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Discrimination of four marine crabs and one freshwater crab through mt-COI gene

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Abstract
This study dealt with DNA barcoding of five species of Brachyuran crabs, of which four marine species (*Portunus sanguinolentus*, *Charybdis natator*, *Portunus pelagicus* and *Portunus trituberculatus*, and one freshwater species, *Travancoriana napaea*, served as an out group. The amplified DNA sequences against the universal primers, LCO1490 and HCO2198 for mt-COI gene revealed ~700 bp in each species, and they showed 89-98% similarity. The phylogenetic information revealed that nucleotide substitutions occurred at different levels than that of nucleotide saturation. All the subjected marine crab species were aligned in one cluster along with retrieved species. And some other retrieved marine crab species were aligned in two separate clusters. *T. napaea* was alone aligned in an independent cluster at the base of the phylogenetic tree. Therefore, these sequences are conserved and less subjected to evolutionary forces and thus these species are genetically distinct, but closely related. Hence, all the subjected crab species have originated from a common ancestor. *T. napaea* was also subjected to phylogenetic divergence with six retrieved freshwater crab species and showed 89% similarity. The phylogenetic information revealed that the retrieved freshwater crab species were aligned in one cluster, and the subjected *T. napaea* was alone sat in a separate cluster as in the previous case. Hence, all the freshwater crab species have originated from a very close and common ancestor.

Keywords: *Portunus sanguinolentus*, *Charybdis natator*, *Portunus pelagicus*, *Portunus trituberculatus*, *Travancoriana napaea*, mt-COI gene, Divergence, Phylogeny

1. Introduction
In India crabs are an important exportable fishery items and hidden resources [1]. Out of about 640 species of marine crabs so far recorded from Indian waters only 15 species are commercial importance, which are inhabit the coastal waters and adjoining brackish water environments, support a fairly good fisheries [2-4]. The Indo-Burma hotspot hosts 182 known species of freshwater crabs in 55 genera belonging to two families, the Gecarcinucidae (45 species, and10 genera) and the Potamidae (136 species, and 45 genera) [5-7]. These crabs are highly endemic, which accounts for 76% of the Gecarcinucid species and 92% of the Potamid species [6, 7]. The taxonomic diversity of crabs inhabiting in marine and freshwater ecosystems have increased considerably [6-17].

Species identification by morphological characters is difficult because of various reasons behind in phenotypical expression, such as genetic variation, sexual dimorphism, geographical variation and mimicry etc. Morphological features are sometime useless and misleading when trying to determine the species and identity of various larval stages. Based on morphology the larval stages of some species groups often cannot be assigned to the correct species [18]. During handling some animals may get damaged so there is a chance for fish fraud. The morphological identification is more complicated when the species were damaged [19]. Morphological identification of crustacean is difficult, time-consuming and very often requires highly trained taxonomists. A technique that combines DNA sequencing and phylogenetic analysis is used to identify samples based on informative nucleotide sequences. The concept of forensically informative nucleotide sequencing (FINS) was first proposed by Bartlett and Davidson [20] to identify the origin of animal food products and has since been extensively applied in forensic investigations. The ideal DNA-based identification system that employed with a single gene and found suitable for any organism in the taxonomic hierarchy are 12S rRNA and 16S rRNA [21], Cyt c and Cyt b [22] and 18S rRNA [23]. Therefore, DNA barcoding was found to be a useful tool for species identification [24, 25]. Morphologically cryptic species

have been increasingly revealed by this technique [26]. Folmer *et al*. [27] designed a universal primer for the mitochondrial cytochrome oxidase subunit I (mt-COI) gene, which subsequently became a popular marker to study invertebrates. Latter, Tautz *et al***.** [26]**,** Hebert *et al*. [28], Blaxter [29], Lefebure *et al*. [30] and Costa *et al*. [25] suggested that the COI gene appeared as an appropriate molecular marker on several taxonomic scales, particularly at the species level. This statement has also been proved by us with crabs, prawns and planktons of freshwater and marine species [31-34].

In this study, DNA barcoding of five species of Brachyuran crabs, including four marine species (*Portunus sanguinolentus*, *Charybdis natator*, *Portunus pelagicus* and *Portunus trituberculatus* inhabiting in the Coromandel coastal region of Tamil Nadu, India, and one freshwater species, *Travancoriana napaea* inhabiting in the Kallar River, Mettupalayam, Tamil Nadu, India, which served as an out group was studied. These crabs were first morphologically identified/ discriminated and then subjected to molecular identification based on mitochondrial cytochrome C oxidase subunit-I gene (mt-COI gene). Molecular analyses, such as sequence similarity, amino acids residues, base composition, sequence divergence, and phylogenetic information like synonymous and non-synonymous substitutions, transitional and transvertional substitutions, and saturations were calculated. Finally the phylogenetic tree was constructed and based on the phylogenetic tree topology the evolutionary significance was analyzed.

2. Materials and Methods

2.1. Sample collection and species identification

Along the small portion of the Coromandel Coastal region of Tamil Nadu, India, the marine crabs, *P. sanguinolentus* and *C. natator* were collected from Rameswaram (9.28º N' 79.30º E') during March, 2015, *P. pelagicus* was collected from Mandabam (9.27º N' 79.12º E') during January, 2015 and *P. trituberculatus* was collected from Kattumavadi (10.20º N' 79.23º E') during June, 2015. One freshwater crab, *T. napaea* was collected from the Kallar River, Mettupalayam (11.30º N' 76.95 º E'), Tamil Nadu, India, during March, 2014, was served as an out group. The crab samples were collected by trawl and hoop net. Triplicate samples of each species were collected from each place depending upon their availability. They were first morphologically identified/ discriminated based on the body color, size, shape, carapace length and width, length of the chelate leg, walking leg, swimming leg, antenna, cervical groove, abdominal segments and lateral spines, and numbers of antero-lateral teeth [35-42] (Figs. 1-5 of Plate 1; Table 1). These crabs species were authenticated by Dr. M. Kathirvel, Former Principal Scientist, Central Institute of Brackish water Aquaculture, ICAR, Chennai, India.

2.1.1. Portunus sanguinolentus (Herbst, 1783) [35] (Fig. 1 of Plate 1)

Common name: Three-spot swimming crab

Colour: Olive to dark green, with 3 prominent maroon to red spot on posterior 1/3 of carapace.

Genera: Carapace broad, much broader than long. Antero-lateral teeth not alternately large and small; last one much larger than the others.....…….. *Portunus.*

Species: No spine on posterior margin of merus of chelipeds. Carapace marked with 3 large blood red Spots..…….. *P. sanguinolentus.*

Descriptions: Carapace finely granulose, region just discernible; 9 teeth on each antero-lateral margin, the last is 2 to 3 times larger than preceding teeth. Chelae elongated in males; larger chela with conical tooth at base of fingers; polex ridged [36].

2.1.2. Charybdis natator (Herbst, 1789) [37] (Fig. 2 of Plate 1).

Common name: Ridged swimming crab.

Colour: Orangish red overall, with ridges on carapace and legs dark reddish brown.

Genera: Antero-lateral margin of carapace divided into 6 teeth, of which at least 5 are large.....…… *Charybdis.*

Species: Carapace with distinct ridges or granular patches behind level of last pair of antero-lateral teeth.…. *C. natator.*

Descriptions: Carapace densely pubescent, granulate in postfrontal area and in the vicinity of the antero-lateral borders; transverse granular lines on protogastric and mesogastric regions, epibranchial line interrupted at the cervical groove and across midline, cardiac region with one, each mesobranchial region with 3 such lines; front with six teeth, medians and sub-medians truncate, laterals triangular with rounded tips; antero-lateral borders with 6 teeth, first truncate, second and third with rounded lateral borders and tips, fourth and fifth acute; postero-lateral junctions rounded. Antennal flagellum excluded from orbit. Chelipeds covered with large granules and/or squamiform markings; merus with 3 strong spines and numerous granules on anterior border, posterior border granulate; carpus with a strong internal spine, outer border with 3 spinules; palm with 4 spines on upper border, lower border longitudinally sulcate. Merus of swimming leg with a sub-distal posterior spine, propodus with a variable number of denticles on posterior border [38].

2.1.3. Portunus pelagicus (Linnaeus, 1758) [39] (Fig. 3 of Plate 1).

Common name: Flower crab.

Colour: Males with blue marking, females dull green/greenish brown.

Genera: Carapace broad, much broader than long. Antero-lateral teeth not alternately large and small; last one much larger than the others..…….. *Portunus.*

Species: Carapace covered with scattered, coarse granules, and meshwork pattern. Front cut into 4 teeth, besides the teeth of dorsal orbital margins.…… *P. pelagicus.*

Descriptions: Carapace rough to granulose, front with 4 acutely triangular teeth; 9 teeth on each antero-lateral margin, the last tooth 2 to 4 times larger than preceding teeth. Chelae elongate in males; larger chela with conical tooth at base of fingers [36].

2.1.4. Portunus trituberculatus (Miers, 1876) [40] (Fig. 4 of Plate 1).

Common name: Horse crab

Colour: Greenish-brown.

Genera: Carapace broad, much broader than long. Antero-lateral teeth not alternately large and small; last one much larger than the others..…….. *Portunus.*

Species: Carapace covered with much smaller granules, without any meshwork pattern. Front cut into 2 teeth, besides the teeth of dorsal orbital margins …….. *P. trituberculatus.*

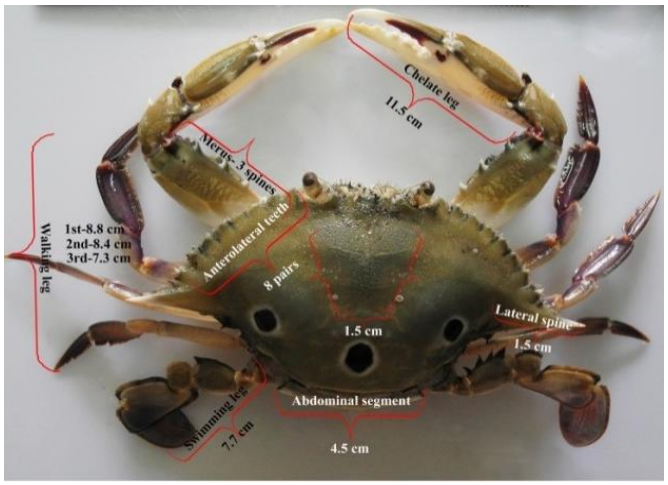

Fig 1: *Portunus sanguinolentus* (three-spot swimming crab).

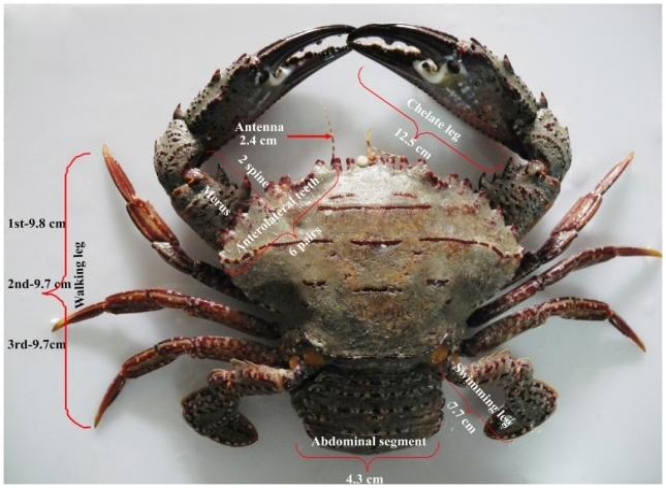

Fig 2: *Charybdis natator* (ridged swimming crab).

