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All of a Piece: Identification of the Different Life Stages of *Scylla serrata* (Forsskål, 1775) using DNA Barcodes

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Abstract

DNA barcoding is an effective tool for the precise identification of a species. This method has so far been employed in only a few studies related to larval identification of marine animals. Such studies are rather rare as far as the identification of their eggs is concerned. The present study tests the efficacy of DNA barcoding using the partial mitochondrial cytochrome oxidase *c* subunit-I (COI) gene in identifying different life stages (eggs, larvae and adults) of a portunid crab, *Scylla serrata* (Forsskål, 1775). From the genetic distance analysis, it is clear that the COI gene for the eggs and larvae of *S. serrata* remained stable and shared a relatively minimal variation in relation to their adult counterparts when compared to that of a congener, *Scylla olivacea* (Herbst, 1796). The eggs, larvae and adults of *S. serrata* all form a single clade in the phylogram, which was very distinct from *S. olivacea*. Furthermore, BLAST analysis revealed that eggs, larvae and adults of *S. serrata* obtained during the present study shared a maximum similarity in the COI gene with that of a previously sequenced adult *S. serrata*. The similarity of the gene was relatively low in the congener and a confamilial, *Callinectes sapidus* (Rathbun, 1896). We concluded that DNA barcoding can be safely applied in quantifying standing stock of eggs and larvae, which is useful for forecasting of commercial fisheries. Moreover, DNA barcoding makes ecological research independent of future taxonomic changes of a species and keeps the ecological studies comparable forever.

Keywords: DNA barcoding; India; Portunidae; *Scylla* spp.; COI gene

1. Introduction

Scylla being one of the important genus of Portunidae and contains economically important species of mud crab, *Scylla serrata* is vast, diverse and preferred widely for its size and meat (Marichamy and Rajapackiam, 2001). Along with Portunids, Brachyura is bestowed with more than 900 species of crabs in Indian waters (Trivedi *et al.*, 2018). A vast number of species were recorded from East coast and west coast represents only 49 percent of more diverse East coast of Peninsular India. Recent studies from west coast suggested the possibility of occurrence of more species on brachyuran diversity (Biju Kumar *et al.*, 2007; Trivedi *et al.*, 2012; Biju Kumar *et al.*, 2013; Lakshmi Devi *et al.*, 2015; Pati *et al.*, 2017; Trivedi *et al.*, 2018). In biodiversity studies, the species identification is often restricted to genera or families or even orders, especially in complicated groups (Gaston and O'Neill, 2004). The correct identification of species can only be confirmed by a taxonomist. The number of taxonomic experts, however, is declining nowadays (Wilson, 1994; Lai *et al.*, 2010; Puillandre *et al.*, 2011; Kumar *et al.*, 2012). Marine fauna inventories have been failed in species-level identification of about one-third of specimens (Schander and Willassen, 2005), and the existence of cryptic species in the marine ecosystem further impedes the biodiversity assessments (Etter *et al.*, 1999; Knowlton, 2000; Bezeng and Bank, 2019).

Hebert *et al.* (2004) proposed DNA barcoding, in which, a short nucleotide sequence of the mitochondrial genome may act as a DNA barcode for species identification of all eukaryotes, particularly animals. This technology has proven to be a rapid tool for precise identification of biological specimens. DNA barcoding has turned out to be a precise tool in larval identification of marine animals (Barber and Boyce, 2006; Pegg *et al.*, 2006; Webb *et al.*, 2006; Victor *et al.*, 2009; Hubert *et al.*, 2010; Ko *et al.*, 2013; Brandão *et al.*, 2016; Wibowo *et al.*, 2016; Collet *et al.*, 2018; Lona *et al.*, 2019). On the other hand, studies related to the identification of eggs of marine animals using DNA barcoding are quite rare. Unlike traditional methods, the DNA barcoding method has a great advantage of identifying an organism accurately at any stage of development because the mitochondrial genome and its sequence orders remain stable during the entire life cycle of an animal. Of the 26,000 crustacean species, 53% species pass through an indirect life cycle that involves more than three larval stages (Pandian, 1994). We hypothesize that the partial mitochondrial cytochrome oxidase *c* subunit-I gene (COI) sequences of eggs and larvae of *Scylla serrata* (Family:Portunidae) will share relatively minimal variations in relation to their adult counterparts when compared to that of a closely related species of the genus. DNA barcoding will, therefore, facilitate accurate identification of species' eggs and larvae. Previous studies showed that DNA barcoding is an important tool for the identification of the life stages of a various marine organisms (Barber and Boyce, 2006; Pegg *et al.*, 2006; Victor *et al.*, 2009; Hubert *et al.*, 2010;

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Raupach and Radulovici, 2015). The present study was undertaken to test the hypothesis in a brachyuran crab for the first time in India.

