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Diversity, phylogeny, and DNA barcoding of brachyuran crabs in mangrove environments created artificially

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Abstract

Globally, mangrove coverings are disappearing at the rate of 1–2% per annum and 35% have been lost in the last 20 years. Changes in climate and human activities are affecting the mangrove habitats significantly. When the mangroves were transplanted artificially 25 years ago in the Vellar estuary, no mangrove-associated crabs were found. We sampled this mangrove ecosystem and intent to estimate the diversity, species abundance, composition and phylogenetic relationships of barchyuran crabs. We also intend to evaluate the efficacy of DNA barcoding technique in precisely identifying species of brachyuran crab associated with mangroves. Mangrove species such as, *Avicennia marina*, *A. officinalis*, *Rhizophora apiculata*, *R. mucronata* and *R. annamalayana*, *Acanthus ilicifolius* and salt marshes; *Suaeda maritima* and *Prosopis juliflora* constituted the artificially created mangrove ecosystem. A total of 2844 crabs were collected, representing 35 species belonging to 20 genera within 8 families. The four species of brachyuran crab, that is, *Uca lactae*, *U. triangularis*, *Selatium brockii*, and *Neosarmatium asiaticum* contribute >70% of total abundances. The present

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study recovered an estimated 87.5% of crab species. The maximum association index value (97.7%) was observed between *Uca lactea* and *Uca triangularis*. Cluster analysis, grouped the sampled stations according to the type of mangrove species present. It was clear that the type of mangrove species influences brachyuran crabs' structure and species composition. Clustering analysis also clearly distinguished the mangrove stations and salt-marsh station (control) based on the composition of the brachyuran crab species. In general, the abundances of all collected species of crabs, and particularly *Neosarmatium asiaticum*, prefers vegetative cover composed of multiple species of mangroves. DNA barcoding analysis shows that 40% of the species collected in this study was barcoded for the first time. In near future, the advent of new high-throughput sequencing technologies will dramatically change bio-monitoring applications and surveys. This will make reference datasets such as ours important. Using the array of diversity and species estimator indices we presented useful data on brachyuran crab diversity associated with artificially created mangrove ecosystem, which will be useful for marine policy makers, coastal ecosystem designers and climate researchers.

Keywords: Mangrove afforestation, brachuyuran carbs, DNA barcoding, barcode gap, Marine policy

1. Introduction

Mangrove habitats are of ecological and economic significance (Lee et al., 2014). With high biomass and economic values (Alongi, 2015), globally, mangroves cover 15,000,000 ha (Giri, 2011). These forests provide food, breeding grounds and nursery sites at the land-sea interface, for a range of terrestrial and marine organisms, including many commercial species and juvenile reef fish (FAO, 2007; Igulu et al., 2014). Mangrove forests are highly productive ecosystems, primary production rates comparable to those of evergreen tropical forests (Alongi, 2014). They sequester carbon in tree biomass, and much of that carbon is lost to neighbouring habitats through decomposition and export (Algoni, 2012). Mangroves, which are extensively used for food, timber, fuel and medicine (Alongi, 2002; Saenger, 2003), also play also a key role in human survival and livelihoods. They provide protection against devasting events such as tsunami, tropical cyclones and tidal bores and can dampen the erosion of the shoreline (Alongi et a., 2014; Igulu et al., 2014). Despite their value, mangroves are disappearing at a global loss rate of 1–2% per year (Spalding et al., 2010), and the loss rate has reached 35% over the last 20 years (FAO, 2007; Polidoro et al., 2010). Changes in climate (increasing sea level and altering rainfall events) and human

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activities (urbanization, aquaculture, mining, and overexploitation of timber, fish, shellfish and crustaceans) pose significant threats to mangrove ecosystems (McLeod et al., 2006, Gilman et al., 2008, Van Lavieren et al., 2012, Ellison et al., 2012, Carugati et al., 2018).

The anthrogenically contaminated mangrove area reported a 20% loss of benthic biodiversity, with the local extinction of major macrobenthic phyla, a loss of 80% of microbial-mediated rates of decomposition, benthic biomass and trophic resources (Carugati et al., 2018). Unlike Tampa Bay, Florida, mangroves, where tidal salt marshes were formed that were naturally transposed into mangrove wetlands (Osland et al., 2012), Vellar estuary mangroves were planned and risen from mangrove saplings (see, Ajmal Khan et al., 2005; Kathiresan & Rajendran, 2005). The Vellar estuary at Parangipettai (lat. 11°29'N; long. 79°46'E) flowing over the southeast coast of India at is one of the fertile estuaries in Tamil Nadu. A mangrove plantation covering an area of 10 ha was developed in 1991 nearly 1.5 km upstream of the mouth at the tidal zone and on the northern bank of the estuary (Kathiresan and Rajendran, 2005 and references therein). During 2004, Vellar estuary mangroves played a significant role in reducing major tsunami impact.

Indo-Pacific mangrove forests host abundant molluscs and crab species, both critically important regulating ecosystem's energy and food web (Plaziat, 1984; Fratini et al., 2004; Cannicci et al., 2008). In terms of diversity, brachuyuran crabs are as diverse as molluscs (Cannicci et al., 2008). For example, 149 brachyuran crab species belonging to 75 genera have been found living in mangroves in the Indian subcontinent (Dev Roy, 2008), >100 species are known to colonise peninsular Malaysia mangroves (Tan and Pg, 1994).

When the mangroves were artificially transplanted in the Vellar estuary (Kathiresen and Rajendran, 2005), there were no mangrove-associated crabs (Ajmal Khan et al., 2005). Pichavaram mangrove ecosystem (11.4319° N, 79.7810° E) located parallel to transplanted Vellar mangroves, is a heterogenous mixture of mangrove elements spread over 10Km^2 and is the main supplier of brachyuran crabs (38 species found) to Vellar mangrove transplants. In 2005, few brachyuran crab species (n=8) were sampled in the Vellar estuary due to their extended breeding period, high fecundity, higher rate of larvae survival, transport, settling and its colonizing ability (Ajmal Khan et al., 2005). Ajmal Khan (2005) hypothesized that, in due course of time, the remaining species of Pitchavaram mangrove could also be sampled, but how long was unknown then. Developing mangrove saplings affects the soil biogeochemistry. Since soil organic matter, total nitrogen, and redox potential increased with stand age to about 11 years in Philippines' *Rhizophora mucronata* plantations, and subsequently began to level off with progressive stand maturity (Salmo et al., 2013). These

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plantations were projected not to reach full maturity until approximately 25 years (Salmo et al., 2013), considering the initial soil conditions. Since sampling was done exactly 25 years after mangrove sampling transplantation, we intent to check the biodiversity of brachyuran crab species in Vellar mangroves. In this study, we intent to estimate the diversity, species richness, composition and phylogenetic relationships of barchyuran crab species inhabiting in artificially created Vellar mangrove ecosystems. We also intend to evaluate the efficacy of DNA barcoding technique in precisely identifying brachyuran crab species of Vellar mangroves.