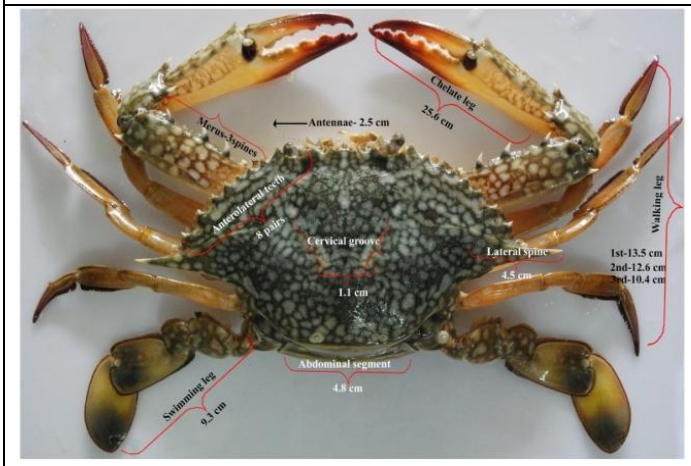

Fig 3: *Portunus pelagicus* (flower crab).

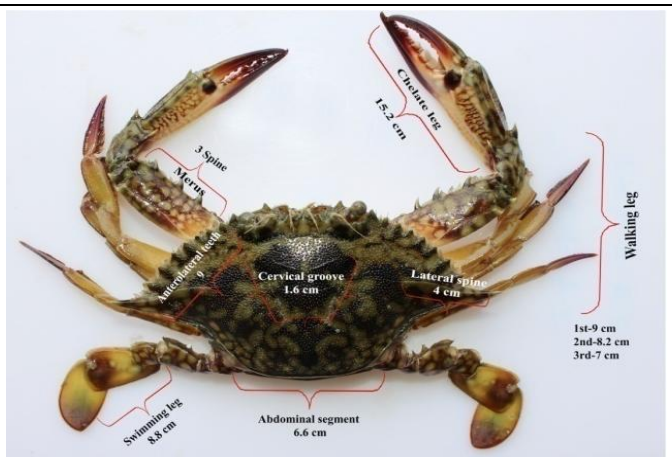

Fig 4: *Portunus trituberculatus* (horse crab).

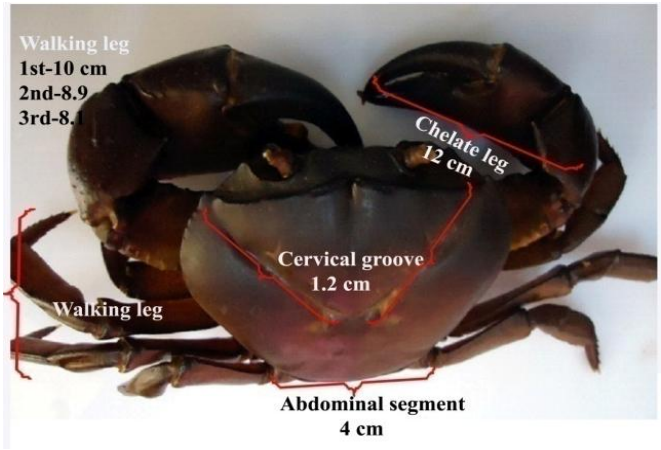

Fig 5: *Travancoriana napaea* (the freshwater crab).

Plate 1: Morphological features of subjected Brachyuran (four marine and a freshwater) crab species.

Descriptions: Carapace very broad (breadth just over 2-2 1/3 times length); surface finely granulated; usually with recognizable mesogastric, epibranchial, and indistinct metagastric ridges, cardiac and mesobranchial ridges with low granular eminences; front with 2 acute teeth; antero-lateral borders with 9 teeth, last one very large and projecting straight out laterally; postero-lateral junction rounded. Cheliped merus with postero-distal border spinous, anterior border with 3-4 (usually 4) sharp spines; carpus with inner and outer spines; lower surface of palm smooth. Posterior border of swimming leg without spines or spinules [38].

2.1.5. Travancoriana napaea (Alcock, 1909) [41] (Fig. 5 of Plate 1).
Common name: -Nil-
Colour: Brownish red.
Descriptions: The groove is generally shallow, covers the entire lateral side, and only has the ventral margin broadened

Table 1: Morphometric characteristics of four subjected marine and a freshwater crab species

Characters	Marine crabs				Freshwater crab
	P. sanguinolentus	*C. natator*	*P. pelagicus*	*P. trituberculatus*	*T. napaea*
Colour	Olive to dark green	Orange red	males with blue marking, females dull green/ greenish brown	Greenish brown	Brownish red
Shape	Carapace very broad with 3 red spots in posterior half	Carapace densely pubescent, granulate in postfrontal area	Carapace very broad, surface coarsely granulated	Carapace very broad, covered with fine granules	Carapace hard
Weight (g)	74.0	78.0	120.0	98.0	70.0
Length (cm)	17.0	11.0	15.5	12.2	10.0
I-Antenna (cm)	1.5	2.4	2.3	1.6	--
II-Antenna (cm)	1.5	1.5	1.4	1.5	--
Cervical groove (cm)	1.5	--	1.0	1.6	1.2
Anterolateral teeth (Nos.)	8.0	6.0	8.0	9.0	--
Lateral spine (cm)	1.5	--	4.5	4.0	--
Chelated leg (cm)	11.5	12.5	20.6	15.2	12.0
Walking leg -1 (cm)	8.8	9.8	13.5	9.0	10.0
Walking leg -2 (cm)	8.4	9.7	12.6	8.2	8.9
Walking leg -3 (cm)	7.3	9.7	10.4	7.0	8.1
Swimming leg (cm)	7.0	7.7	9.3	8.8	--
Abdominal segment (cm)	4.1	4.3	4.5	6.2	4.0

Whereas, the dorsal margin remains smooth and indistinct. Its ventral margin and the gonopod itself forming a continuous, leaf like distal part (*Travancoriana* sp.) [42]. This species is known only from its type locality in India (1909). Data pertaining to its extent of occurrence, ecological requirements, population size, population trends, and long-term threats are not available. Bott [43] included this species in the subfamily Liotelphusinae of the Gecarcinucidae. According to Cumberlidge [44] *T. napaea* is placed in the IUCN Red List of Threatened Species.

2.2. Molecular analysis

Genomic DNA was isolated from the adductor muscle by using Qiagen Dneasy Blood and Tissue Kit (Germany) and 1% Agarose Gel Electrophoresis was performed to resolve the genomic DNA, which was detected under a Gel documentation system (Medicare, India). DNA amplification of mt-COI gene was carried out with universal primers, LCO1490 and HCO2198 [27] of forward and reverse in nature respectively using ABI Thermo Cycler. These primer sets were worked well for crabs and prawns and other crustaceans [31-34]. Amplification was performed in a total volume of 50 µl containing 4 µl of DNA template, 20 p.mol of each primer (forward primer, 0.30 µl; reverse primer, 0.30 µl), 36 µl of 2X polymerase chain reaction (PCR) master mix (MBI Fermantas) containing 0.05 units/ µl *Taq* DNA polymerase in reaction buffer, 4 mM $MgCl_2$, 0.4 mM dATP, 0.4 mM dGTP, 0.4 mM dTTP and 0.4 mM dCTP, and 9.4 µl of DNase - RNase free water. The thermo cycler condition was as follows: pre-running for 5 min at 95 °C; denaturation of 35 cycles of 30 s each at 95 °C; annealing for 45 s at 57 °C; extension for 90 s at 72 °C; final extension for 10 min at 72 °C. The amplified product was resolved with 2% Agarose Gel Electrophoresis. Sequencing was performed with a total volume of 20 µl reaction mixture containing 3 µl of Template DNA, 3.2 pM/ µl of primers (forward, 0.50 µl and reverse, 0.50 µl), 2 µl of 5X BigDye sequencing buffer and 4 µl of 2.5X Ready Reaction Premix (Tris-HCL, pH 9.0 and $MgCl_2$) and 10 µl of DNase - RNase free water. The PCR Sequencing cycling condition was as follows, 30 cycles of 20 s each at 95 °C for denaturation, followed by 30 cycles of 20 s each at 50 ºC for annealing and 30 cycles of 4 min each at 60 ºC for extension. After completion of the PCR program, the sample was processed for ethanolic precipitation. From the PCR tubes, the samples were transferred to 96 well microlitre plates and 5 µl of 125 mM EDTA was added to each well. 60 µl of ice cold 100% ethanol (stored at -20 ºC) was added to each reaction, the plate was sealed and mixed by vortexing for 20-30 seconds and incubated at room temperature for 15 minutes. The sample plate was spined at 3,000 × g for 30 min at 4ºC. The supernatant was carefully removed by inverting the plate and spined up to 180 × g for 1 min then removed from the centrifuge. The pellet was rinsed once with 60 µl of ice cold 70% ethanol (stored at -20 ºC) by centrifugation at 1650 × g for 15 min at 4 ºC. Again the plate was inverted and spined up to 180 × g for 1 minute, and then removed from the centrifuge. The sample was re-suspended in 10 µl of Hi-Di formamide and incubated for 15 min at room temperature. The re-suspended samples were transferred to the appropriate wells of the sample plate. Ensured each sample was positioned at the bottom of its tube or well. The samples were denatured at 95ºC for 5 min with snap chill and the plate was loaded into Sequencer, after completion of run the data was analyzed (ABI 3500 XL Genetic Analyzer, Chromous Biotech, Bangalore, India).

The forward and reverse sequences were aligned pair wise by using CAP3. BLAST was performed for sequence similarity identification and removal of internal stop codons. The

reading frame shift was deducted by open reading frame (ORF) finder. The trimmed sequence was authenticated with GenBank. The multiple sequence alignment (MSA) was done by using T-Coffee and the aligned sequence was highlighted with multiple align show (MAS) as identical, similar and variable sites of amino acids. The nucleotide composition (AT and GC biases), nucleotide divergence (K2P model [45]) and some phylogenetic information were calculated by using MEGA v.6.01.Assessment of species-wise synonymous (Ks) and non-synonymous (Ka) substitutions for 3rd codon positions were calculated by using Li93 method of DAMBE [46]. Similarly, the overall site-wise inferred synonymous (dS) and inferred non-synonymous (dN) substitutions for 3rd codon positions were predicted by using Muse-Gaut model [47]. The transitional (Ts) and transvertional (Tv) substitutions were determined by using Felsenstein model [48]. Analysis of sequence saturation, index of substitutional saturation (Iss) and critical value of index of substitutional saturation (Iss.c) was done by using Xia method of DAMBE [49]. Finally the phylogenetic tree was reconstructed by Maximum Likelihood model [50].

