International Journal of Academic Research and Development
ISSN: 2455-4197
Impact Factor: RJIF 5.22
www.academicsjournal.com
Volume 3; Issue 1; January 2018; Page No. 654-658

Study on DNA divergence of selected crabs from coastal Andhra Pradesh based on Cytochrome Oxidase I (COI) sequences

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Abstract
Effective stock assessment and fisheries management require clearness in their taxonomy for any species. Crabs are well known for its morphological plasticity and complex life cycles for which they became much contentious in their identification. Although crab fauna of Andhra Pradesh studied using different morphological and molecular approaches, still there were ambiguities with some species in India. The present study was carried out to find out genetic divergence and to validate phylogenetic position crab fauna of Andhra Pradesh by analyzing six partial Cytochrome Oxidase I (COI) sequences of mitochondrial origin. These COI barcodes were utilized to analyze the nucleotide frequencies, and evolutionary genetic divergence by following Kimura 2-parameter model employed in MEGA 6.0. A pair wise genetic distance among the species was found increasing with higher taxonomic levels. Nearest Neighbour distance was calculated using NCBI-BlastN, clearly demarcates its nearby species. Neighbour-Joining tree generated was incongruous with morphological taxonomy based on morphology emphasizing that multiple specimen inclusion in barcoding studies. Findings of this study demonstrated the efficiency of DNA Barcoding as molecular identification tool. So, it could provide us leverage for a better understanding in management and monitoring of crab diversity.

Keywords: cytochrome oxidase I (COI), crabs, DNA divergence, Andhra Pradesh

Introduction

Crabs are well known for their high nutritional value also for delicious taste. They are good source of proteins, lipids, carbohydrates and minerals [1]. Crab meat was proved as good supplement for protein-mineral balance in human nutrition to eradicate nutritional deficiencies [2]. Sometimes, the difficulty in identification of a crab species leads to controversial because of their morphological differences caused by not only the environmental variations at species level [3, 4, 5] but also due to processed or semi processed raw food [6]. Studies reported that suppression of crab food was being carried out by replacing superior quality crab with inferior quality crab in restaurants of India [7]. In this scenario, alternative methods for specimen identification using advanced tools became a great concern for enhanced food safety as some crabs also have poisonous nature [8]. In this direction DNA Barcoding gained advantage and proved to be efficient in sea food authentication [7, 9]. Decapods belongs to an order of crustaceans estimated to contain 15000 species belongs to 2700 genera with 3300 fossil species, of which half of the species are known to be crabs [10]. In India 705 brachyuran crab species belongs to 28 families, 270 genera have been reported [11]. There were 15 edible crab species in Indian coastal region, of which 12 were commercially valuable [12]. Previous studies reported that the distribution of crabs extends up to 6000 meters depth [13]. Collection of specimen from such extreme conditions for taxonomical studies was a herculean task. Brachyuran diversity of coastal Andhra Pradesh is less explored and no previous reports found on overall species richness of crab species by following integrative approaches. Some scientists worked on particular species (e.g. *Scylla*) of crab fauna [14], fishes [15], shrimps [16] and lobsters [17]. In the context of food demand for fast growing population, marine diversity is assuring people with reliable potentiality for future needs.

Whole DNA sequence based diversity assessment, whether directly or indirectly by analysis of proteins was used for species discrimination studies almost 40 years ago [18]. Afterwards, single gene based analysis of ribosomal DNA was used extensively to investigate evolutionary relationships [19]. Recently, Mitochondrial DNA (mtDNA) dependent molecular systematic studies were gained importance over other markers [20]. mtDNA with the characteristic feature of fast evolution rate than the nuclear DNA, has recently been used to elucidate genetic relationships for many species [21, 22]. Mitochondrial Cytochrome Oxidase I (COI) gene based DNA Barcoding technique also gaining importance as a useful tool for investigating the genetic structure of species [23] apart from its identification application [24]. DNA Barcoding data enabled the researchers to read the genetic information which can be used efficiently in accurate management of species of ecosystem importance (e.g. Wetlands) [25].

In the present study, we have examined the effectiveness of COI sequence data in assessing the genetic distance between interspecific and intraspecific datasets and to calculate the nucleotide frequencies along with GC content in different codon positions for selected crabs. Finally, we verified the phylogenetic signal carried by COI data in deciphering species at different hierarchical levels.