2. Materials and methods

2.1. Sample collection and identification

Between February 2015 and 2016, extensive collection was carried out on mangrove patches along the shores of the Vellar estuary at Parangipettai ((lat. 11°29'N; long. 79°46'E) in Southeast coast of India (fig. S1). *Rhizophora* spp., *Avicennia* spp., and *Acanthus illicifolius* were major species distributed along the estuary shore as single and mixed patches of vegetation. Eight species of brachyuran crabs with a density of 27-40/m^2 have been reported after a decade of their establishment (Ajmal Khan et al., 2005). The present study samples brachyuran crabs along the estuary shore at 10 sampling stations. Based on the mangrove species composition at each station, the sampled stations were classified into 4 types; *viz*., 1. Mixed mangrove patch (M) (*Avicennia marina, A. officinalis, Rhizophora apiculata*, *R. mucronata* and *R. annamalayana*), 2. *Avicennia marina* patch (A), 3. *Acanthus ilicifolius* patch (Ai) and 4. Non-mangrove patch (N) dominated by the salt marshes (*Suaeda maritima* and *Prosopis juliflora*).

Random sampling method (using 5 square meter quadrate randomly positioned low tide) was used to estimate crab density (Ajmal Khan et al., 2005). Crabs were washed in ambient waters, transported to lab, where it was preserved in -20ºC until further analysis. Crabs were identified based on the keys issued by Sakai (1976), Sethuramalingam and Ajmal Khan (1991), Ragionieri et al., (2012) and Lee et al., (2015).

2.2. **Abundance and diversity estimation**

An approximation of the abundance and diversity was made using Plymouth Routines in Multivariate Ecological Research (PRIMER) version 6.1.10 (Clarke and Warwick, 2001). Dominance plot (Warwick, (1986) which includes the plotting of separate k - dominance curves (Lambshed *et al*., 1983) on X- axis (logarithmic scale) with percent dominance on the Y- axis (cumulative scale), was used to compare species diversity between stations.

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We deployed a number of diversity indices to get more accurate data on bracyuran crab diversity. We used the Margalef index (based on the number of species at a site; -species richness), the Pielou's evenness index (calculates evenness based on the observed diversity; Pielou, 1969), the Brillouin alpha index (based on total number of individuals in the samples and number of individuals per species), the Fisher alpha index (based on the assumption that species abundance follows the log series distribution), rarefaction index (predicts species richness based on the number of samples), Shannon – Wiener index (based on the proportion of individuals in the individual species), Simpson's index (based on the number of individuals per species and the total number of the individuals in the sample), taxonomic diversity index (based on the taxonomic relation between different organisms in a community), taxonomic distinctness index (based on the taxonomic relatedness and evenness), total phylogenetic diversity (based on phylogeny) to estimate the crab diversity.

Similarly the following estimators were used to obtain estimates of the total number of species likely to occur with intensive collections in the study area: Sobs (based on the total number of species found in a sample or sample sets), Chao 1 (based on the approximate true species diversity of a sample) and Chao 2 (based on the presence-absence data of a species in the sample), Jacknife 1 (based on the number of samples and the number of species in any one sample), Jacknife 2 (based on the number of species only found in one sample and number of species only found in two samples), Bootstrap (based on the presence of individual species in the proportion of sample randomly resampled), Michaelis Menton (based on the species diversity and richness) and Ugland-Gray-Ellingson (based on how many taxa would have been found at each site if a specific number of sampling units (= quadrats) had been sampled at each site). The measure of the extent of linkage equilibrium within the collected species, sampled stations and influences of the mangroves types were estimated using the index of association (Smith et al., 1993) in PRIMER ver. 6.1.10.

2.3. Statistical analysis

Multivariate methods; cluster analysis, multi-dimensional scaling

Unlike diversity indices, the multivariate methods conserve the identity of species and are more sensitive in detecting community patterns and thus detecting subsequent effects (Warwick and Clarke, 1991). Using the Bray – Curtis coefficient (Bray and Curtis, 1957) to generate the dendrogram, cluster analysis was used to cluster related groups by hierarchical agglomerative method. The cophenetic correlation coefficient was used to compare the diversity between sampled stations. Non-metric Multi Dimensional Scaling (MDS) was used

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to evaluate the similarities (or dissimilarities) between each pair of entities in order to create a 'map' to show the interrelationships between all entities. Shepard's plot was drawn to get a sense of how well the two-dimensional data is structured and fits the original data patterns. Bubble plot was synthesised to reveal relationships between types of mangrove and crab species. SIMPER analysis was performed to measure the contribution (%) of each species to the dissimilarity values between types of mangroves in the sampled stations. SIMPER uses Bray-Curtis dissimilarity to assess the respective sampled station's strong discriminators.

2.4. DNA barcoding and analysis

We tested the efficacy of DNA barcoding in identifying 15 brachyuran crab species *viz*., *1- Neosarmatium asiaticum, 2-Episesarma versicolor, 3-Perisesarma bidens, 4-Parasesarma plicatum, 5-Nanosesarma minutum, 6- Plagusia dentipes, 7- Ocypode platytarsis, 8-Cardisoma carnifex, 9- Macrophthalmus depressus, 10- Metopograpsus frontalis, 11-Metopograpsus latifrons, 12- Grapsus albolineatus, 13-Uca lacteal, 14-Uca triangularis and 15- Ocypode brevicornis* collected in this study.

For DNA barcoding, 15 brachyuran crab species that were morphologically difficult to differentiate were picked. Following the manufacturer's guidance, about 50-100 mg of tissue (from the second pereopod) was used for DNA extraction using NucleoSpin® Tissue Kit (Macherey-Nagel). The tissues with lysis buffer and proteinase K were incubated at 56°C until complete tissue lysis, after which 5 µl of RNase A (100 mg/ml) was added and incubated for 5 minutes at room temperature. Using spin columns provided with the kit, DNA was eluted and stored at -20 ^{o}C until further analysis. For polymerase chain reaction (PCR), the eluted DNA in 50 µl buffer was used as such (without dilutions). Folmer's primer pairs; LCO1490 (5'- GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994) were used to amplify the COI gene fragments using PCR conditions; initial denaturation at 98°C (30 seconds), 10 cycles of 98°C (5 seconds), 45°C (10 seconds), 72°C (15 seconds), followed by 30 cycles of 98°C (5 seconds), 50°C (10 seconds), 72°C (15 seconds) and the final extension at 72°C (60 seconds). Sangar's dideoxy sequencing (two ways) was performed using DNA Analyzer 3730xL (Applied Biosystems, USA) in Rajiv Gandhi Centre for Biotechnology, Trivandrum (India).

The tested the sequence quality using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and editing were performed using Geneious Pro v5.1 (Drummond et al., 2010). Basic Local Alignment Search Tool (BLAST) algorithms

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(Altschul et al., 1997) were used for DNA sequences identification by comparing with GenBank database. The reference sequences from the Genbank were selected based on 2% cut-off values for phylogram construction. When 2 or more sequences (of same species) of identical similarity values is present, only one is selected based on the highest query coverage (q) or lowest error (e) value. For those 2 or more sequences with equal similarity values, and representing exact q and e values, the first sequences was selected for phylogram construction. Neighbor- Joining trees were constructed and pair-wise distance was calculated using Kimura-2 parametric (K2P) distance model (Kimura, 1980) using 1000 bootstrapping (Nei and Kumar, 2000).