3. Results and Discussion

The isolated genomic DNA was measured greater than 10 kb nucleotides (Fig. 1) in each sample, and the PCR amplified products showed ~700 bp DNA each (Fig. 2). Actually, the sequence analyses showed 682 bp, 695 bp, 637 bp, 675 bp and 414 bp for *P. sanguinolentus*, *C. natator*, *P. pelagicus* and *P. trituberculatus*, and *T. napaea* respectively.

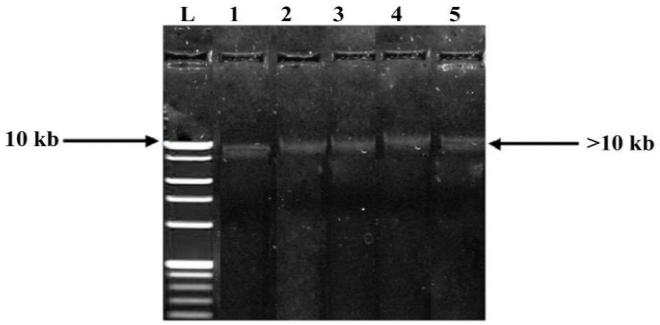

Fig 1: AGE (1%) of four marine and a freshwater crab species genomic DNA.
L, Ladder (10 kb); **1,** *P. sanguinolentus*; **2,** *C. natator*; **3**, *P. pelagicus*; **4,** *P. trituberculatus*; **5,** *T. Napaea*

The BLAST result revealed that the similarity of data available in the NCBI database against each sequence generated was identified as 97% for *P. sanguinolentus* with *P. sanguinolentus* reported from China, 97% for *C. natator* with *Charybdis variegate* reported from Philippines, 98% for *P. pelagicus* with *P. pelagicus* reported from China, 97% for *P. trituberculatus* with *P. pelagicus* reported from China, and 89% for the freshwater crab *T. napaea* with the freshwater crab, *Barytelphusa cunicularis* reported from Belgium (Table 2).The sequences generated in this study have been authenticated with the GenBank accession numbers (Table 3).

The results of MSA with MAS for identification of identical, similar and variable sites of amino acids are shown in Plate 2. On the whole the sequences of marine crabs with the out group showed 135 identical amino acids residues, 42 similar amino acids residues and 533 variable amino acids sites (Table 4; Fig. 1 of Plate 2).

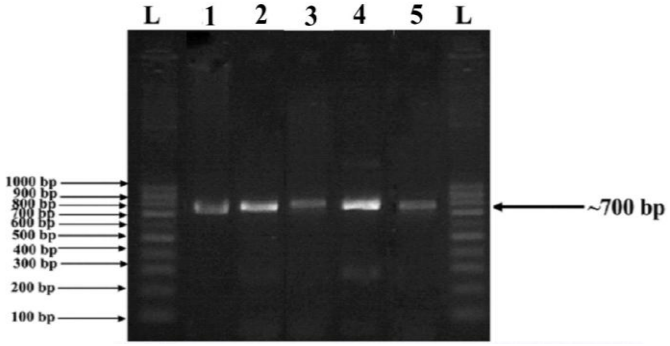

Fig 2: AGE (2%) of PCR amplified DNA product of four marine crabs and a freshwater crab species.
L, Ladder (100 bp); **1,** *P. sanguinolentus*; **2,** *C. natator*; **3**, *P. pelagicus*; **4,** *P. trituberculatus*; **5,** *T. napaea*

The sequences of marine crabs without the outgroup showed 414 identical amino acids residues, 19 similar amino acids residues and 264 variable amino acids sites (Table 4; Fig. 2 of Plate 2). These data revealed with more numbers of identical amino acids when the out group was not included and the reverses were seen in numbers of similar amino acid residues and variable amino acid sites. It indicates discrimination of marine crabs from the freshwater crab. The MAS for *T. napaea* with six retrieved freshwater crab species showed 152 identical amino acids, 43 similar amino acid residues and 525 variable amino acid sites. This data revealed that more number of variables sites than that of the identical and similar amino acid residues, which indicate more discrimination between the freshwater crabs (Table 4; Fig. 3 of Plate 2).

In this study, the base compositions of the COI gene fragment varied among the species, AT biases were ranged between 58.7-63.3% (*P. sanguinolentus* and *C. natator* respectively) and the GC bias were ranged between 36.7-41.3% (*C. natator* and *P. sanguinolentus* respectively). The marine crabs with the out group showed 60.76% of AT bias (A=26.38; T=34.4) and 39.04% of GC bias (G=18.18; C=21.06). Similarly, the marine crabs without the out group showed 61.15% of AT bias (A=24.7; T=36.4) and 38.85% of GC bias (G=17.52; C=21.32). These data revealed that the AT biases were higher than that of the GC biases in all subjected species, overall, which was more than 60% (Table 5). Moreover, the AT biases of marine crabs without the out group showed little higher value than that of with the out group. The higher AT bias recorded indicates the lower abundance of nuclear copies of mt-DNA (NUMTs) genes known as pseudogenes, homologs or paralogs. The higher AT bias has also been reported in a freshwater ostracod, *Eucypris virens* (AT=60.4% (A=27.4; T=33.0); GC=39.6 (G=17.3; C=22.3) [51].

The base composition of the subjected *T. napaea* and retrieved freshwater crabs showed AT biases ranged between 59.2–69.7% (*T. napaea* and *S. hydrodroma* respectively) and GC biases of 21.1-40.8% (*B. jacquemontii* and *T. napaea* respectively) with an average value of 64.8% AT biases and 35.3% GC biases (Table 6). Similar type of information has also been studied by us in crabs, prawns and zooplanktons [31-34].

3.1. Interspecies divergence

The divergence rate of different subjected marine crabs with the out group, *T. napaea* was ranged between 0.64–0.86 (*P. pelagicus* vs. *T. napaea*, and *C. natator* vs. *T. napaea* respectively) (Table 7; Fig. 1 of Plate 3).The inter species divergence rate calculated between different subjected marine crab species was ranged between 1.49-4.03 (*P. sanguinolentus*

vs. *C. natator*, and *P. pelagicus* vs. *P. trituberculatus* respectively) (Table 8; Fig 2 of Plate 3). The divergence rate of *P. sanguinolentus* with other marine crabs was ranged between 1.49–1.79 (*P. sanguinolentus* vs. *P. pelagicus*, and *P. sanguinolentus* vs. *P. trituberculatus* respectively). The divergence rate of *C. natator* was ranged between 1.56-2.07 (*C. natator* vs. *P. sanguinolentus*, and *C. natator* vs. *P. trituberculatus* respectively) and the divergence rate of *P. pelagicus* was ranged between 1.49-4.03 (*P. pelagicus* vs. *P. sanguinolentus*, and *P. pelagicus* vs. *P. trituberculatus* respectively (Table 8; Fig. 2 of Plate 3).

The inter species divergence rate between different marine crabs without the out group was higher than that of with the out group. This may be due to the fact that freshwater species have originated from marine recently. The genetic distances of the mt-COI gene sequence have been reported for various animal taxa mainly from Vertebrata and Arthropoda, and the general ranges for the intra and inter species distances were 0.0001-0.05 and 0.04-0.21 respectively [52]. The interspecies divergence rates of 1.4% (in the genus *Alpheus*) and 2.6% (in

Table 2: BLAST identification of COI gene sequences of four subjected marine and a freshwater crab species for similarity data available with the NCBI database

Queried sequences	Collection site & Country	Identity (%)	Gap (%)	Matched Strand	Matched Organism	Reference Country
P. sanguinolentus	Rameshwaram, India	97	2	Plus	*P. sanguinolentus*	China
C. natator	Rameshwaram, India	97	2	Plus	*C. variegata*	Philippines
P.pelagicus	Mandapam, India	98	1	Plus	*P. pelagicus*	China
P.trituberculatus	Kattumavadi, India	97	2	Plus	*P. pelagicus*	China
T. napaea	Mettupalayam, India	89	0	Minus	*B. cunicularis*	Belgium

***P. sanguinolentus* (682 bp, KR781515)**

GGTCAACAAAACATAAAGATATTGGTACATTATATTTCATTTTTGGAGCATCAGGGATAGTAGGAACCTCACTAAGTCT
GATCATTCGAGCTGAGTTAGGGCAGCCAGGAACTCTTATTGGTAACGATCAAATTTATAACGTTGTTGTCACCGCTCAT
GCCTTTGTTATAATTTTCTTTATAGTTATACCAATTATAATTGGTGGATTTGGTAACCTTGTACCCTTAATGCTTGGGGCC
CCTGATATAGCATTCCCTCGTATAAATAATATAAGATTTCTCCTTCCTCCCTCACTCACCCTCCTTCTTATAAGAGGTATG
GTTGAAAGTGGTGTAGGTACTGGTACTGTCTACCCTCCTCTTGCTGCCGCTATTGCCCACGCTGGAGCTTCAGTCGATTT
GGGGATTTTCTCCTTGCATTTAGCTGGTGTATCCTCTATTCTAGGTGCCGTAAACTTTATAACTACTGTAATTAACATACG
ATCCTTCGGCATGAGAATGGACCAGATACCGTTATTTGTGTCAGTCTTCATTACCGCTATTCTGCTCCTTCTCTCCCTACC
TGTTCTTGCAGGAGCTATTACTATGCTTTTAACAGATCGTAACCTCAACACCTCCTTCTTTGATCCTGCAGGTGGTGGAGA
CCCTGTTTTATATCAACACTTATTCTTTTTTGGTCACCC

***C. natator* (695 bp, KR781514)**

GGTCAACAAAACATAAAGATATTGGTACATTATATTTTATTTTTGGAGCTTCAGGAATAGTTGGAACTTCATTAAGATTGA
TTATTCGAGCCGAGCTCGGTCAGCCTGGTACTTTAATCGGCAACGATCAAATTTATAATGTTGTAGTTACAGCCCACGCAT
TCATTATAATTTTCGTTATAGTTATACCAATTATAATTGGAGGATTTGGTAATCTTGTTCCCCTAATATTAGGGGCTCCTGA
TATAGCATTTCCCCGTATAAACAACATAAGATTTCTTCTTCCTCCTTCCTTAACCTTGTTATTGATAAGAGGAATAGTTGAA
AGAGGAGTAGGAACAGGTACTGTATACCCTCCTTTATCGGCCGCTATTGCCCATGCAGGTGCCTCAGTAGATTTAGGAATT
TTTTCACTTCACTTAGCAGGTGTCTCCTCCATCTTAGGAGCCGTTAATTTTATAACCACCGTTATTAATATACGTTCCTTTGG
TATAAGTATAGACCAAATACCTTTATTTGTATGGTCAGTCTTTATTACTGCAATTCTTTTACTCCTCTCCCTCCCTGTTTTAG
CAGGAGCTATTACTATGTTATTAACAGACCGAAACTTAAATACCTCATTTTTTGATCCTGCTGGAGGAGGAGATCCTGTCC
TTTATCAACATTTATTTTTTTTTGGTCACCTGGGAAGTTTA