Materials and Methods

In total, seven partial gene sequences obtained for six species (Table 1) as on 24th December, 2018. The COI partial gene

sequences obtained for each species were manually verified for presence of internal stop codons using the translate tool in ExPASy Bioinformatics resource portal with inputs related to invertebrate mitochondrial genome. Verified sequences submitted to NCBI-GenBank to get accession numbers. COI partial gene sequences obtained for each species were assembled and end-trimmed to a homologous region to avoid errors during sequencing and those sequences subjected to aligned using ClustalW analysis tool [26]. Sequences with sufficient length *i.e.* >600bp only were considered with the view of bringing uniformity in analysis across all species. To ensure homology in heterogeneous sequences, some bases were trimmed. To bring this homogeneity in some sequences, missing sequence parts were adopted from most conserved regions of the sequences available in NCBI GenBank for the same species.

Table 1: List of barcodes along with their Coordinates and NCBI-GenBank accession numbers

S. No.	Species	Family	Subfamily	Lat-long	Accession No.
1	*Portunus pelagicus*	Portunidae	Portuninae	17° 69" N, 83° 30" E	KP666120
2	*Charybdis lucifera*	Portunidae	Thalamitinae	17°69" N, 83° 30" E	KP317980
3	*Charybdis feriatus*	Portunidae	Thalamitinae	17° 24" N, 82° 32" E	MG575215
4	*Demania baccalipes (2)*	Xanthidae	Xanthinae	17° 69" N, 83° 30" E 17° 24" N, 82° 32" E	KP070740 MG575216
5	*Calappa japonica*	Calappidae	Calappinae	16°77" N, 82° 64" E	KP666121
6	*Atergatis integerrimus*	Xanthidae	Zosiminae	16°77" N, 82° 64" E	MG575217

Nucleotide composition (A, T, G, C, GC1, GC2 & GC3) calculated for homologous end-trimmed sequences using MEGA V.7.0 (Molecular Evolutionary Genetic Analysis) [27] software (Arizona). Inter and intra species evolutionary divergences in various hierarchical levels were analysed using Kimura 2 Parameter method [28]. The variation was estimated following the bootstrap method with 1000 bootstrap replicate values. The pair-wise deletion option was selected to treat the gaps or missing data between each compared specimen. The Nearest Neighbour distance was calculated for the studies crabs by performing BlastN analysis using the sequence data. The same species with highest similarity which were previously submitted were not considered. Different sequence in a row of BlastN results for a species was considered. Finally, the Neighbour-Joining (NJ) tree among species was created to give distance values using K2P method and those values are in the units of the number of base substitutions per site [29]. To verify the robustness of the nodes of the N-J tree, bootstrap analysis was carried out using 5000 pseudo replicates [30]. Both transitions and transversions were cumulated and included as substitutions. Missing bases or gaps were treated by adopting pair wise deletion method employed in MEGA V 7.0.

Results

COI gene sequence analysis

A total of seven COI sequences were analyzed for six crab species covering three families and five genus (Table 4). PCR amplified bands for COI gene was shown in Figure 4. Sequence alignment resulted in 624bases per taxon after exclusion of the primer sequence. No complexity or ambiguities were observed among all the sequencing results. Out of seven, one sequence is identified as NUMT *i.e.* nuclear DNA sequence that is originated from Mitochondrial DNA as it contains internal stop codons. It is evident that DNA Barcoding may overestimate the number of species, if nuclear mitochondrial pseudogenes are co-amplified. Results of this study recommended that utility of COI gene as DNA barcode in identification marine crabs for coastal Andhra Pradesh and also aiding in taxonomical identification using molecular tools. Because Decapods were known to be distinguished easily in their adult forms but it is difficult to identify then during larval and juvenile forms [31].

Average genetic divergence within species was 0.143% compared with 0.446% for species within genera, while genetic divergence within family is 0.834% (Table 2). Increased genetic divergence was observed with increasing taxonomic levels, indicating noticeable change in genetic divergence at the species boundaries (Figure 1). It is supporting the robustness of genetic divergence at the species boundaries [32]. The average congeneric distance is approximately 4- fold to average conspecific distance.