3. Results

3.1. Collection, classification and diversity estimation

Station 1 was known as *Avicennia marina* patch (and indicated as 1A) based on the presence of mangrove species; Station 2 was non-mangrove zone containing *Suaeda maritima* and *Prosopis juliflora* (2N); station 3 was mixed mangrove zone (3M) (containing 5 mangrove species; *viz*., *Avicennia marina, A. officinalis, Rhizophora apiculata, R. mucronata* and *R. annamalayana*); Station 4 had single species of *Avicennia marina* (4A); Station 5 was mixed mangrove (5M) types (composition similar to station 3M excerpt the absence of *R. mucronata* and *R. annamalayana*); Station 6 had single species of *Acanthus ilicifolius* (6Ai); Station 7 represented non- mangrove zone (7N) (similar of 2N); Station 8 had single species of *Avicennia officinalis* (8A); Station 9 had single species of *Acanthus ilicifolius* (9Ai) and Station 10 had single species of *Avicennia marina* (10A).

A total of 2844 crabs were collected, representing 35 species (Table S1). These 35 species belonged to 20 genera within 8 families (Sesarmidae, Portunidae, Ocypodidae, Grapsidae, Dotillidae, Varunidae, Macrophthalmidae and Gecarcinidae). Barchyuran crabs were richer in station 3 (M) and meagre in station 1 (A) (fig. 1).

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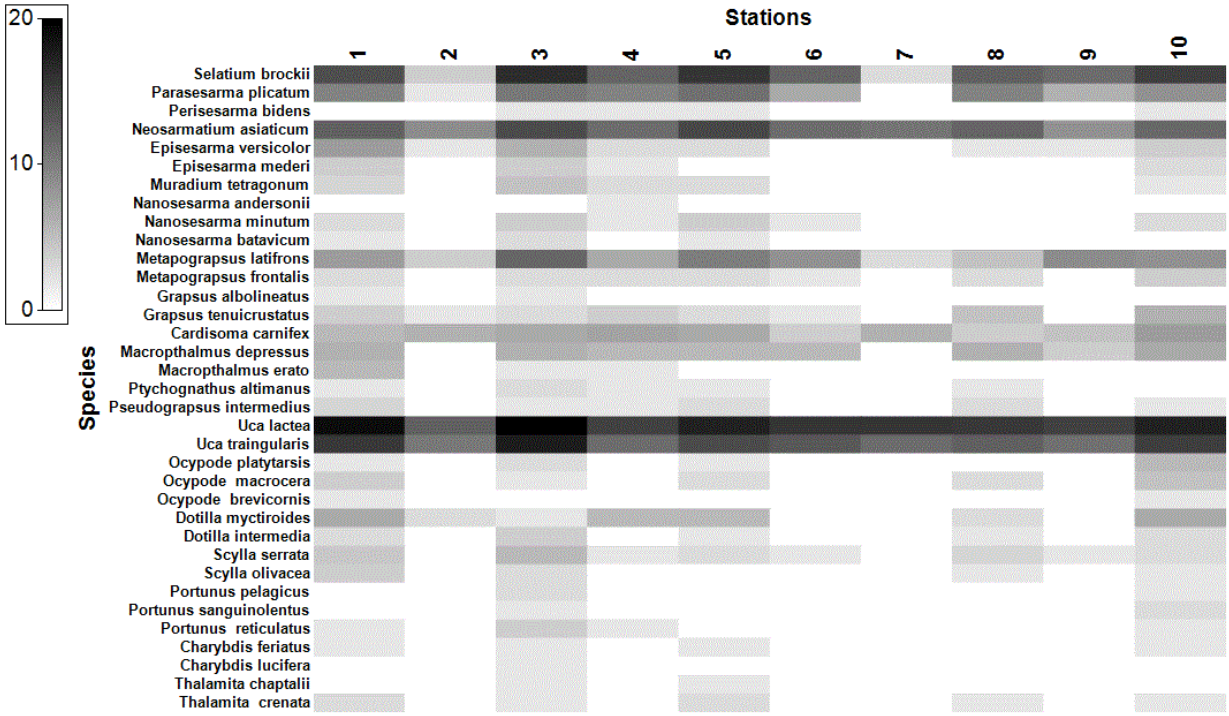

Fig. 1: Shade plot represents the abundance abundance of brachyuran crab species. Note in all sampled stations, species such as *Uca lactea*, *Uca triangularis* and *Neosarmatium asiaticum* were consistently abundant.

At station 3M the dominance plot revealed higher diversity as the natural curve representing station (3M) dropped at the bottom (fig. S2). Also alpha diversity indices such as number of species (S); abundance (N); Margalef index (d), Brillouin, Fisher; rarefaction index (ES - 25, 50, 75, 100, 150), Shannon-Wiener diversity index (H'(log2), total taxonomic distinctness index (sDelta+) and the total phylogenetic diversity index (sPhi+) highlighted the higher diversity in mixed mangrove patch at 3M (fig. S2.A, Table S2). However, the Pielou's evenness index (J'), the Simpson dominance index (Lambda'), the Simpson richness index (1-Lambda') showed higher diversity at 1A and highest taxonomic diversity index (Delta) at 2N (table S2). But based on the shade plot and dominant plot curves, these exceptions could be rejected.

The 8 families, *viz*., Sesarmidae, Portunidae, Ocypodidae, Grapsidae, Dotillidae, Varunidae, Macrophthalmidae and Gecarcinidae were represented by 10, 9, 5, 4, 2, 2, 2 and 1 species, respectively (Table S1). Four crab species cumulatively contributes >70% of total abundance, *viz*., *Uca lactae* (~26%), *U. triangularis* (17.3%), *Selatium brockii* (14.8%), and *Neosarmatium asiaticum* (13.2%) (Table S1). The number of brachyuran crab species varied

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between the minimum of 6 species at 7N and maximum 33 species at 3M. The abundance varied from the minimum of 117 crabs at 2N and maximum of 480 crabs extracted at 3M (Table S1). Four sampled stations, *viz*., 3M (~17%), 1A (~14%), 5M (13.6%) and 10A (13.2%), contributed >57% of the abundance. Less than 10% abundance was contributed by each other stations. Among all the sampled stations, the estimated species (ES) numbers for 25, 50, 75,100 and 150 crabs were below 33 species. Thus the sampled sizes were found to be reasonably adequate for the number species reported (n=35) (table S2).

However, different species estimators, *viz*., Sobs, Chao 1, Chao 2, Jacknife 1, Jacknife 2, Bootstrap, Michaelis Menton (MM) and Ugland-Gray-Ellingsen (UGE), used to estimate the true total number of species that would have been observed with intensive sampling (assuming a closed population is sampled successively), predicted a maximum of 39.86 (by Chao 2 in 3M) (fig. S2B, Table S2). With intense sampling there is a possibility to obtain 40 crab species from the ten stations. While the present study recovered 87.5% (n=35) of a total species estimated.

3.2. Index of association of among the sampled species and stations

The index of association analysis between the sampled species suggested the maximum association value (97.7%) between *Uca lactea* and *Uca triangularis* (table S3). When grouping sampled stations to determine the index of association, 9Ai and 6Ai showed the highest association value (89.8%) (table 1). The minimum association value (43.1%) was recorded between the stations; 7N and 1A (table 1). Excerpt for 6 species (*viz*., *Selatium brockii, Neosarmatium asiaticum, Metapograpsus latifrons, Cardisoma carnifex, Uca lactea,* and *U. triangularis*), all other species present in 1A were absent in 7N (table S1). Similarly, when mangrove types were grouped on the basis of similarity 9Ai and 6Ai were very similar (88.96%). The stations 7N and 3M (44.15%) were very dissimilar (table 1). Interesting to note that the sampled stations were grouped according to the mangrove types (A, Ai, M & N). However such patterns of groupings were not noticed among the brachyuran crabs species (excerpt *Uca* spp.). In this regard, further investigations could be directed to determine the factors shaping the association indices of brachyuran crabs in artificially created mangrove ecosystems.

