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DNA testing of edible crabs from seafood shops on the Odisha coast, India

https://doi.org/10.1515/bmc-2018-0002
received July 3, 2017; accepted February 21, 2018.

Abstract: Seafood consumption is highly demanding due to the important source of protein it contains, as well as being rich in omega-3 fatty acids. However, the adulteration of seafood is an alarming issue worldwide, including India. This study deals with edible crabs from seafood shops on the Odisha state coast in eastern India. The generated DNA barcode sequences successfully identified most of the studied brachyuran crab species by similarity search results in global databases. The species were also delimited by significant genetic divergence and Neighbour-Joining phylogeny. Additionally, the study detected the contamination of unknown organisms in the commercialized crab recipes from seafood shops. The DNA based species detection of brachyuran crab may be useful to resolve many ambiguities in species identification and monitoring of commercialized seafood concerning food safety.

Keywords: Seafood; Adulteration; Decapoda; DNA barcoding; Eastern India.

Introduction

Seafood is one of the 'highly dealt lucrative commodities' around the world (1). Due to the high consumption rate, importation and the globalization of the seafood industry, over 8000 tons of marine species (i.e. fishes, crabs, prawns, squids etc.) were captured by fishermen communities during 2010-2011 in India (2). However, in food safety concern of each commodity; food regulators are enforcing proper labeling of food products based on the composition and purity (3). The economically motivated food fraud is a risk that is gaining recognition and concern in recent days (4). The adulteration of seafood products is gradually increasing in various coastal regions throughout the globe and is posing a greater risk to consumer health (5,6).

The sea coast of the Odisha state lies in between 21.61N, 87.48E and 19.12N, 84.79E and is known as a popular tourist place in India. The total length of the coastline is about 480 kilometres, covering six districts. The region has a unique biodiversity composition, from wide spread mangroves, to many endemic and rare faunal elements (7). The coastal and offshore waters also form a rich abode of many crustacean resources (8). After marine fishes, the brachyuran crabs and prawns are some of the most highly consumed seafood, with a high demanding market value. Every year many people visit this place for recreation purposes and for seafood consumption. Unfortunately, the consumers are often unable to detect the cooked or untagged seafood recipes offered by the locals due to the missing of external morphology.

The survey of brachyuran crabs in Odisha state was started long back ago (7). A total of 140 species of brachyuran crabs belonging to 79 genera of 30 families has been recorded from a wide range of habitats in the Odisha coast (8,9). Earlier, the classifications of brachyuran crabs were described based on the shape, size, texture of carapace and buccal frame (9). Later on, the position of gonopore was charaterized as an important taxonomic character for accurate species identification (10). However, the study with gonopore is difficult for a non-taxonomist. The partial fragment (~650bp) of mitochondrial DNA (mtDNA), the Cytochrome C oxidase subunit I (mtCOI) gene, has been standardized to identify the brachyuran crabs (11). So far, very limited studies have been conducted for generating DNA barcode data from taxonomically identified brachyuran crabs in India (12). The molecular tools have