Table 2: Summary of COI genetic divergence values for 7 crabs of coastal Andhra Pradesh

S. No.	Comparision	Min. distance (%)	Max. Distance (%)	Mean±S.E.
1	Within species	0.00	0.143	0.143±0.016
2	With genus	0.143	0.749	0.446±0.035
3	Within family	0.667	1.001	0.834±0.105

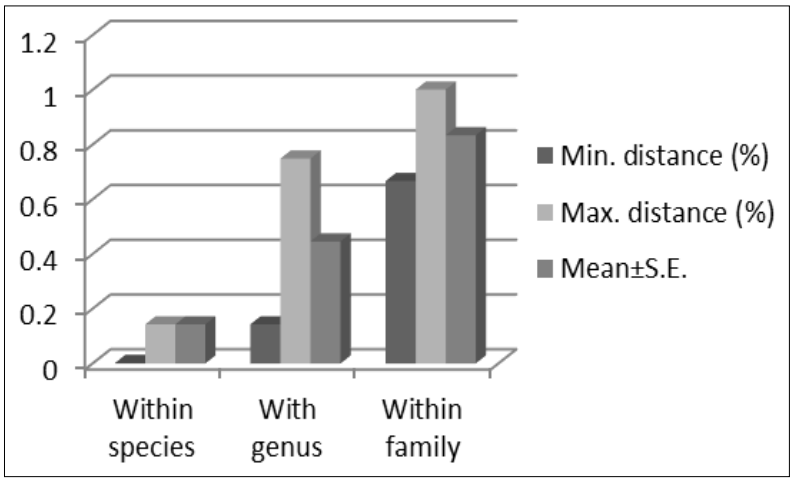

Fig 1: COI genetic divergence values at various hierarchical levels

The average nucleotide frequencies for all species are as follows: A= 25.1%, T= 29.8%, G= 21.7%, C= 23.4%. The minimum, mean and maximum GC% content for three codon positions of 6 species were 43.0, 49.8 and 42.4 respectively. (Tabe 3). Among three, GC2 content is dominated followed by GC1 and GC3. This indicating that the synonymous mutations occur mostly at GC2 followed by GC1 and GC3. Synonymous mutates The highest percentage of GC content was noticed in *Atergatis integerrimus* for all the three codon positions (70.8, 69.7 & 61.3) whereas the lowest value was observed in *Charybdis lucifera* for GC1, GC3 and *Demania baccalipes* for GC2 content. Average GC content was higher than the COI gene GC content of Arthropod dataset suggested by Keskin & Atar [33].

Table 3: Nucleotide frequencies for COI sequences of 6 commonly available crabs

S. No.	Name of species	T%	C%	A%	G%	(G+C)1	(G+C)2	(G+C)3
1	*Calappa japonica*	32.0	23.9	26.4	17.7	40.9	47.2	36.5
2	*Charybdis lucifera*	36.7	18.9	26.6	17.8	32.8	43.7	33.5
3	*Demania baccalipes*	34.5	21.9	26.4	17.0	37.2	42.9	36.8
4	*Portunus pelagicus*	36.4	21.5	24.1	18.0	34.3	45.7	38.6
5	*Atergatis integerrimus*	12.2	30.2	20.5	37.1	70.8	69.7	61.3
6	*Charybdis feriatus*	27.2	24.1	26.4	22.3	41.8	49.8	47.5
Average		29.8	23.4	25.1	21.7	43.0	49.8	42.4

Pair wise genetic distance matrix for COI gene sequences of Crab datasets were represented in Table 4, including seven sequences belongs to 6 species. The average Kimura's 2-parameter calculated genetic distance was calculated as 0.846. The maximum evolutionary genetic divergence value in COI gene was observed is 1.937 (*Atergatis integerrimus* and *Calappa japonica*) whereas the lowest value observed as 0.123 (*Demania baccalipes* and *Portunus pelagicus*).