***P. pelagicus* (637 bp, KT158620)**

GGTCAACAAATCATAAAGATATTGGTACATTATATTTTATTTTTGGAGCATGATCAGGAAGGACTTCACTTAGTCTTATTCG
AGCAGAACGACAACCTGGCACTCTTATTGGCAATGATCAAATTTACAACGTTGTTACAGCTCATGCTTTTGTTTTCTTTATTA
TACCAATTATTGGAGGATTTGGTAACTGACTTCCTTTGCGGGCCCCTGATATGGCTTTTCCCCGTAACAACAGATTTTGACTT
CTCCCTCCTTCTTCTCTACTTCTTAGAGGTATGGCAAAAAGAGGTGTTGGTACGGGCTGAACCGTATACCCTCCTCTTTCGGC
AGCGATCGCTCATGCAGGAGCTTCTGATCGTATTTTCTCTTTACATCCAGGTGTTTCCTCTATTTGTGCAGATTTCACCACCGT
TATTAATATGCGATCTTTTGGGAGAAACCAAATACCATTATTCGTTTGATCAGTATTTATCACTGCTATTCTTCTCCTCTTATC
TCTCCCTGTTCTTGCTGAAGCTATTACTATACTTCTTACAGATCGAAATCTCAATACTTCGATCTTTGACCCTGCCGGGGGTGG
TGACCCTGTACTCTACCAACGCTTATTTTGATTTTTTAGTCACCCTGCAGTTTA

***P. trituberculatus* (675 bp, KR781516)**

AAAGATATTGGTACATTATATTTTATTTTTGGAGCATCAGGAATAGTAGGGACTTCTCTTAGTCTTATTATTCGAGCAGAACTAGGTC
AACCTGGTACTCTTATTGGTAATGACCAAATTTACAATGTTGTAGTTACAGCTCATGCTTTTGTAATAATTTTCTTTATAGTTATACCA
ATTATAATTGGGGGATTTGGTAACCTAGTACCATTAATGTTAGGAGCCCCTGACATGGCTTTTCCTCGTATAAACAACATAAGATTTC
TTCTCCCTCCTTCTCTAACTTTACTTCTTATAAGAGGTATAGTGGAAAGAGGTGTTGGTACAGGTACCGTCTATCCTCCTCTTTCAGCA
GCCATCGCTCACGCAGGAGCTTCTGTAGATCTAGGTATTTTCTCTTTACATCTGGCAGGTGTTTCCTCTATTCTAGGTGCAGTAAATTT
CATGACCACCGTTATTAACATGCGATCTTTTGGTATAAGAATGGACCAAATGCCATTATTCGTTTCAGTATTTATCACTGCTATTCTTC
TACTTTTATCTCTCCCTGTTCTTGCTGGAGCCATTACTATACTTCTTACAGACCGAAATCTAAATACTTCATTCTTTGACCCTGCCGGA
GGTGGTGACCCTGTACTCTACCAACACTTATTCTTTTTTGGTCACCCTGGAAGT

***T. napaea* (414 bp, KT290872)**

AGTGATAGCCCCGGCAAATACTGGAAGAGAAAGTAAAAGTATAACTACCGTAGTAAATATTGCTCAAACTAATAAAGG
TATTTGGTCTATAATTGTACCAAAAGAGCGTATATTAATTACCGTGGTTATAAAATTTACGGGACCGAGAATTGAAGAT
ACCCCAGCATAATGGAGTGAAAAAATTCCCATCTCCACTGAAGCTCCGGCTTGGGCGATGACGGCTGCTAATGGAGGAT
AAACCGTTCACCCCGGCCCCACCCCTCTTTCTACTATTCCTCTCATAAGAAGTGAGGTTAATGAACGAGGTAAAAGTCAG
AATCTTATATTGTTTATTCTTGGGAAGGCTATATGCCGGGCTTCTAATATAAACGGAAGGAGTCAATCACCAAATCCCCC
AATTATTAGGGGTATTAC

Table 3: GenBank accession numbers of the COI gene sequences generated for four subjected marine and a freshwater crab species, and retrieved species from the NCBI database

Species Name	Country	Author	Accession number
Portunus sanguinolentus	**India**	**Paper authors 2015**	**KR781515**
Portunus sanguinolentus	Sri Lanka	Senevirathna, 2015	KM528129
Portunus sanguinolentus	China	Xing, 2007	EU284144
Portunus sanguinolentus	South Korea	Kim, 2012	JX502943
Portunus sanguinolentus	India	Narasimmalu, 2014	KC760160
Portunus sanguinolentus	Viet Nam	Bui, 2009	AM410510
Charybdis natator	**India**	**Paper authors 2015**	**KR781514**
Charybdis natator	Sri Lanka	Senevirathna *et al.* 2015	KM528125
Charybdis natator	India	Jijith *et al*., 2014	KF736897
Charybdis natator	Moscow	Spiridonov *et al*., 2013	JX398102
Charybdis natator	New Zealand	Smith *et al*. 2003	AY351873
Portunus pelagicus	**India**	**Paper authors 2015**	**KT158620**
Portunus pelagicus	Sri Lanka	Senevirathna *et al*., 2015	KM528127
Portunus pelagicus	Philippines	Yambot *et al*., 2014	KF604896
Portunus pelagicus	India	Seshendra *et al*., 2015	KP666120
Portunus pelagicus	China	Chu, 1998	AF082732
Portunus pelagicus	Singapore	Lai, 2008	EF661947
Portunus pelagicus	Moscow	Spiridonov, 2013	JX398106
Portunus pelagicus	Australia	Teske, 2009	FJ812293
Portunus pelagicus	UK	Costa, 2012	DQ889124
Portunus pelagicus	Viet Nam	Bui, 2009	AM410517
Portunus trituberculatus	**India**	**Paper authors 2015**	**KR781516**
Portunus trituberculatus	China	Liu, 2010	GU321230
Portunus trituberculatus	Philippines	Yambot, 2014	KF604899
Portunus trituberculatus	South Korea	Kim, 2012	JX502944
Portunus trituberculatus	Egypt	Galal-Khallaf, 2015	LN624573
Portunus trituberculatus	Viet Nam	Bui, 2009	AM410516
Trvancoriana napaea	**India**	**Paper authors 2015**	**KT290872**
Travancoriana sp.	Belgium	Beenaerts *et al*., 2009	GQ289636
Travancoriana schirnerae	Belgium	Beenaerts *et al*., 2009	GQ289635
Barytelphusa cunicularis	India	Vartak *et al*., 2013	KC928397
Barytelphusa jacquemontii	India	Bhavan *et al*., 2014	KJ684063
Spiralothelphusa hydrodroma	India	Bhavan *et al*., 2014	KJ652336
Geothelphusa monticola	Taiwan	Shy *et al*., 2009	AB535481

Table 4: Number of identical, similar and variable amino acids in four subjected marine crabs, and a subjected and retrieved freshwater crabs

Crab species	No. of identical amino acid residues	No. of similar amino acid residues	Variable amino acid sites
Marine crabs with the out group	135	42	533
Marine crabs without the out group	414	19	263
Freshwater crabs alone	152	43	525

Table 5: Nucleotide composition of four subjected marine and a freshwater crab species

Crab species	A	T	AT bias (%)	G	C	GC bias (%)
P. sanguinolentus	23.3	35.3	58.7	18.3	23.0	41.3
C. natator	26.8	36.5	63.3	17.3	19.4	36.7
P. pelagicus	23.8	36.9	60.8	17.2	22.0	39.2
P. trituberculatus	24.9	36.9	61.8	17.3	20.9	38.2
T. napaea	33.1	26.1	59.2	20.8	20.0	40.8
Marine crabs with the out group	26.3	34.4	60.7	18.1	21.0	39.0
Marine crabs without the out group	24.7	36.4	61.1	17.5	21.3	38.8

the order Euphausiacea) per million years in crustaceans have been reported for mt-COI gene [53, 54]. Silva *et al*. [55] have reported that the divergent rate of 0.0-4.6% within species, and 2.5-32.7% within different genera. Vartak *et al.* [17] reported lowest divergent rates between the different marine crab species, the divergent rate of *Menippe rumphii* was 0.08 and 0.18% against *Myomenippe hardwickii* and *Atergattis integerimus* respectively. Low mean intra species divergence rates of 0.3% have been reported between the two species of *P. pelagicus* recently [56].

The subjected *T. napaea* with other retrieved freshwater crabs showed interspecies divergence values between 1.059 - 1.780 (*T. napaea* vs. *B. cunicularis*, and, *T. napaea* vs. *G. monticola* respectively) (Table 9; Fig. 3 of Plate 3). The divergent rate of mangrove crab, *Neosarmatium asiaticum* was 0.497% and it was found to be overlap against freshwater crabs, *S. hydrodroma* and *B. jacquemontii* [33]. According to Hebert *et al*. [28] genetically distinct species with low divergence (0.6-2%) suggests their recent origin. Therefore, in the present study the very low level of divergence (1.059-1.780) recorded suggests that the freshwater crabs might have originated very recently. Similar type of information has also been studied by us in crabs, prawns and zooplanktons [31-34].

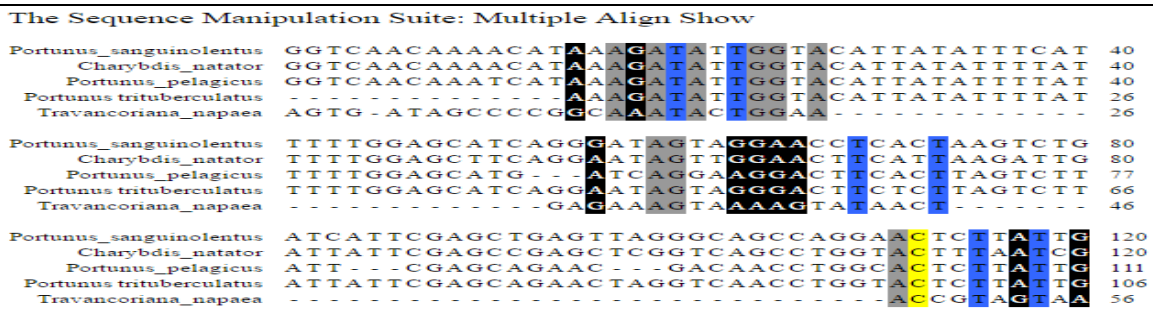

Fig 1: Multiple sequence alignment of COI gene sequences generated in four subjected marine crabs with the out group. An alignment is formatted by using multiple align show (MAS) with coloured background and a consensus setting of 100%. Identical residues are indicated by amino acid colour and similar residues are black in colour. Gaps and other residues are given in white background.