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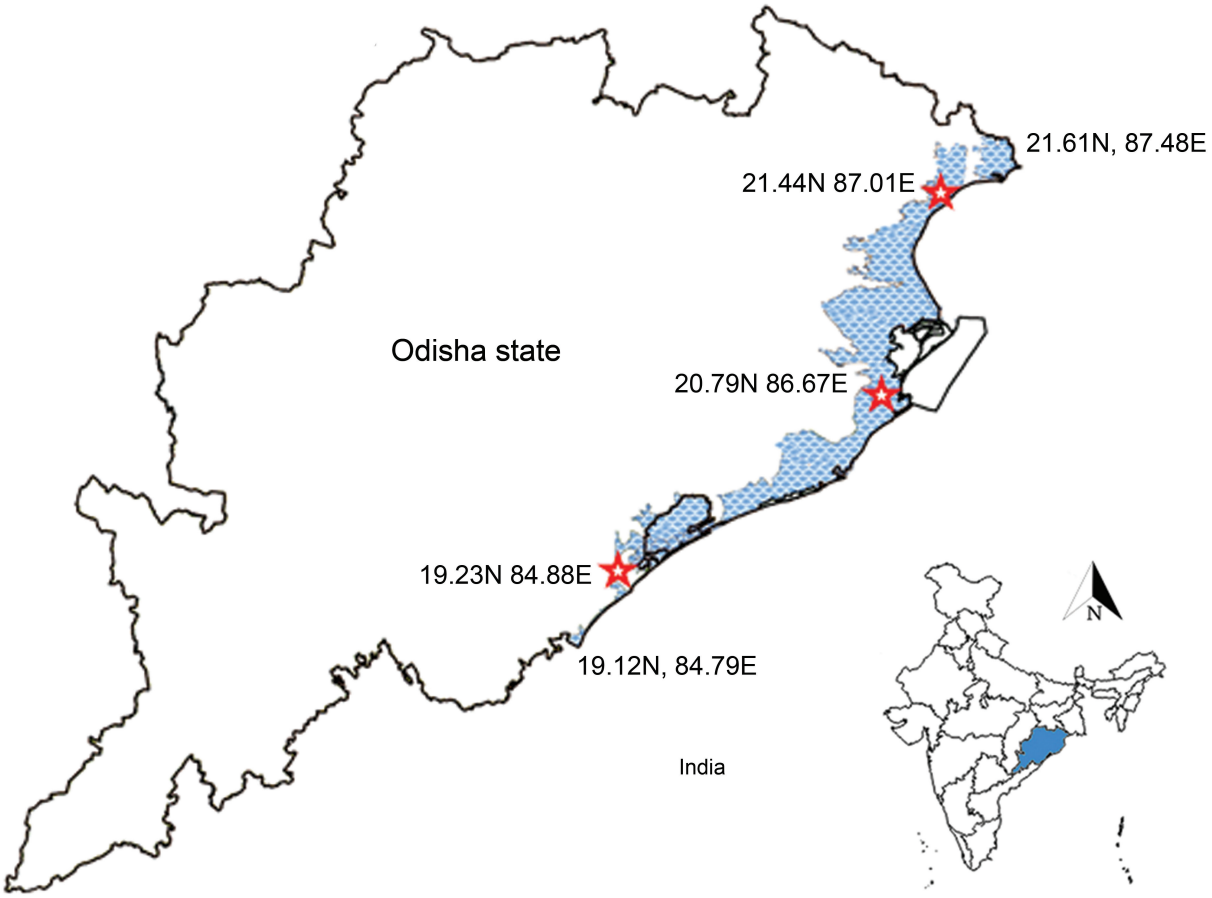

Figure 1: Map showing the coastal region of Odisha state by Blue dots and collection localities by Red star marks. The geographic coordinates of the coastal region and the sampling sites are superimposed on the map. The map of India is added on inset with marking of the Odisha state by blue color.

also not been used to detect the seafood products being commercialized in the coastal regions of India. Therefore, this research aimed to determine the efficacy of mtCOI gene to identify the brachyuran crabs from a seafood shop on the Odisha coast. This baseline DNA data would enrich the global database and substantiate the identification of brachyuran crabs from India, as well as help to detect the commercial seafood fraud.

Materials and Methods

Sampling and laboratory analysis

A total of nine samples (six whole body brachyuran crabs and three amorphous fresh tissue samples processed for crab recipes) were collected from seafood shops in three different locations: Chandipur (21.44N 87.01E), Chandbali (20.79N 86.67E) and Gopalpur (19.23N 84.88E) in Odisha state (Figure 1). The whole body specimens were preliminary identified by available taxonomic keys (10,13). The muscle tissues were obtained from the chelate leg of whole specimens, preserved in 70% alcohol and deposited in the Crustacea Section of Zoological Survey of India, Kolkata. Further, the amorphous fresh tissue samples were directly collected in 500µl ATL buffer containing 50mM Tris-HCl (pH 8.0), 25mM EDTA (pH 8.0), and 150mM NaCl, for DNA analysis. The total genomic DNA was extracted by standard Phenol-chloroform extraction method with Proteinase K (200µg/ml) (14). The extracted DNA was checked by 1.5% Agarose gel electrophoresis using standard protocol. The published primer pair was used to amplify the partial mtCOI gene segment (15). The 25µl PCR reaction mixture contains 10 pmol of each primer, 10-20 ng of DNA template, 1x PCR buffer, 1.0-1.5 mM of $MgCl_2$, 0.25 mM of each dNTPs, and 0.25 U of high-fidelity TaqDNA polymerase. The thermal profile for PCR was set as initial denaturation at 94ºC for 2 minutes, followed by 30 cycles at 94ºC for 45 seconds, 50ºC for 45 seconds and 72ºC for 1 minute, and subsequent

Table 1: Similarity search results of the studied sample in both GenBank and BOLD database.