Table 4: Estimates of Evolutionary Divergence using Kimura 2-Parameter model for Sequences of commonly available crabs in coastal Andhra Pradesh. (The number of base substitutions per site among sequences is shown below the diagonal with standard error estimates are shown above the diagonal. The first letters of the genus and species are combined to represent the species above the matrix)

S. No.	Species	CJ	CL	DB	PP	AI	CF	DB[a]
1	*Calappa japonica* (CJ)		0.021	0.021	0.024	0.742	0.074	0.025
2	*Charybdis lucifera* (CL)	0.241		0.022	0.020	0.509	0.067	0.024
3	*Demania baccalipes* (DB)	0.226	0.222		0.020	0.551	0.072	0.016
4	*Portunus pelagicus* (PP)	0.279	0.222	0.209		0.536	0.076	0.015
5	*Atergatis integerrimus* (AI)	1.937	1.754	1.858	1.832		0.236	0.522
6	*Charybdis feriatus* (CF)	0.924	0.866	0.930	0.928	1.653		0.077
7	*Demania baccalipes* (DB[a])	0.292	0.283	0.143	0.123	1.870	0.970	

The nearest-neighbor distance (NND) analysis revealed that all NND values are above 5% (Table 5).The average NND value was 11.7 which is almost 75 times higher than the mean intra- specific distance value (0.143%). The minimum NND value was observed as 06 between *Portunus pelagicus* and *P. segnis* whereas the maximum NND value observed as 18 between *Charybdis feriatus* and *Charybdis sagamiensis*. Hubert *et al.* [34] identified 20% of NND values below 1% when barcoded Canadian fresh water fish fauna. This might be due to more number of samples included. However, among all sequences COI gene of *Atergatis integerrimus* was identified as NUMT or pseudogene, emphasizing the need for more number of specimens to be included in barcoding.

Table 5: Nearest species based on Blastn search and their nearest neighbor distance values

S. No.	Species	Nearest neighbor	Nearest neighbor distance (%)
1	*Calappa japonica*	*Calappa gallus*	16
2	*Charybdis lucifera*	*Charybdis helleri*	11
3	*Demania baccalipes*	*Pulcratis reticulatus*	07
4	*Portunus pelagicus*	*Portunus segnis*	06
5	*Atergatis integerrimus*	Identified as pseudogene	
6	*Charybdis feriatus*	*Charybdis sagamiensis*	18
7	*Demania baccalipes*	*Portunus pelagicus*	12
Average NND value			**11.7**

The Neighbor-Joining tree derived by using sequences of all 7 sequences of 6 crabs with MEGA 6.0 is shown in figure 2. The N-J tree formed the clades with species belongs to same genus (*Demania* sps.; *Charybdis feriatus* and *Charybdis lucifera*) under different clades with significant boot strap values. Subsequently, even at the familial level also, the scenario was appeared as expected after genus level clades. Except for *Demania baccalipes* and *Demania baccalipes*, all the remaining species formed separate clusters with bootstrap values ranging from 39 as minimum up to 98 as maximum values. *Atergatis integerrimus* which was identified as pseudogene formed as distinguished clade below the tree which was also given inference as NUMT gene.

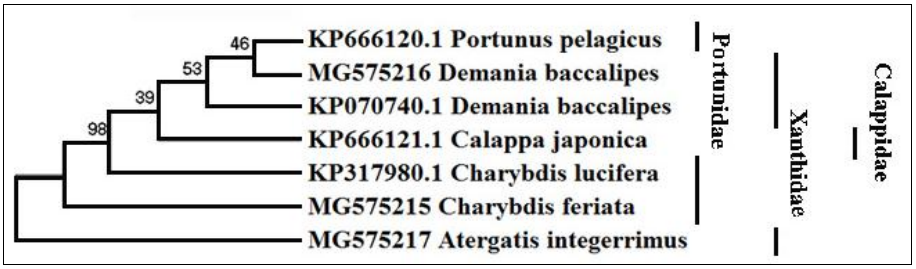

Fig 2: Neighbor-Joining tree for partial sequence of mitochondrial COI gene of seven crabs

Conclusion

The current study, data on species diversity and abundance showed that coastal area of Andhra Pradesh harbors a diverse group of bracyuran crabs. Several destructive fishing practices and anthropogenic activities like over fishing, habitat degradation, disposal of industrial and domestic sewage which affects the coastal plant ecosystem as they provide habitat, breeding grounds and feeding for many brachyuran crabs. Overall, this data would helps in further monitoring of anthropogenic inputs on crabs from coastal Andhra Pradesh. All the species analyzed in current study has both economical and ecological impact on biodiversity of fishery biodiversity in coastal Andhra Pradesh. These results will also improve our knowledge on population structures, revealing cryptic species of the coastal aquatic diversity of Andhra Pradesh.

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