The ghost crabs and the fiddler crabs both display burrowing activity where they create holes and reside in them. Thus, these earlier reports on the resemblance of habitat of the fiddler crabs and the ghost crabs further bolster the reports of this study.

Also, with the help of the phylogenetic analysis the genetic diversity among the Ocypodidae and Portunidae crabs was deduced. The crabs belonging to the Portunidae family can be distinguished easily with the help of morphological and habitat variation from the Ocypodidae family (Cruz, 2014). Ocypodidae is the family of semiterrestrial crabs (Heilemann, 2010) while the Portunidae is the family of the swimming crabs(Rafinesque, 2017) which includes *Portunus pelagicus*, *Charybdis feriata*, *Carcinus maenas* and *Scylla serrata*. The study undertaken in this project serves as a foundation in distinguishing both families at a molecular level. Thus, this study concludes that the species of Ocypodidae and Portunidae are clearly delineated from each other based on phylogenetic information obtained through mt-COI and 16S DNA sequences.

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