Voucher ID	Morphological identification	Accession No.	Highest similarity search in GenBank		Highest similarity search in BOLD	
			%	with	%	with
ZSI_CR1	*Portunus pelagicus*	MF043861	99	*P. pelagicus*	99.69	*P. pelagicus*
ZSI_CR2	*Tubuca rosea*	MF043862	98	*U. rosea*	98.08	*T. rosea*
ZSI_CR4	*Neosarmatium asiaticum*	MF043863	99	*N. asiaticum*	99.34	*N. asiaticum*
ZSI_CR7	*Charybdis vadorum*	MF043864	100	*C. vadorum*	100	*C. vadorum*
ZSI_CR8	*Galene bispinosa*	MF043865	100	*G. bispinosa*	99.85	*G. bispinosa*
ZSI_CR10	Tissue sample	MF043866	83	*Octolasmis hawaiense*	83.46	*Balanus balanus*
ZSI_CR11	*Calappa* cf. *lophos**	MF043867	96	*C. lophos*	96.02	*C. lophos*
ZSI_CR12	Tissue sample	MF043868	84	*Sacculina sp.*	83.02	*Sacculina sp.*
ZSI_CR14	*Matuta planipes*	MF043869	98	*M. planipes*	99.5	*M. planipes*

**Calappa* cf. *lophos* indicates that the specimen is in the genus *Calappa*, and believed to be *Calappa lophos* but the actual species-level identification cannot be certain.

storage at 4ºC. The amplification was performed using a Veriti® Thermal Cycler. The PCR products were purified by using QIAquickR Gel extraction kit and cycle sequencing products were cleaned using standard BigDye X Terminator Purification Kit. The bidirectional sequencing was performed by the 48 capillary array, Applied Biosystems 3730 DNA Analyzer, in the in-house sequencing facilities of Zoological Survey of India, Kolkata.

Similarity search, genetic distance and phylogenetic analysis

The low quality regions were trimmed at both end and ambiguous bases greater than 2% were discarded, using a quality value of >40 for both bidirectional chromatograms reads. The nucleotide BLAST (BLASTn) program was used to further evaluate the sequences with no gaps, and the ORF finder to examine the complete alignment of protein coding genes without stop codons (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Finally the generated sequences were submitted in global database (GenBank) to acquire the specific accession numbers. Further, based on the BLASTn results, eight closely related sequences were acquired from the GenBank database. The generated and published database sequences were aligned using ClustalX software to form a combined dataset (16). The generated sequences were identified through the online identification system, in GenBank with 'Highly similar sequences (megablast)' and BOLD databases with 'All Barcode Records on BOLD' search engine. The genetic distances of the dataset sequences were analyzed through the Kimura 2 parameter (K2P) model in MEGA6, due to the transitional and transversional substitution rates. Further, to test the monophyletic clustering of the studied species, Neighbour-Joining (NJ) phylogeny was established by using MEGA6 with the K2P model and 1000 bootstrap replications (17).

Results and Discussion

The DNA barcode sequences detected five brachyuran crabs, *Portunus pelagicus* (family Portunidae), *Tubuca rosea* (family Ocypodidae), *Neosarmatium asiaticum* (family Sesarmidae), *Galene bispinosa* (family Xanthidae), and *Matuta planipes* (family Matutidae) with 98% to 100% similarity to the same species in both GenBank and BOLD database (Table 1). However, one sample (ZSI_CR11) showed 96% similarity with the *Calappa lophos* (Accession no. KX757761, generated from the southern coast of India) in GenBank. The *C. lophos* (family Calappidae) is a highly argued species widely distributed in the Indian Ocean to the western Pacific, including the Andaman Sea, Japan, Taiwan, and Australia (18). Due to the low identity, as depicted in the similarity search result, we further examined the genetic divergence of generated and publicly available database sequences of *C. lophos*. The incongruency in genetic divergences between the two published sequences of *C. lophos* and the generated sequence (high genetic divergence with AY579999: collected from Phuket, Pichai Fish Port, Thailand and low genetic divergence with KX757761: collected from the southern coast of India) depicts inconclusive identification of *C. lophos* in this study. This study remarks that the generation of more DNA barcode data of *C. lophos* from different geographical regions would further authenticate the understanding about the species.