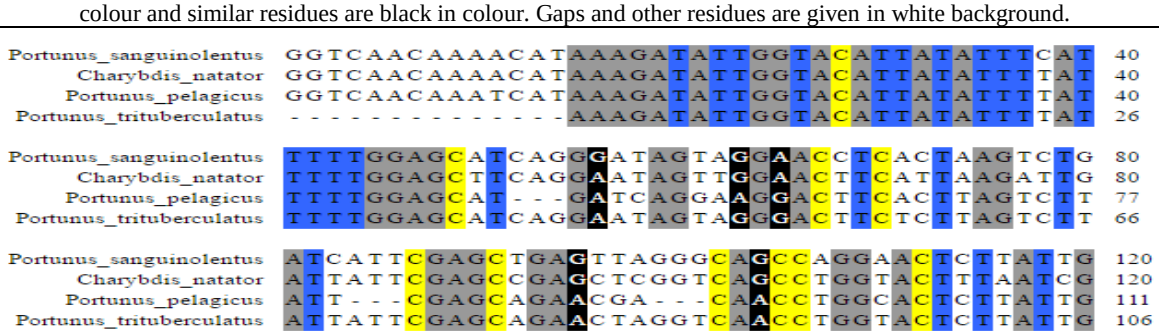

Fig 2: Multiple sequence alignment of COI gene sequences generated in four subjected marine crabs without the out group. An alignment is formatted by using multiple align show (MAS) with coloured background and a consensus setting of 100%. Identical residues are indicated by amino acid colour and similar residues are black in colour. Gaps and other residues are given in white background.

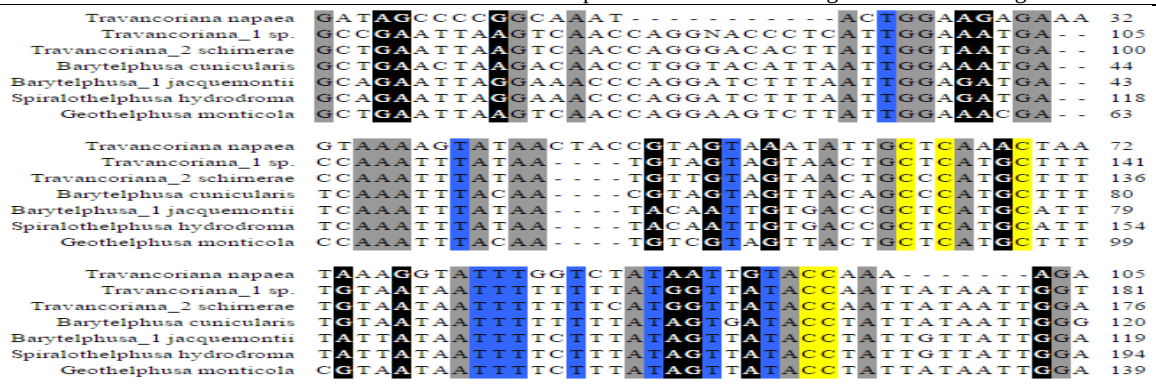

Fig 3: Multiple sequence alignment of COI gene sequences generated for a subjected freshwater crab, *T. napaea* and six retrieved freshwater crab species. An alignment is formatted by using multiple align show (MAS) with coloured background and a consensus setting of 100%. Identical residues are indicated by amino acid colour and similar residues are black in colour. Gaps and other residues are given in white background.

Plate 2: Number of amino acid residues in marine and freshwater crab species.

Table 6: Nucleotide composition of a subjected (*T. napaea*) and six retrieved freshwater crab species

Crab species	A	T	AT bias (%)	G	C	GC bias (%)
T. napaea	33.1	26.1	59.2	20.8	20.0	40.8
Travancoriana sp.	26.5	35.8	62.3	17.0	20.8	37.8
T. schirnerae	26.5	37.0	63.5	17.4	19.0	36.4
B. cunicularis	28.6	35.3	63.9	15.9	20.3	36.2
B. jacquemontii	29.6	39.3	68.9	15.2	15.9	21.1
S. hydrodroma	30.8	38.9	69.7	15.2	15.1	30.3
G. monticola	28.4	35.2	63.6	15.7	20.6	36.3
Average	28.9	35.9	64.8	16.6	18.7	35.3

3.2. Phylogenetic information

The predicted phylogenetic information without and with the retrieved species of marine and freshwater crabs, such as species-wise synonymous (Ks) and species-wise non-synonymous (Ka) substitutions, overall inferred synonymous(dS) and overall inferred non-synonymous (dN) substitutions, transitional (Ts) and transvertional (Tv) substitutions, and saturation, index of substitutional saturation (Iss) and critical value of the index of substitutional saturation (Iss.c) are presented in Table 10; Plates 4-9. When the subjected marine crabs (without retrieved species) compared with the out group, *T. napaea*, the following values in the phylogenetic information were observed.

Table 7: Inter species divergence of four subjected marine crabs with the out group, the freshwater crab, *T. napaea*

Between species	Divergence (%)
P. sanguinolentus vs. *T. napaea*	0.75
C. natator vs. *T. napaea*	0.86
P. pelagicus vs. *T. napaea*	0.64
P. trituberculatus vs. *T. napaea*	0.67

Table 8: Inter species divergence within four subjected marine crab species without the out group

Between species	Divergence (%)
P. sanguinolentus vs. *C. natator*	1.56
P. sanguinolentus vs. *P. pelagicus*	1.49
P. sanguinolentus vs. *P. trituberculatus*	1.79
C. natator vs. *P. pelagicus*	1.93
C. natator vs. *P. trituberculatus*	2.07
P. pelagicus vs. *P. trituberculatus*	4.03

Table 9: Inter species divergence between a subjected (*T. napaea*) and six retrieved freshwater crabs

Between species	Divergence (%)
T. napaea vs. *Travancoriana sp.*	1.429
T. napaea vs. *T. schirnerae*	1.277
T. napaea vs. *B. cunicularis*	1.059
T. napaea vs. *B. jacquemontii*	1.337
T. napaea vs. *S. hydrodroma*	1.337
T. napaea vs. *G. monticola*	1.780

The Ks (occurrence of silent mutation, i.e. no change in the amino acid sequences of a polypeptide) was 0.147, and the Ka was 0.455 (occurrence of deleterious mutation), which indicates occurrence of more deleterious mutation and less silent mutation (Table 10; Fig. 1 of Plate 4). The same trend was found in dS and dN value, the dS was 92.051 (39 sites) and dN was 107.232 (62 sites), which confirm the fact that the deleterious mutation was greater than that of the silent mutation. The dN-dS showed more positive signed values (represents inferred non-synonymous) than that of the negative signed values (represents inferred synonymous) (Table 10; Fig. 1 of Plate 5). The Ts (replacement of one nitrogenous base by another type of the same group of nucleotide) was 0.22 and the Tv (replacement of one type of nitrogenous base by another group) was 0.33.Therefore, there were occurrences of more transversional substitution and thus more phylogenetic information were there (Table 10; Fig. 1 of Plate 6). This indicates the fact that the sequences are not highly conserved. The value of Iss.c was more (0.787) than that of the value of Iss (0.565), which indicates the fact that there was no substitutional saturation occurred in these sequences.

When the subjected marine crabs (without retrieved species) compared without the out group, the following values in the phylogenetic information were observed. The recorded Ks and Ka values were 0.000 and 0.380 respectively, which indicates occurrence of more deleterious mutation and less silent mutation (Table 10; Fig. 2 of Plate 4). The same trend was found in dS and dN value, the dS was 42.43(24 sites) and dN was 74.14 (146 sites), which indicates the fact that there was less deleterious mutation and more silent mutation. The dN-dS showed more positive sign (represents more inferred non-synonymous) than that of the negative sign (Table 10; Fig. 2 of Plate 5). The Ts and Tv was 0.12 and 0.07 respectively, which confirmed the occurrences of less transversional substitution and thus less phylogenetic information (Table 10; Fig. 2 of Plate 6), which in turn indicates the fact that the sequences are more conserved. However, the value of Iss.c was more (0.807) than that of the value of Iss (0.295), which indicates the fact that there was no substitutional saturation occurred in these sequences, therefore it could be subjected to evolutionary forces.

The above assessed less occurrence of sequence saturation without the retrieved species turned towards occurrence more saturation when retrieved species were included (Table 10; Plates 8 and 9). Therefore discrimination was wider, which showed occurrence of more inferred non-synonymous substitution with more negative values of dN-dS (524.703 and 452.258 for with the out group and without the out group respectively) (Table 10; Figs. 1 and 2 of Plate 8) and more transversional substitution (0.46 and 0.46 for with the out group and without the out group respectively) (Table 10; Figs. 1 and 2 of Plate 9) and thus substitutional saturation occurred (Iss of 1.010 and 0.953 for with the out group and without the out group respectively). Moreover, the sequence saturation was more when the out group was included and thus more discrimination was resulted. However, species-wise, only more silent mutations were evident (Table 10; Plates 7-9).

The subjected freshwater crab, *T. napaea* along with retrieved freshwater crabs showed more species-wise silent mutation and less deleterious mutation when one to one comparison alone was considered (Ks=0.756 and Ka=0.733) and as well as overall comparison were done including between retrieved species (Ks=1.640 and Ka= 0.068) (Table 10; Fig. 3 of Plates 4 and 6). The overall inferred non-synonymous substitution (dN-dS showed more positive values) and transversional substitution were occurred more in both cases of comparison (94.2 (111 sites) and 0.15 respectively) (Table 10; Fig. 3 of Plates 5 and 8; Fig. 3 of Plates 6 and 8).This indicates the fact that these sequences are not highly conserved.

Table 10: Overall average phylogenetic information of four marine and a freshwater crabs

Phylogenetic information		Ks	Ka	dS	dN	Ts	Tv	Iss	Iss.c
Without the retrieved species	Marine crabs with the out group	0.147	0.455	92.05(62)	107.23(39)	0.22	0.33	0.565	0.787
	Marine crabs without the out group	0.000	0.380	42.43(24)	74.14(146)	0.12	0.07	0.295	0.807
With the retrieved species	Marine crabs with the out group	2.348	0.559	524.70(77)	284.41(13)	0.32	0.46	1.010	0.741
	Marine crabs without the out group	2.494	0.566	452.25(87)	153.05(7)	0.38	0.46	0.953	0.744
T. napaea Vs. Six retrieved freshwater crabs (one to one comparison alone, subjected Vs. retrieved)		0.756	0.733	80.91(33)	94.20(111)	0.10	0.15	0.416	0.775
T. napaea Vs. Six retrieved freshwater crabs (over all comparison, subjected Vs. retrieved, and retrieved Vs. retrieved)		1.640	0.068	80.91(33)	94.20(111)	0.10	0.15	0.416	0.775

Ks, Synonymous substitution; **Ka**, Non-synonymous substitution; **dS**, Inferred synonymous substitutions; **dN**, Inferred non-synonymous substitutions; **Ts**, Transitional substitution; **Tv**, Transversional substitution; **Iss**, Index of substitution saturation; **Iss.c**, Critical value of index of substitution saturation. Values in parenthesis indicates No. of sites.