Table 1. Results for index of association index (lower diagonal) and mangrove types (upper diagonal) for all sampled stations. In both cases, the overall association value of 9Ai and 6Ai was >88%. Higher and lower statistical values were highlighted in bold.

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Stations	1A	2N	3M	4A	5M	6Ai	7N	8A	9Ai	10A
1A		49.94	84.64	78.97	83.77	68.42	46.74	78.76	61.83	85.13
2N	58.26		46.49	64.51	55.93	68.14	83.66	65.06	74.74	52.47
3M	86.13	57.01		73.77	84.25	65.10	**44.15**	71.98	59.11	81.33
4A	79.61	66.62	78.67		82.46	80.90	59.63	82.77	76.05	79.68
5M	82.90	63.12	83.39	85.36		75.53	52.53	81.89	69.07	84.17
6Ai	64.00	72.17	66.69	74.89	75.86		70.64	82.10	**88.96**	71.46
7N	**43.00**	81.11	44.37	50.54	48.55	63.12		60.87	72.61	49.20
8A	77.79	64.74	73.91	80.66	82.90	78.37	51.63		76.43	78.08
9Ai	61.29	73.77	64.36	73.22	72.05	**89.89**	64.37	75.85		64.79
10A	85.10	60.83	84.02	82.31	83.24	67.29	45.06	77.94	64.00	

3.3. Statistical analysis

Multivariate methods; cluster analysis, multi-dimensional scaling

The cophenetic correlation was found to be strong (0.809). All of the grouping patterns observed found in the MDS plot had higher similarity levels (>80%) (fig. 2). The first grouping was of 88.96% similarity between 6Ai and 9Ai, which revalidated the reliability of the aggregation of index. So, for the given mangrove species (like *Acanthus ilicifolius*) there was an established pattern of bracyuran crab community. In other words, type of mangrove species influences the structure and species composition of brachyuran crabs. Similar patterning at non-mangrove (2N and 7N) stations was also observed. However, the 4 sampled stations that contains *Avicennia* (*viz*., 1A, 4A, 8A and 10A) have been segregated into two distinct groups (fig. 2.A). Non-metric multidimensional scaling (MDS) was plotted to account for this distinct grouping pattern of *Avicennia* stations. MDS shoawed that stations with mangroves (mixed species and *Avicennia*) fell to the left side of plot, stations with Acanthus *ilicifolius* (6Ai and 9Ai) fell to the middle and non-mangrove zone (2N and 7N) fell to the right side of the MDS plot (fig. 2B). Even though while clustering all stations, >80% similarity level was observed, the stations with mangroves (mixed, *Avicennia* and *Acanthus*) and non-mangroves formed two distinct clusters in the plot when the similarity level was reduced to 60%.

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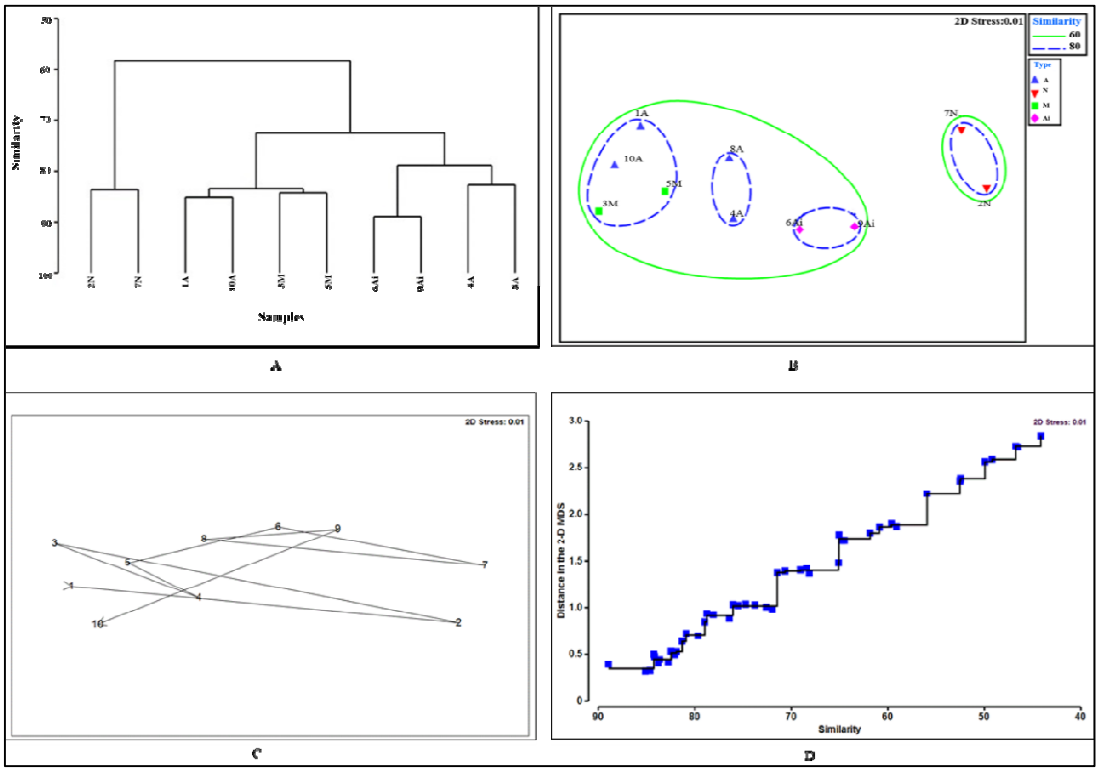

Fig. 2: Cluster analysis results (A) are represented by a dendrogram with the x- axis representing the collections of samples, and the y-axis describing the degree of similarity at which the samples or groups are fused. Combined MDS and cluster plot (B) shows that closer lying samples were similar in species composition and vice versa. The MDS trajectory plot (C) interconnects the sampled stations where the line's direction and length indicates similarity (or distance) between stations. Shepard plot (D) shows a good fit (stress value; 0.01) between the distances of the community (similarity) and the distances of ordination (indicated by the monotonic black regression line).

Stations with mixed mangrove species; 5M (with dominant *Avicennia* spp. populations (*A. marina* and *A. officinalis*) and a *Rhizophora apiculata*) was positioned relatively closer to other *Avicennia* mangrove stations (6Ai and 9Ai) than mixed mangrove station 3M (dominated by 3 *Rhizopora* spp. (*Rhizophora apiculata*, *R. mucronata* and *R. annamalayana*) and *Avicennia* spp. (*A. marina* and *A. officinalis*)) (fig. S1). *Rhizopora* spp. richer in 3M than 5M was also noticed. So it was found that, *Avicennia* spp. dominating 5M station was the reason for distinct clustering patterns of 1A, 10A and 4A, 8A in the dendrogram. But it has been unclear how a mangrove species (in a mixed mangrove community) affect a crab species (in mixed population).

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This does not, however, alter the observed fact that the type of mangrove species affects the structure and species composition brachyuran crabs, because the pattern of crab species composition depends on the presence and absence of mangrove species (fig. 2.B). The MDS trajectory plot (fig. 2.C) also showed that the types of mangrove species (rather than its geographical distances) determines the species compositions of colonising brachyuran crabs. This trend was reconfirmed by the Shepard stress plot (fig.2.D), as a good fit of ordination was observed. The stress value was very poor (0.01), and the points dropped nearer to the line of regression line (fig. 2.D).