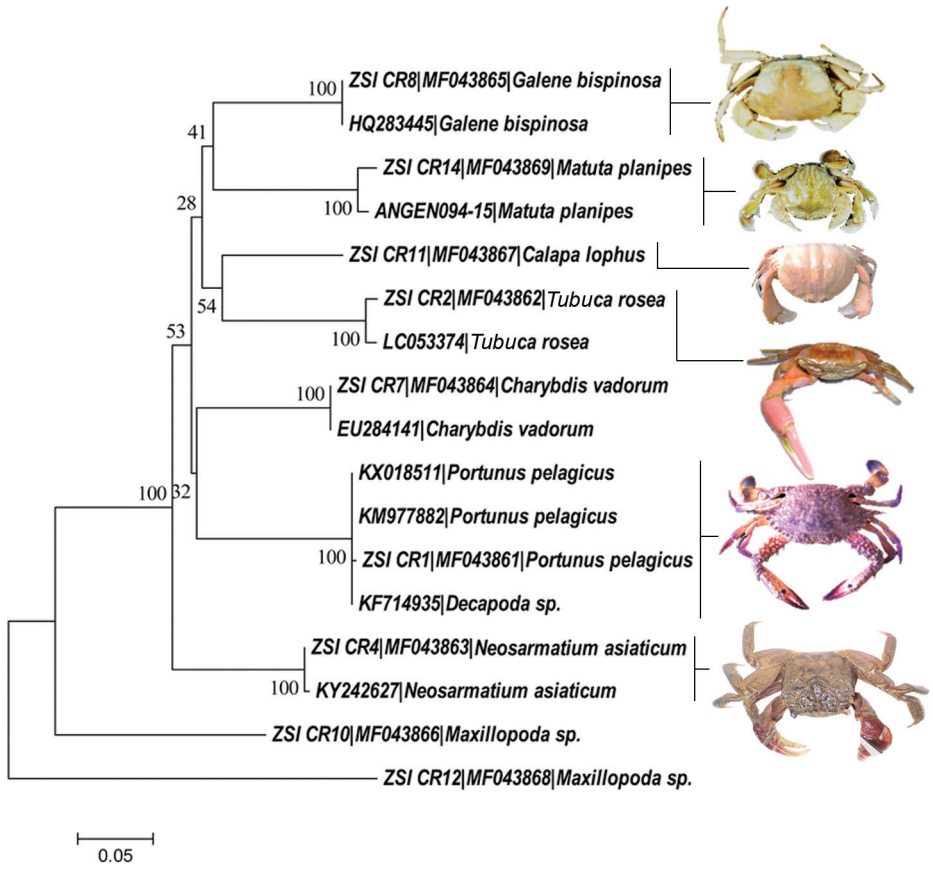

Figure 2: Neighbour-Joining tree of the studied crab species. Bootstrap supports are mentioned in each node. Voucher IDs, GenBank accession numbers, and species name are indicated within parentheses. Photographs of Crab species taken by the first author are superimposed on the tree.

The *N. asiaticum* is distributed from Sri Lanka through the Andamans, ending in Indonesia and Taiwan (19). In this study, we detected the genetic data of *N. asiaticum* from the sample (ZSI_CR4) obtained from seafood shops. We assumed the occurrence of *N. asiaticum* from the same locality, since the fishermen community caught the marine fauna near the seafood shop location, and sold the same products to the seafood shops (personal observation). Hence, the study provides the genetic record of *N. asiaticum* in the coastal region of the Odisha state, and assumes a new distribution record of the species. With this preliminary observation, further rigorous taxonomic studies can be completed to confirm the distribution of *N. asiaticum* in Odisha. Further, the sequence generated from the amorphous fresh tissue sample (ZSI_CR7) shows 100% similarity with *Charybdis vadorum* (family Portunidae) in both NCBI and BOLD databases. The DNA data of two more tissue samples (ZSI_CR10 and ZSI_CR12) shows 83% to 84% highest similarity with the species (*Octolasmis hawaiense*, *Balanus balanus*, and *Sacculina sp.*) of class Maxillopoda in both GenBank and BOLD database, and thus the crab species identification remains unknown in this case. Nevertheless, this study suspected contamination of unknown organisms in the commercialized crab tissue samples, which are being processed for crab recipes. Therefore, this study suggests adoption of proper management to avoid the seafood contamination in the coastal region of India.

In conclusion, this study detected a total of six edible brachyuran crab species from the seafood shops. The intra-species K2P genetic divergence among the dataset ranged from 0% to 1.9%. The NJ tree resulted monophyletic clade of each studied species with 100 bootstrap supports. In this dataset, *G. bispinosa* and *C. lophus* show a close relationship with *M. planipes* and *T. rosea* respectively. Further, the *C. vadorum* show a close relationship with *P. pelagicus* and *N. asiaticum*, forming a distinct clade from all studied species (Figure 2). The generated sequences of the unknown organisms here associate as an out-group. Thus, the utility of DNA barcodes to detect seafood products in this case study revealed a number of implications pertaining to correct species identification

and management efforts (20). The contributed DNA data in the global database further helps to strengthen the species identification library. Further, the tagging of DNA data offers proper regulatory compliance in the export of seafoods in international markets (21). Moreover, the technique is emerging as a reliable tool to assure all consumers concerning food authentication or food safety.

Acknowledgement: We thank the Director of ZSI, MoEF&CC for providing necessary permissions and facilities. We acknowledge the research funding from ZSI, MoEF&CC in-house project to the second author VK and DST-SERB National Post-Doctoral fellowship (F. No. PDF/2015/000302) to the third author SK. The funders had no role in study design, data collection and analysis or preparation of the manuscript.

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