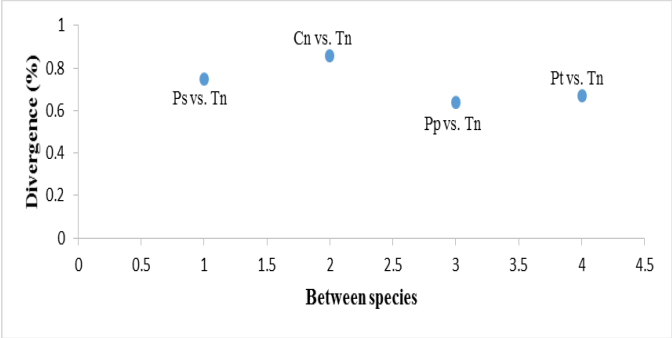

Fig 1: Inter species divergence of four subjected marine crabs with the out group, *T. napaea*. Ps, *P. sanguinolentus*; Cn, *C. natator*; Pp, *P. pelagicus*; Pt, *P. trituberculatus*; Tn, *T. Napaea*

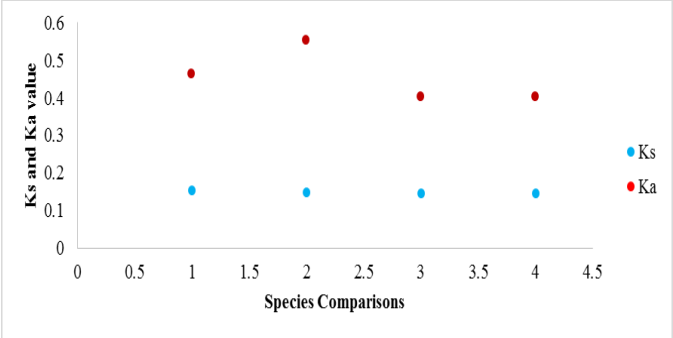

Fig 1: Number of synonymous (Ks) and non-synonymous (Ka) substitutions of nucleotides at 3rd codon position for COI gene sequences generated for four subjected marine crabs with the out group

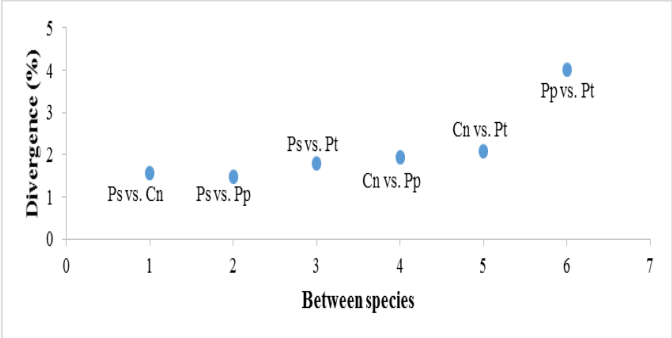

Fig 2: Inter species divergence between subjected marine crabs without the out group, *T. napaea*. Ps, *P. sanguinolentus*; Cn, *C. natator*; Pp, *P. pelagicus*; Pt, *P. trituberculatus*

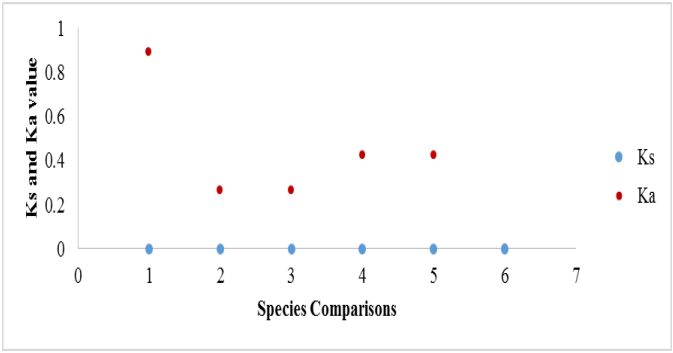

Fig 2: Number of synonymous (Ks) and non-synonymous (Ka) substitutions of nucleotides at 3rd codon position for COI gene sequences generated for four subjected marine crabs (inter species comparison) without the out group

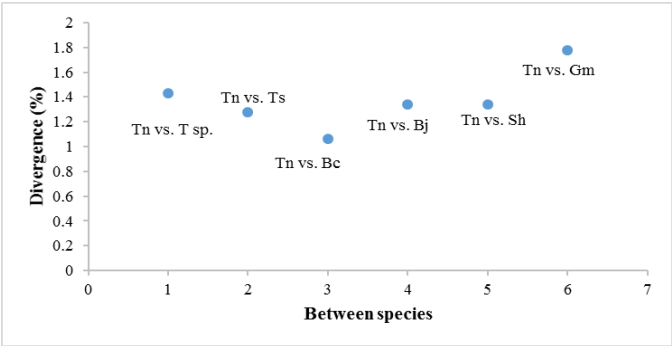

Fig 3: Inter species divergence between a subjected freshwater crab, *T. napaea* and six retrieved freshwater crabs. Tn, *T. napaea*; T sp., *Travancoriana sp.*; Ts, *T. schirnerae*; Bc, *B. cunicularis*; Bj, *B. jacquemontii*; Sh, *S. hydrodroma*; Gm, *G. Monticola*

Plate 3: Nucleotide divergence of subjected marine crabs, and subjected and retrieved freshwater crabs.

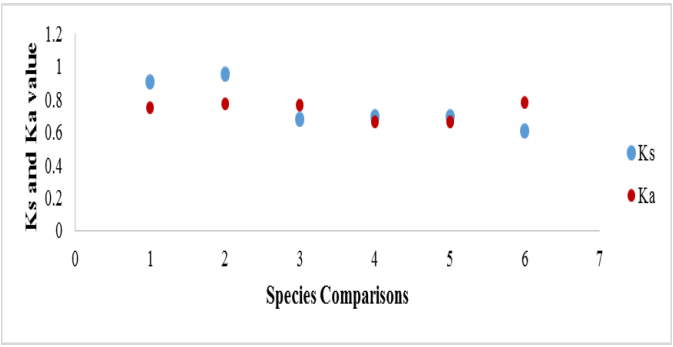

Fig 3: Number of synonymous (Ks) and non-synonymous (Ka) substitutions of nucleotides at 3rd codon position for COI gene sequences of a subjected freshwater crab, *T. napaea* and retrieved freshwater crab species

Plate 4: Number of synonymous (Ks) and non-synonymous (Ka) substitutions of subjected marine crabs, and subjected and retrieved freshwater crabs.

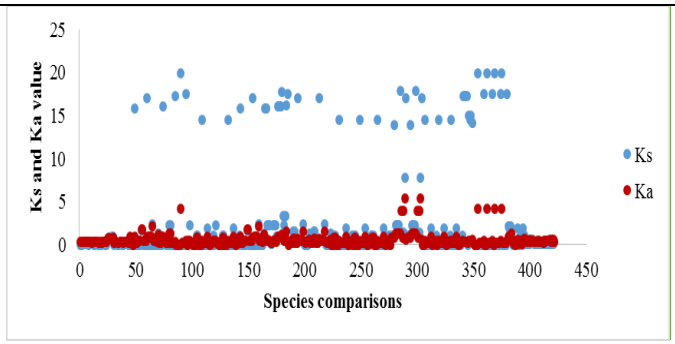

Fig 1: Number of synonymous (Ks) and non-synonymous (Ka) substitutions of nucleotides at 3rd codon position for COI gene sequences generated for four subjected and twenty three retrieved marine crabs with the out group

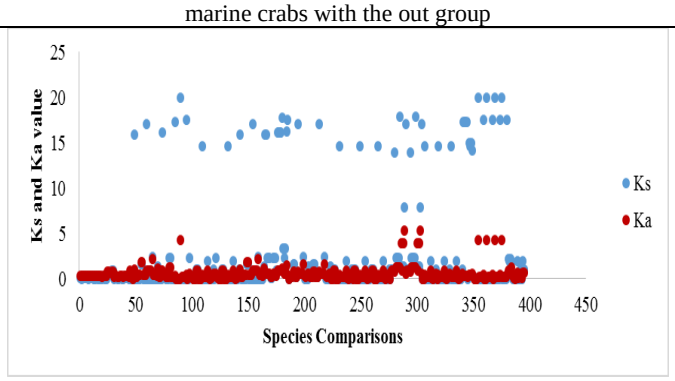

Fig 2: Number of synonymous (Ks) and non-synonymous (Ka) substitutions of nucleotides at 3rd codon position for COI gene sequences generated for four subjected and twenty three retrieved marine crabs without the out group

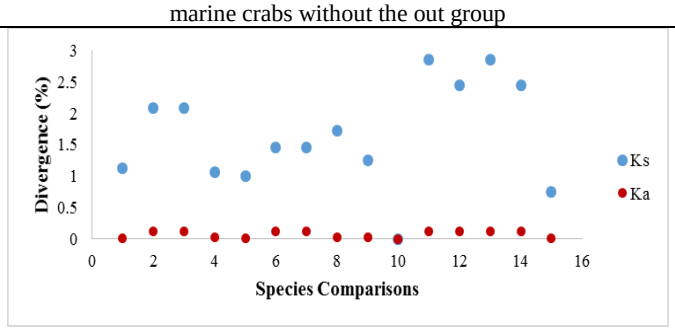

Fig 3: Number of synonymous (Ks) and non-synonymous (Ka) substitutions of nucleotides at 3rd codon position for COI gene sequences of a subjected freshwater crab, *T. napaea* and six retrieved freshwater crab species

Plate 5: Number of synonymous (Ks) and non-synonymous (Ka) substitutions of both subjected and retrieved marine and freshwater crabs.

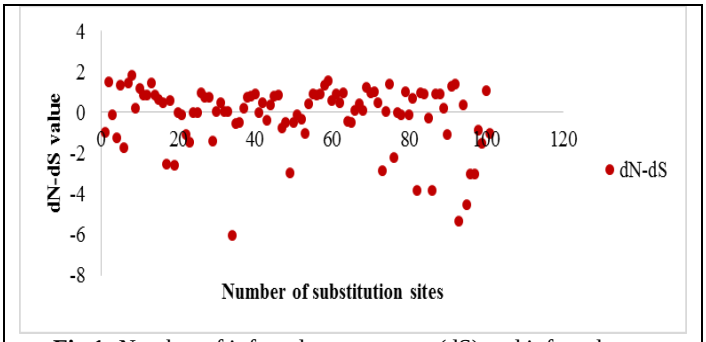

Fig 1: Number of inferred synonymous (dS) and inferred non-synonymous (dN) substitutions of nucleotides at 3rd codon position for COI gene sequences generated for four subjected marine crabs with the out group.

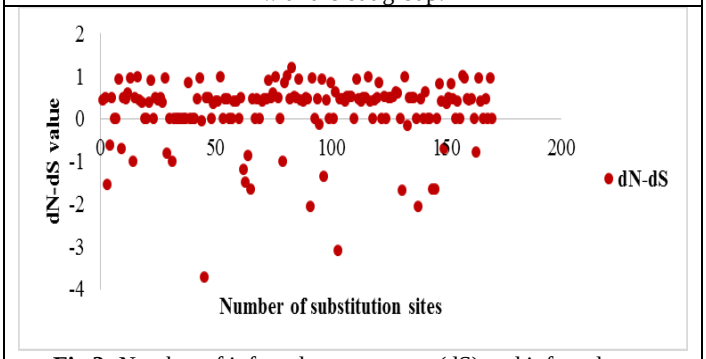

Fig 2: Number of inferred synonymous (dS) and inferred non-synonymous (dN) substitutions of nucleotides at 3rd codon position for COI gene sequences generated for four subjected marine crabs without the out group.