3.4. Bubble segmented plot and SIMPER analysis

We used bubble segmented plot in order to understand the essence of inter-relationship between mangroves and crab population at the given sampled stations. The plots were drawn from all all sampled stations for the top 4 abundant crab species (A - *Selatium brockii*, B - *Neosarmatium asiaticum*, C - *Uca lactea* and D - *Uca triangularis*) (fig. 3). When the number of *Rhizopora* spp. decreases in the stations of mixed mangrove population (5M > 3M), decrease in *Uca lactea* and *U. triangularis* abundances were observed. *Selatium brockii* abundances have been significantly reduced at non-mangrove stations (7N and 2N), exposing this species' heavy dependence on mangrove vegetation (fig. 2). In general, the abundances of all collected crab species and *Neosarmatium asiaticum* in particular, prefers mixed mangrove vegetation, followed by *Avicennia* spp., *Acanthus ilicifolius* and non-mangrove vegetation.

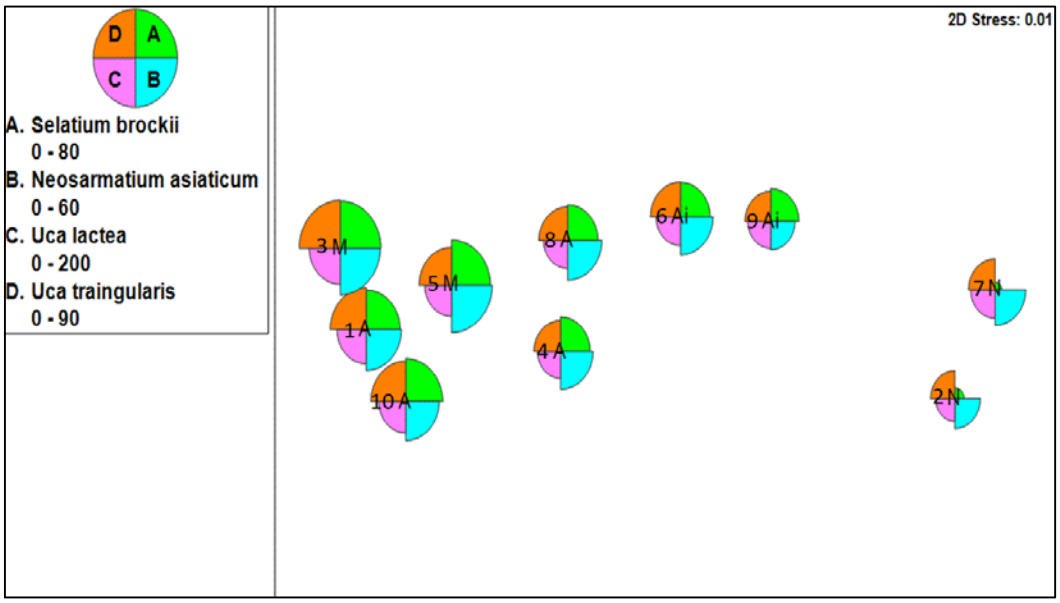

Figure 3: Segmented bubble plot showing distribution of top four abundant brachyuran crab species between the sampled stations. The abundances of crab species declines in the

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following patterns; mixed mangrove types (highest abundances and species diversity), followed by *Avicennia* spp. types, *Acanthus ilicifolius* types and non-mangrove types.

The average similarity for the sampled stations; M, A, Ai and N were 84.26%, 80.57%, 88.96% and 83.66%, respectively (fig. S3). While *Uca lactea* contributed maximum average similarity for all four sampled stations types (between 11.04% in M and 23.95% in N), different species contributed minimum average similarity for each sampled mangrove types (fig. S2). *Macropthalmus depressus* (3.45%), *Cardisoma carnifex* (3.7%), *Metapograpsus latifrons* (10.84%) and *Neosarmatium asiaticum* (17.33%), respectively contributed the minimum average similarity values for types M, A, Ai and N (fig. 3). The factors driving the minimum contribution values for different brachyuran crab species at a given mangrove type station is currently unknown. Maximum average dissimilarity (MAD) was observed between M and N stations (50.22%) (table 2). *Selatium brockii* is the only species to contribute to MAD observed between N and all types of mangrove stations.

Table 2: Average dissimilarity between the mangrove types was shown in percentage. Percentage values in the parenthesis represents the average percentage dissimilarity values. Values in lower and upper diagonals represents the maximum and minimum average dissimilarity percentage contribution of brachyuran crab species, respectively.

	M	**A**	**Ai**	**N**
M		*Ocypode macrocera* (0.56%)	*Metapograpsus latifrons* (0.94%)	*Pseudograpsus intermedius* (1.13 %)
A	19.49% *Metapograpsus latifrons* (1.47%)		*Ocypode platytarsis* (0.79 %)	*Neosarmatium asiaticum* (1.11%)
Ai	32.79% *Parasesarma plicatum* (1.92%)	27.25% *Dotilla myctiroides* (2.49%)		*Cardisoma carnifex* (1.36 %)
N	50.22% *Selatium brockii* (6.19%)	43.94% *Selatium brockii* (5.49%)	28.46% *Selatium brockii* (6.53%)	

3.5. DNA barcoding and pairwise analysis

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BLAST analysis revealed that 40% of the species (*viz*., *Plagusia dentipes*, *Ocypode platytarsis*, *O. brevicornis*, *Metopograpsus frontalis*, and *M. latifrons* and the genus *Macrophthalmus* sp. (*M. depressus*)) was barcoded for the first time (table 3). The maximum similarity values of the aforementioned species was ≤95% with that of all barchyuran COI sequences in the Genbank database, and individual searches for these species revealed their absence in the Genbank. *Macrophthalmus depressus* shared a similarity value of only 86% (with *Uca* spp.) and the COI sequences of none of the species belonging to this genera was found in the Genbank database suggesting that, the genus *Macrophthalmus* sp. was first time barcoded.

Table 3. Accession numbers of 15 species and the species barcoded for the first time were provided with their corresponding percentage of similarity and closest species matches.

No.	**Name of the species**	**Accession Number**	**Family**	**First time sequenced specimens with closest Genbank reference**
1.	*Neosarmatium asiaticum*	KY242627	Sesarmidae	-
2.	*Episesarma versicolor*	KY284641	Sesarmidae	-
3.	*Perisesarma bidens*	KY436763	Sesarmidae	-
4.	*Parasesarma plicatum*	KY284646	Sesarmidae	-
5.	*Nanosesarma minutum*	KY284644	Sesarmidae	-
6.	***Plagusia dentipes***	KY284645	Plagusiidae	92% matched with *P. immaculata*
7.	*Cardisoma carnifex*	KY398729	Gecarcinidae	-
8.	***Macrophthalmus depressus***	KY427938	Macrophthalmidae	86% similar with Uca stenodactylus
9.	***Metopograpsus frontalis***	KY284642	Grapsidae	94% similar with *M. thukuhar*
10.	***Metopograpsus latifrons***	KY284643	Grapsidae	94% similar with *M. thukuhar*

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11.	*Grapsus albolineatus*	KY436762	Grapsidae	-
12.	*Uca lactea*	KY406231	Ocypodidae	-
13.	*Uca triangularis*	KY284640	Ocypodidae	-
14.	***Ocypode brevicornis***	KY284647	Ocypodidae	89% similar with *O. platytarsis*
15.	***Ocypode platytarsis***	KY284648	Ocypodidae	95% similar with *O. macrocera*

The COI Sequences were grouped into 3 major clades (Sesarmidae-Plagusiidae-Macrophthalmidae (SPM) clade; Ocypodidae-Grapsidae (OG) clade and Gecarcinidae-Grapsidae (GG)) in the constructed phylogram (fig. 4) by clearly distinguishing the out group (*Penaeus monodon*). The SPM clade grouped all Sesarmidae members (*Perisesarma bidens*, *Parasesarma plicatum*, *Neosarmatium asiaticum*, *Episesarma versicolor* and *Nanosesarma minutum*) into one major clade, and members of Plagusiidae (*Plagusia dentipes*), Macrophthalmidae (*Macrophthalmus depressus*) in the neighbouring clade. GG clade also separated Gecarcinidae (*Cardisoma carnifex*) from other members of the Grapsidae (*Metopograpsus frontalis*, *M. latifrons*). However, OG clade grouped Ocypodidae members (*Uca lactea*, *U. triangularis*, *Ocypode brevicornis* and *O. platytarsis*) into single clade while placing a Grapsidae member (*Grapsus albolineatus*) in the neighbouring clade. The reason *Grapus albolineatus* does not clade group with other members of the same family (in GG clade) is unknown, and therefore the phylogenetic relationships between Grapsidae members which need further investigation.

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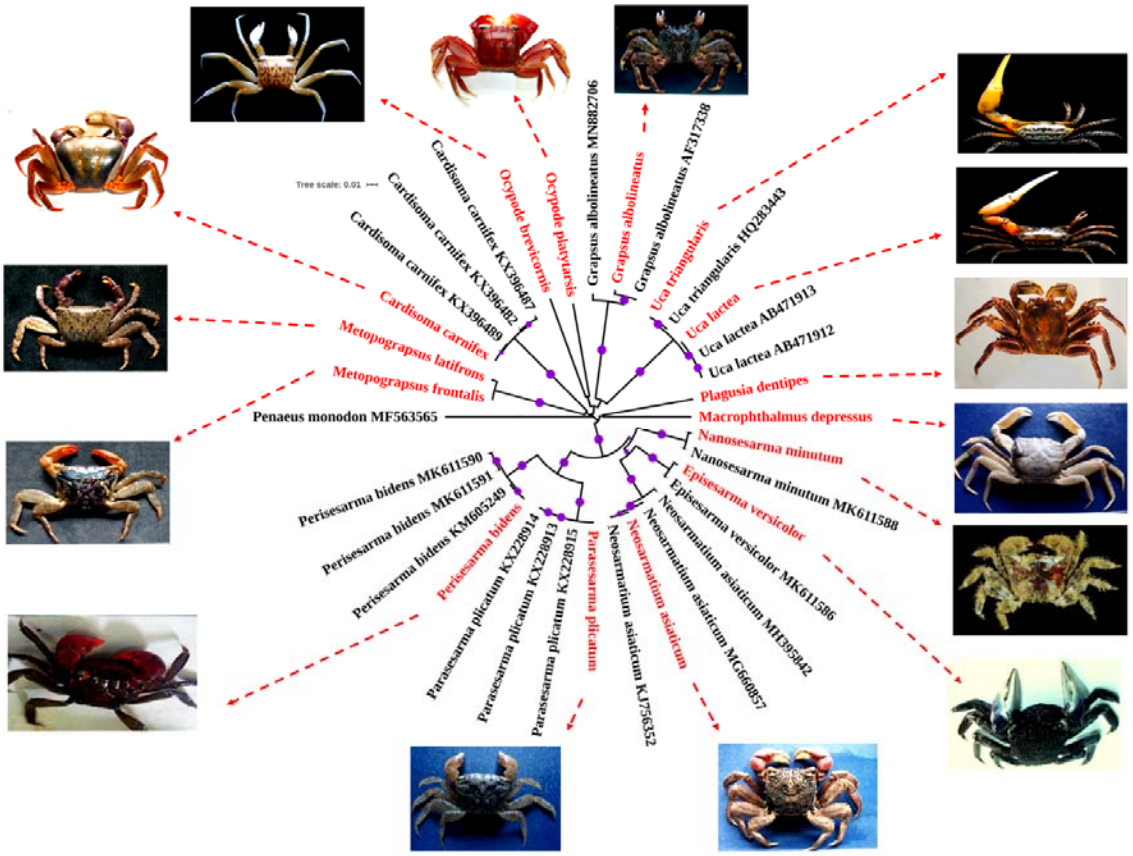

Fig. 4: Neighbour-joining tree was drawn using Kimura-2 parametric distance model. Species marked in red and black were sequences of this study and reference Genbank sequences (with accession numbers), respectively. Bootstrap values >70 were shown in purple circles. *Penaeus monodon* (Genbank Acc. No.: 563565) used as an out-group. Species without reference sequences in the phylogram indicates the first-time barcoded species.

The K2P distance showed that *Grapsus albolineatus* had been genetically closer to *Uca* spp. (0.21) excluding the *Ocypode* spp. (0.22) between members of the OG clade (table S4). When K2P distance was compared between *G. albolineatus* and other Grapsidae members (*Metopograpsus latifrons* and *M. frontalis*), the values were slim (~0.2) (table S4). The mis-placement of *G. albolineatus* in NJ tree and higher genetic variations among the members of Grapsidae may deserve further investigation. The average K2P distances among all the brachyuran crab species barcoded in this study were 0.2.

4. Discussion

Diversity is a measure of the complexity of the community structure (example: Cisneros et al., 2011; Menta, 2012) and the mangroves ecosystem's biodiversity changes due to physical, chemical and biological factors (Kathiresan and Bingham, 2001; Carugati et al.,

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2018). High diversity within mangrove ecosystems suggest that a community is balanced, stable and responsive (Carugati et al., 2018). Many habitats are highly dependent on their faunal communities which are dominated by brachyuran crabs. Brachyuran crabs are among the most important components of both natural and artificially created mangrove ecosystems (Cannicci et al., 2008, Kristensen et al., 2008), but their occurrences and preferences in mangrove ecosystems artificially created are not well established.

Unlike Tampa Bay, Florida, where tidal salt marshes created that were naturally transposed into mangrove wetlands (Osland et al., 2012), Vellar estuary mangroves were planned and resurrected from mangrove saplings (see, Ajmal Khan et al., 2005). Vellar estuary mangroves played an important role in Tsunami mitigation during 2004 (Kathiresan & Rajendran, 2005). During flooding and competent larvae settling, brachyuran crab larvae are transported into mangrove ecosystems (Epifanio, 1995). Most brachyuran crab larvae were highly competent in locating conspecific adults colonizing the forest area (Cannicci et al., 2019). *Parasesarma capensis* is more abundant in the *Rhizophora mucronata* characterized areas (Fratini et al., 2019). During settlement and colonization, different species of brachyuran crab larvae prefers different mangrove species (Cannicci et al., 2019).