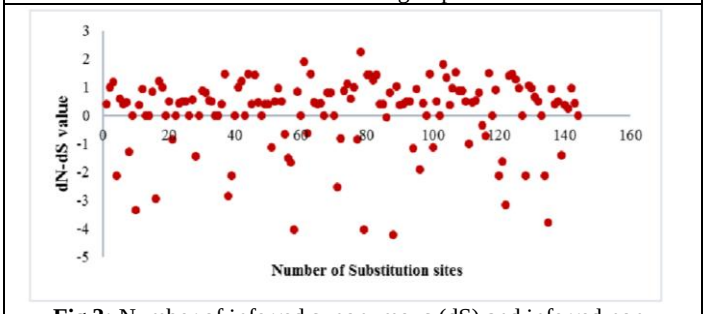

Fig 3: Number of inferred synonymous (dS) and inferred non-synonymous (dN) substitutions of nucleotides at 3rd codon position for COI gene sequences of a subjected freshwater crab, *T. napaea* and six retrieved freshwater crab species

Plate 6: Number of inferred synonymous (dS) and inferred non-synonymous (dN) substitutions of subjected marine crabs, and subjected and retrieved freshwater crabs.

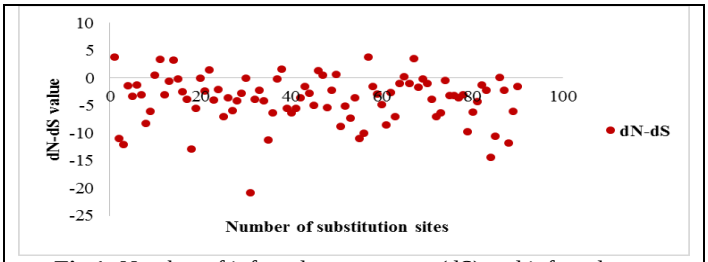

Fig 1: Number of inferred synonymous (dS) and inferred non-synonymous (dN) substitutions of nucleotides at 3rd codon position for COI gene sequences generated for four subjected and twenty three retrieved marine crabs with the out group

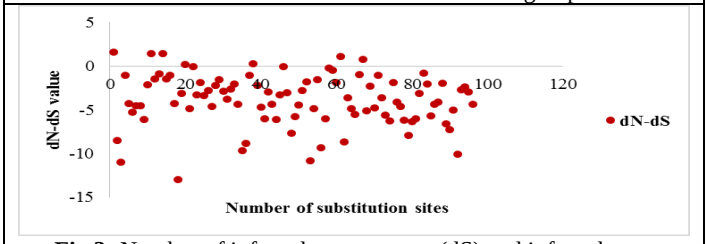

Fig 2: Number of inferred synonymous (dS) and inferred non-synonymous (dN) substitutions of nucleotides at 3rd codon position for COI gene sequences generated for four subjected and twenty three retrieved marine crabs without the out group.

Plate 7: Number of inferred synonymous (dS) and inferred non-synonymous (dN) substitutions of both subjected and retrieved marine crabs.

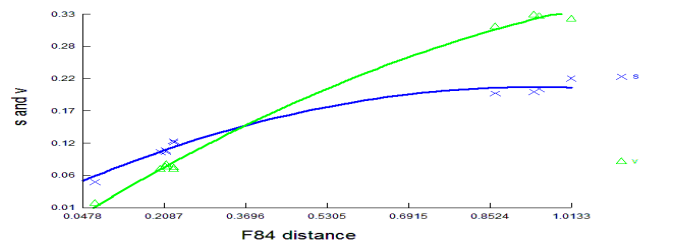

Fig 1: Scattergram shows transitional (Δ, blue) and transversional (X, green) type substitutions occurred in COI gene sequences of four subjected marine crabs with the out group, *T. napaea*

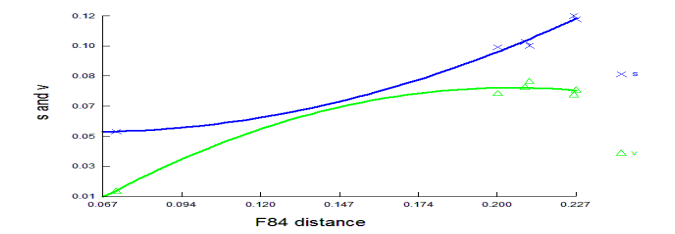

Fig 2: Scattergram shows transitional (Δ, blue) and transversional (X, green) type substitutions occurred in COI gene sequences of four subjected marine crabs without the out group

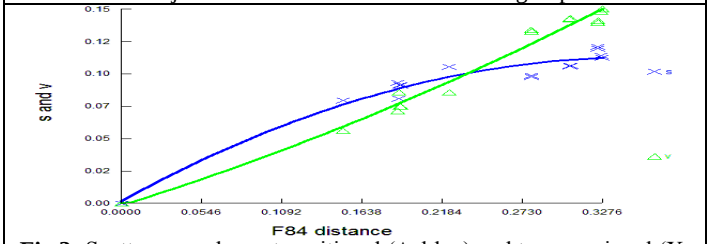

Fig 3: Scattergram shows transitional (Δ, blue) and transversional (X, green) type substitutions occurred in COI gene sequences of a subjected freshwater crab, *T. napaea* and six retrieved freshwater crab species

Plate 8: Number of transitional and transversional substitutions in subjected marine crabs, and subjected and retrieved freshwater crabs.

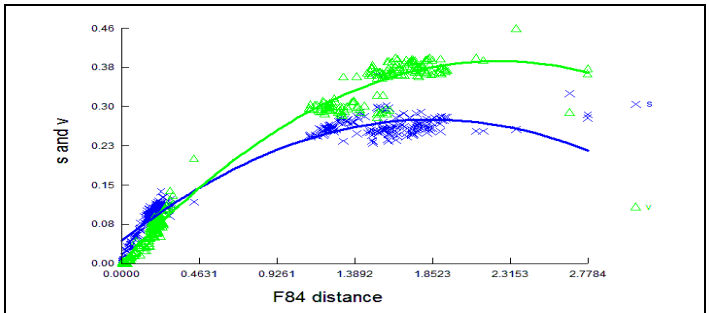

Fig 1: Scattergram shows transitional (Δ, blue) and tranversional (X, green) type substitutions occurred in COI gene sequences of four subjected and twenty three retrieved marine crabs with the out group

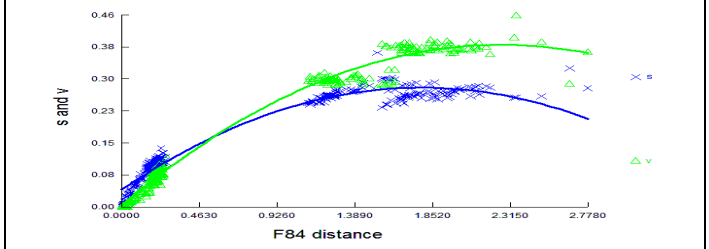

Fig 2: Scattergram shows transitional (Δ, blue) and tranversional (X, green) type substitutions occurred in COI gene sequences of four subjected and twenty three retrieved marine crabs without the out group

Plate 9: Number of transitional and transversional substitutions of both subjected and retrieved marine crabs.

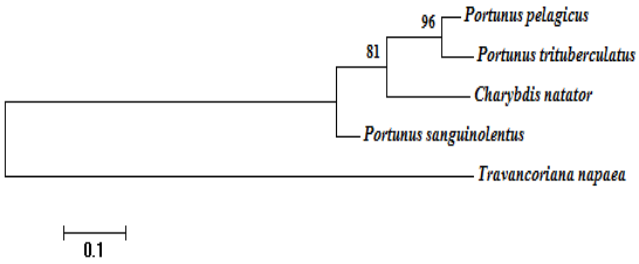

Fig 3: Phylogenetic tree topology of four subjected marine crabs with the out group, the freshwater crab, *T. napaea*).

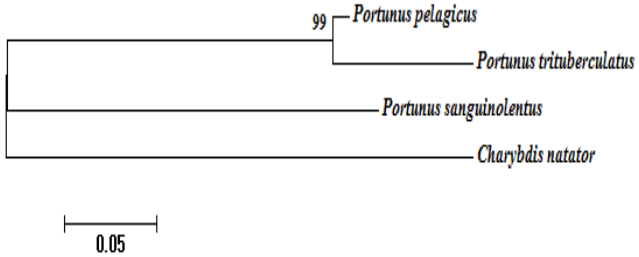

Fig 4: Phylogenetic tree topology of four subjected marine crabs without the out group.

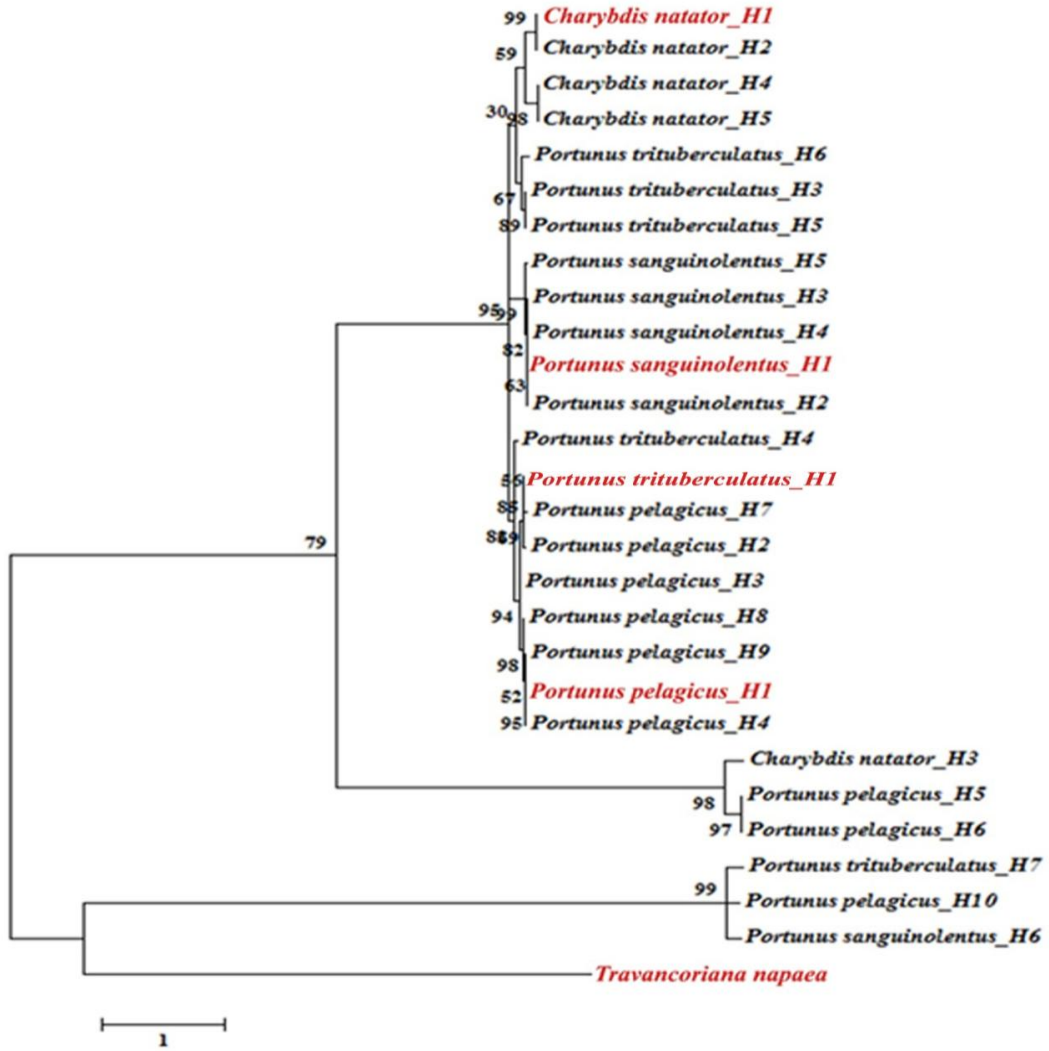

Fig 5: Phylogenetic tree topology of marine crabs (four subjected species given in colored letters, and twenty three retrieved species) with the out group the freshwater crab, *T. napaea* also given in colored letters (*P. sanguinolentus*, 6; *C. natator*, 5; *P. pelagicus*, 10; *P. trituberculatus*, 6; *T. napaea*,1).

The value of Iss.c was more (0.775) than that of the value of Iss (0.416), which indicates the fact that there was no substitutional saturation occurred and thus contained more phylogenetic information. Similar type of information has also been studied by us in crabs, prawns and zooplanktons [31-34]. Synonymous and non-synonymous substitution rates have been found to vary widely within and between taxa [57-60]. Natural selection acts differently on synonymous and non-synonymous mutations in protein coding genes [61]. For almost all proteins, the non-synonymous distance (dN measured by the number of non-synonymous substitutions per non-synonymous site) is lower than the corresponding synonymous distance (dS measured by the number of synonymous substitutions per synonymous site), because the structure and function of a protein imposes constraints on the types of non-synonymous (amino acid) substitutions that can take place, while synonymous substitutions usually accumulate freely with little or no interference from selection.

In contrast, a high non-synonymous distance (dN > dS) is a strong indicator of positive (diversifying) selection acting on the protein [62]. An excess of non-synonymous substitutions (dN/dS > 1) suggests adaptive or diversifying selection, while an excess of synonymous mutations (dN/dS < 1) indicates purifying selection, and no difference between synonymous and non-synonymous mutation rates (dN/dS = 1) is taken as evidence for neutrality [63].

3.3. Phylogenetic tree topology

In the phylogenetic tree created for four subjected marine crabs with the out group, the freshwater crab, *T. napaea* formed two clusters, all the four subjected marine crabs aligned in one cluster with high bootstrap values in the order of *P. pelagicus*, *P. trituberculatus*, *C. natator* and *P. sanguinolentus*, and *T. napaea* alone aligned in a separate cluster (Fig. 3). In the phylogenetic tree created for four subjected marine crabs without the out group formed three clusters with high bootstrap values, *P. pelagicus* and *P. trituberculatus* were aligned in one cluster and *P. sanguinolentus* and *C. natator* were aligned in two separate clusters (Fig. 4).

The phylogenetic information revealed that nucleotide substitutions occurred at different levels than that of nucleotide saturation. These differences have determined the phylogenetic tree structure. The phylogenetic tree topology of marine crabs (four subjected and twenty three retrieved) with the out group of the freshwater crab, *T. napaea* showed four clusters with high bootstrap values (Fig. 5). All the subjected marine crab species were aligned in one cluster in the order of *C. natator*, *P. sanguinolentus*, *P. trituberculatus* and *P. pelagicus* along with retrieved species. And some other retrieved marine crab species were aligned in two separate clusters. The out group, *T. napaea* was alone aligned in one independent cluster at the base of the phylogenetic tree.

Therefore, these sequences are somehow conserved (Iss values were greater than Iss.c values; Table 10) and less subjected to evolutionary forces and thus these species are genetically distinct, but closely related as per information available with mt-COI partial gene. Hence, all the four subjected marine crabs, *P. pelagicus*, *P. trituberculatus*, *C. natator* and *P. sanguinolentus*, and one subjected freshwater crab, *T. napaea* have originated from a common ancestor. The phylogenetic tree topology of marine crabs (four subjected and twenty three retrieved) without the out group showed three clusters (Fig. 6). Here also all the subjected marine crab species were aligned in one cluster along with retrieved species in the order of *C. natator*, *P. sanguinolentus*, *P. trituberculatus* and *P. pelagicus*. And some other retrieved marine crab species were aligned in two separate clusters. But some changes were noticed in the alignment of haplotypes of retrieved species. The subjected *P. trituberculatus* was not aligned with the its retrieved species (Figs. 5 and 6). This may be because of more geographical variation.

The phylogenetic information of freshwater crabs revealed that all the six retrieved species (*Travancoriana* spp., *T. schirnerae*, *B. cunicularis*, *B. jacquemontii*, *S. hydrodroma* and *G. monticola*) were aligned in one cluster, and the subjected *T. napaea* was alone sat in a separate cluster at the base of the phylogenetic tree as in the previous case (Fig. 7).

In this phylogenetic tree the bootstrap value was lower than that of the marine crabs. Therefore, the species are less discriminated, less conserved and more subjected to evolutionary forces (Iss values were lower than Iss.c values; Table 10). However, the sequence generated for *T. napaea* showed some degree of genetic distance with retrieved freshwater crabs and thus aligned in a separate cluster, but very closely related with each other as per information available with mt-COI partial gene. Hence, all the freshwater crab species have originated from a very close common ancestor. According to Klaus *et al.* [42], the phylogenetic position of the genera *Barythelphusa*, *Cylindrothelphusa*, *Gecarcinucus*, *Gubernatoriana*, *Sartoriana* and *Travancoriana* are unstable with respect to the gene trees built with different methods. Further they pointed out that the phylogeny and classification of the freshwater crabs from Africa, Asia and Madagascar has been a controversial topic and the relationship among different freshwater crab taxa has remained uncertain. The phylogenetic tree explains that the early branching of *T. napaea* from the other freshwater crabs and also with the marine crabs which explains its early diversification and thus showed a less phylogenetic information with the retrieved crab species [64-66]. Similar type of information has also been studied by us in crabs [33].

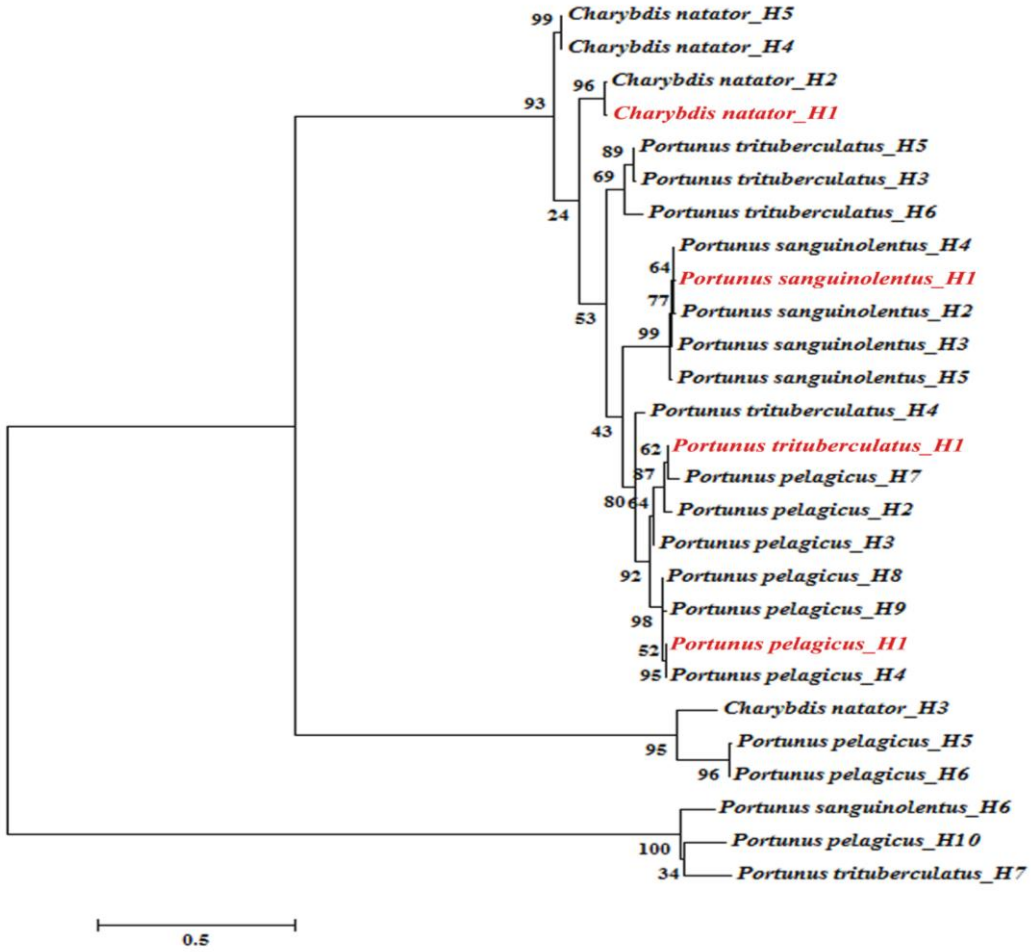

Fig 6: Phylogenetic tree topology of marine crabs (four subjected species given in colored letters, and twenty three retrieved species) without the out group (*P. sanguinolentus*, 6; *C. natator*, 5; *P. pelagicus*, 10; *P. trituberculatus*, 6).

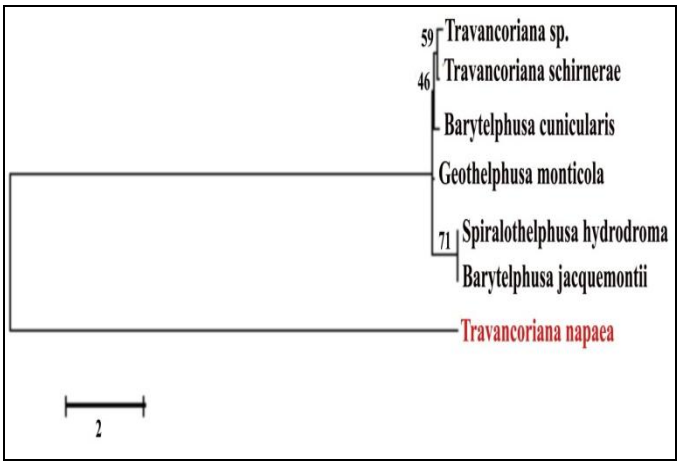

Fig 7: Phylogenetic tree topology of freshwater crabs (one subjected, *T. napaea* given in colored letters, and six retrieved species).

4. Conclusion

The genomic DNA of the crab species studies was greater than 10 kb nucleotides. The amplified sequences of partial mt-COI gene by using universal primers of forward and reverse in natures were revealed ~700 bp nucleotides. These sequences adequately showed similarity and divergence with data available in the NCBI data base. Since literature pertaining to molecular discrimination of crustacean species is scanty, the generated/described phylogentic and evolutionary information in this study can be taken as a vital reference material in the field of genomics of crustacean in general and crabs in particular.

5. Acknowledgements

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