4.1. Mangroves associated brachyuran crab diversity

It is not a comprehensible inference that abundance would contribute to the number of species per unit area. This relation depends on the community level of equity (Glover et al., 2001). In the present study, a total of 2844 crabs representing 35 species representing 8 families (*viz*., Sesarmidae, Portunidae, Ocypodidae, Grapsidae, Dotillidae, Varunidae, Macrophthalmidae and Gecarcinidae) were collected, where over 70% of total abundance was dominated by 4 species of crab, i.e., *Uca lactae*, *U. triangularis*, *Selatium brockii*, and *Neosarmatium asiaticum.*

Station 3M had maximum species diversity that is likely due to increased physical and environmental stability, as stated by Levin and Gage (1998), Ganesh, (2003), Ajmal khan et al. (2005), Bijukumar et al. (2007) and Praveenkumar *et al.* (2013). It was unclear how a mangrove species in a mixed mangrove community influences the colonization and settlement of a crab species. However, this does not alter the observed fact that the type of mangrove species (or community) affects the structure and composition of the brachyuran crab community, since the pattern of distribution of crabs was due to the presence or absence of a particular mangrove species.

Species such as *Uca lactea*, *U. triangularis* and *Neosarmatium asiaticum* have been found to be consistently abundant at all of the study area stations. The bar plot and the shade

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plot thus clearly explained the species-richness of brachyuran crabs of Vellar mangrove, in agreement with earlier works by Bijukumar et al., (2007) and Bharadhirajan (2012). *Selatium brockii* abundances have been substantially reduced at non-mangrove stations (7N and 2N), exposing this species' heavy reliance on mangrove vegetation.

4.2. Comparing brachyuran crab abundances in artificial mangroves between 2005 and 2015

It took about 15 years for 8 species of brachyuran crabs to colonize and the remaining species were colonized in the next 10 years, when compared with near-by Pitchavaram ecosystem. This colonization pattern shows that the effects of transport and settlement are cumulative over time. In the present analysis the sampled stations VI and VII in Ajmal Khan et al, (2005) were 3M and 4A. The present study found that, during 2005, the abundance of 8 species (*Selatium brockii*, *Episesarma mederi*, *Nanosesarma minutum*, *N. batavicum*, *Grapsus tenuicrustatus*, *Uca lactea*, *U. triangularis*, and *Dotilla myctiroides*) colonizing 3M and 4A almost doubled (with few exceptions, such as *G. tenuicrustatus* missing from 3M). The H diversity index also increased by more than 4 times in 3M and 4A, when compared to 2005 sampling (Ajmal Khan et al., 2005). That is, the H diversity index was 8 and 5 during 2005 for the stations 3M and 4A, respectively which is 33 and 22, respectively in the present analysis. This may be clarified due to variations in the mangrove composition observed over various time period at the given sampling station. That is, during 2005, station 3M consisted of *Rhizopora apiculata*, *Avicennia marina*, *Acanthus illicifolius* and station 4A consisting of *Avicennia marina* and *Acanthus illicifolis* (Ajmal Khan et al., 2005). Present study found that, station 3M is composed of *Avicennia marina*, *A. officinalis*, *Rhizophora apiculata*, *R. mucronata* and *R. annamalayana*. The station 4A only composed with *Avicennia marina*. Herbaceous macrophytes may have played a role in influencing the mangrove composition by trapping mangrove propagules from local supply at the given station (McKee et al., 2007; Peterson and Bell, 2012; Lewis, 2005). Therefore, one should be aware of changes in the composition of mangrove spatially and temporally (in the artificially created mangrove patches) in order to identify changes in the composition of the inhabitation brachyuran crab community over time.

4.3. Why DNA barcoding selective brachyuran crab species?

While 8 crab species occurred during 2005 were precisely identified (Ajmal Khan et al., 2005), taxonomic ambiguity was faced in this study (example, in Gecarcinidae; *Cardisoma carnifex*). The key explanation for this was the non-fusion of the supra- and infra-orbital margins along the outer edges which can be called incomplete orbits (Ng et al., 2008). The

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challenging problem was also the distinctive features such as the inclusion versus exclusion of the antennae from the orbital hiatus which is necessary to differentiate between *Pachygrapsus* (includes antennae) from *Metopograpsus* (excludes antennae) (Poupin et al. 2005). Including among the members of Ocypodidae, while the number of features can be used to classify species within this genus Ocypode, many of these are not generally useful for establishing natural groupings (Sakai and Türkay, 2013) and the uncertainty and dispute over the proposed generic, subgeneric, and specific taxonomy of the genus *Uca* (Rosenberg, 2001) has been witnessed.

It was tough to differentiate the members of Plagusiidae and Grapsidae. While the crabs, *Plagusia dentipes* (Plagusiidae) and *Grapsus albolineatus* (Grapsidae) belong to the two separate families, earlier they belonged to the Plagusinae and Grapsinae subfamilies under Grapsidae family, respectively. The subfamilies have now been raised to family-level. Consequently, difficulties in identifying those species using morphological characters are understandable, and molecular identification is necessary. Sesarmidae- the keystone species of mangrove communities (Smith et al., 1991; Lee, 1998), even after many taxonomic revisions (e.g., Davie, 1992; 1994; Schubart et al., 2009; Davie, 2012), there are still several ambiguities and taxonomic issues that call for further studies (see notes on Sesarmidae, Ng et al., 2008). Even after splitting into 5 sub-genera (Barnes, 2010), taxonomic problems prevailed in the genus *Macrophthalmus* was solved (Ng et al., 2008, Davie, 2009).

4.4. DNA barcoding and pairwise analysis

BLAST analysis revealed the species; *Plagusia dentipes*, *Ocypode platytarsis*, *O. brevicornis*, *Metopograpsus frontalis*, and *M. latifrons* and the genus *Macrophthalmus* sp. (*M. depressus*) were barcoded for the first time. We realised that as many more commonly available brachyuran crab species lacked reference barcodes in Ganbank, DNA barcoding needs veen stronger push globally and locally. For example, while *Macrophthalmus depressus* was described as early as the 1830's, (Rüppell, 1830), Genbank was absent from the barcode sequence of this genera as a whole.

Minimal genetic differentiation is expected within the SPM clade between the *Periseserma bidens* and *Parasesarma plicatum*, as most species of *Parasesarma* (De Man, 1895) was transferred from *Perisesarma* (De Man, 1895) (see Shahdadi and Schubart 2017). The distinction of *Parasesarma* and *Perisesarma* was previously due to the presence or absence of a distinct epibranchial tooth, and while this was long suspected of being artificial in nature, Shahdadi and Schubart (2015, 2017) finally demonstrated that character was

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phylogenetically uninformative. Like most sesarmid crabs, the *Parasesarma* genus has marine planktonic larvae (see Flores et al., 2002; Guerao et al., 2004; Lago 1993) and thus a potential for high dispersal capability. Recognition of distinct brachyuran crab family Macrophthalmidae (Dana, 1851) from Ocypodidae (Rafinesque, 1815) on the basis of morphological and molecular evidence (see Kitaura et al., 2002; Mendoza & Ng, 2007; Ng et al., 2008) was reaffirmed in this analysis as *Macrophthalmus depressus* was grouped within the SPM clade, not in OG clade where Ocypodidae members were grouped together. The Grapsidae (MacLeay, 1838) crab family, was originally a large group of thoracotreme crabs, with over 400 species in over 50 genera within the superfamily Grapsoidea (see Guinot, 1978; Bowman & Abele, 1982).

GG clade seperated Gecarcinidae (*Cardisoma carnifex*) from other members of Grapsidae (*Metopograpsus frontalis*, *M. latifrons*), excerpted from *Grapsus albolineatus* grouped within the OG clade. Grouping of Gecarcinidae and Grapsidae was expected as they were under single superfamily Grapsoidea which was also supported previously using molecular data (Schubart et al., 2000; 2002). Grapsidae phylogeny may require re-investigation as OG clade contained the members of Ocypodidae (*Uca lactea*, *U. triangularis*, *Ocypode brevicornis* and *O. platytarsis*), while the member of Grapsidae (*Grapsus albolineatus*) was distanced in the neighbouring clade.

Compared with the Ocypodoidea the Grapsoidea does not seem to be reciprocally monophyletic (see Schubart, Neigel & Felder, 2000b; Kitaura, Wada & Nishida, 2002; Schubart et al., 2006). As such, Schubart et al. (2006) proposed that it would be better to avoid using these taxa until monophyletic superfamilies could be re-defined and in the meantime simply refer to the entire group as Thoracotremata.

The genus *Grapsus* spp. was paraphyletic and would not necessarily be accommodated in the Grapsidae family clade (Ip et al., 2015). The higher similarity between *Metograpsus frontalis* and *M. latifrons* was observed in the previous analysis consisting of 5 different *Metapograpsus* spp. (Ip et al., 2015). The multi-barcode markers approach also verified that *Grapsus albolineatus* would not be identified with members of Grapsidae (see IP et al., 2015). The multiple barcode phylogeny also revealed that *G. albolineatus* was genetically similar to *Pachygrapsus fakaravensis*. Therefore this unique species (*G. albolineatus*) can need further phylogenetic re-examination.

Universal barcode gap proposal would be mandatory to delineate brachyuran crabs from meta- and environmental barcoding outcomes (Weigand et al., 2019). The average genetic distances (K2P) for all brachyuran crab species in this study were 0.2. Proposing a

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2% gap cannot be feasible (based on overall pairwise distance data from this present study) as it was recognised that some species of Plagusiidae (*Perisesarma guttatum*) contain 2.75% K2P distance within various haplotypes along the East African coast (Silva et al., 2010). As new species *Parasesarma austrawati* (in northern Australian mangroves (Shahdadi et al., 2019)) had 4% genetic distances among other species of *Paraseserma* spp., the genetic distance values of 3% species barcode gap, previously proposed by Hebert et al., (2003), may not work for brachyuran crabs. For brachyuran crab species delineation, family specific COI barcode gaps should be defined.

5. Conclusion

A combination of morphological and molecular data will certainly give us the most information (see Klaus et al., 2009) and would allow us to make a major leap towards our ultimate goal of reconstructing the decapod Tree of Life. First time barcodes produced in this study shows the needs to improve the barcode species coverage in reference libraries. It should also be borne in mind that DNA barcoding reimbursement is not restricted to taxonomic or systematic research only. The emergence of modern high-throughput sequencing technologies will in the near future significantly change applications and surveys for bio-monitoring (Fonseca et al., 2010; Hajibabaei et al., 2011; Leray et al., 2015). This will make reference datasets such as ours important for the identification of barchyuran crabs. The current research found it would take approximately 25 years for the Vellar mangroves to absorb almost all brachyuran crab species of Pichavaram mangroves. Using the array of diversity and species estimator indices resulted in useful output data for the period needed for colonization, and preferences of artificially developed mangrove by brachyuran crabs. This is a bench mark data for marine policy makers, coastal ecosystem designers and climate researchers. The hints from the present study on changes in mangrove composition in various artificially created mangrove patches will add value during the blue carbon ecosystem engineering.

6. Acknowledgement

First author thank Ministry of Earth Sciences (Centre for Marine Living Resources and Ecology) for the financial grand (No. 10-IT IS/17/2012 dt.01/10/2012).

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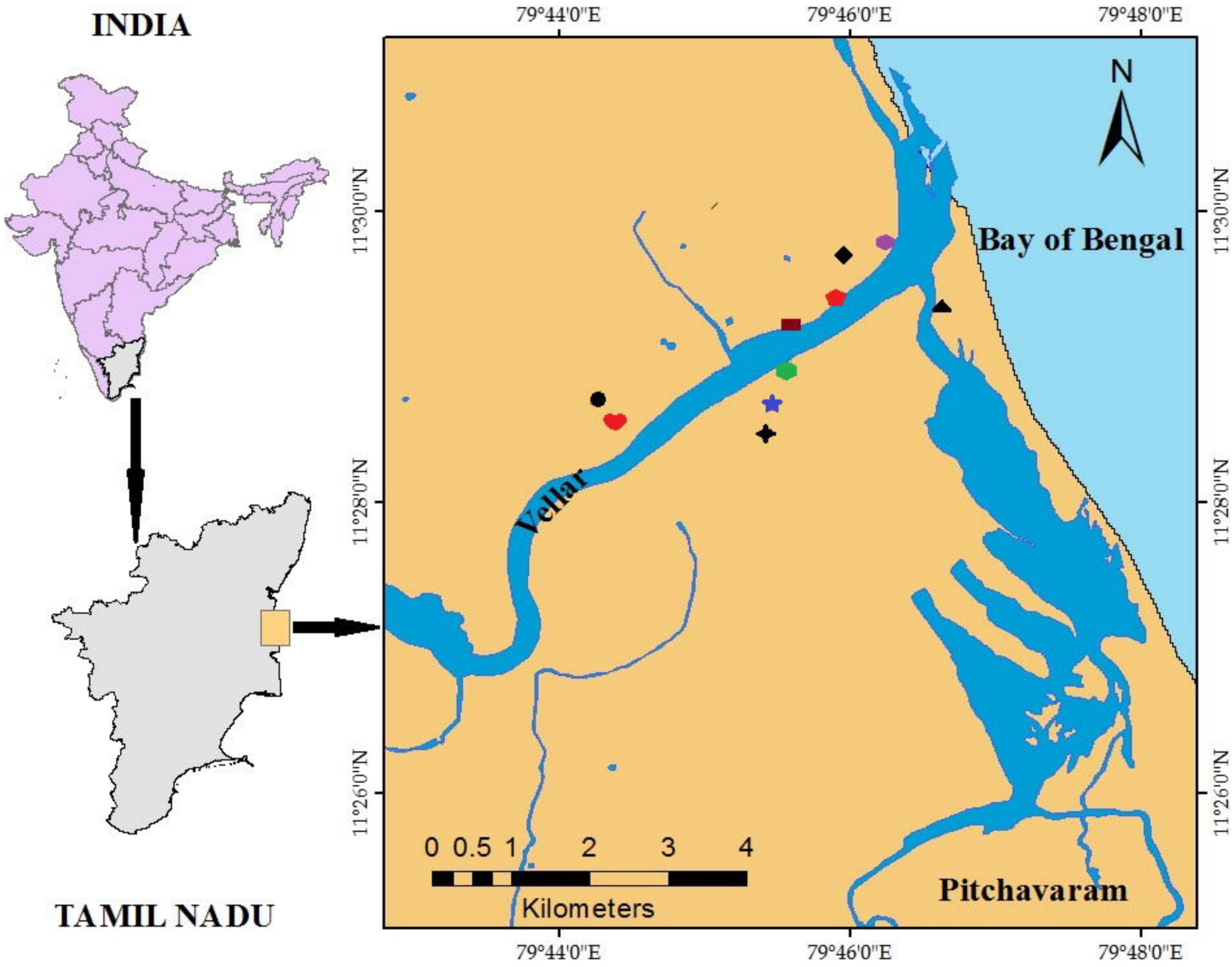
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