2. Materials and Methods

2.1 *Sampling and DNA barcoding*

Adult, larva and egg samples of *S. serrata* were collected from the hatchery facility of Rajiv Gandhi Centre for Aquaculture (RGCA), Sirkazhi, Tamil Nadu, India (Table 1). The egg clusters, larvae (zoea) and adults of *S. serrata* were preserved in 95% ethanol and transported to the laboratory. Samples were replaced with fresh ethanol (95%) when the preservative changed its colour. Samples were maintained at 4°C until DNA isolation. DNA extraction from adult specimens was carried out as per standard protocols (Ward *et al*., 2005). Amplification was made using the forward primer LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and reverse primer HCO2198 (5'-TAAACTTCAGGGTGACC AAAAAATCA-3') (Folmer *et al*., 1994). The PCR was set up to hot start at 94°C for 1 minute followed by 5 cycles of 94°C for 30 seconds. Then annealing was done at 45°C for 40 seconds; extension at 72°C for 1 minute and 35 cycles of 94°C for 30 seconds; succeeded by 51°C for 40 seconds to complete the annealing and extension was carried out subsequently at 72°C for 1 minute. The final extension was conducted at 72°C for 10 minutes. The PCR product was quality checked in 1.2% agarose gel electrophoresis.

All PCR products were purified using the Shrimp Alkaline Phosphatase (SAP) Exonucleose (Exo) method (Werle *et al*., 1994). SAP removes carry-over dNTPs and oligonucleotides from the PCR reaction. Nucleotide sequences were determined by automated bi-directional cycle sequencing using the Big Dye™ terminator sequencing kit using an ABI 3130 automated capillary sequencer ABI 3130 (Bioserve Biotechnologies, Hyderabad, India). In some specimens PCR amplification and sequencing reactions were repeated either to improve the number of base pairs recovered or quality of the final sequence. Only good quality sequences derived from the eggs, larvae and adults have been included in the analysis. All nucleotide sequence chromatograms were initially evaluated for quality using Chromas Pro version 1.5. Forward and reverse chromatograms were aligned using BioEdit (Hall, 1999), and the final sequence length of > 640 bps was considered as near full length sequences and are taken for further sequence analysis. The COI sequences of eggs, larvae and adults of *S. serrata* were submitted to GenBank at the National Center for Biotechnology Information (NCBI) and the accession numbers were given (Table 1).

2.2 *Molecular/genetic distance analysis*

In order to study the species level resolution of the barcode gene in identifying immature forms (especially eggs and larvae), The pairwise estimates of Kimura 2 Parameter (K2P) distances (Kimura, 1980) between different life stages of *S. serrata* were calculated using MEGA version 4.0 software (Tamura *et al*., 2007). Whereas, The phylogram was drawn using Neighbor-Joining (N-J) method in the same software. The outgroup and reference sequences used in the present study (*S. serrata*, *S. olivacea*, *C. sapidus*) were retrieved from GenBank and their accession numbers were given in Table 4.

3. Results

The COI region in all the samples was ranged from 641 to 659 bps. The mean K2P distance of the selected life stages of *S. serrata* was 0.01% (ranging from 0.00% to 0.02%) (Table 2). This indicated that the different life stages of *S. serrata* possess a low variation in COI sequences. *Scylla serrata* appeared to be relatively more close to the congener *S. olivacea* (with 13.5% pairwise distance) than a confamilial, *C. sapidus* (with 18.36% pairwise distance) (Fig. 1). The mean Adenine-Thymine (AT) and Guanine-Cytosine (GC) contents of the selected life stages of *S. serrata* were calculated to 64.48% and 35.52%, respectively (Table 3). The maximum AT content (65.14%) was found in NAT1 (egg) whereas the minimum AT content (63.49%) was for NAT9 (adult). The maximum and minimum GC contents were observed in NAT9 (36.51%) and NAT1 (34.86%), respectively. Overall, the sequences were heavily AT biased.

In the phylogram constructed using N-J method, the eggs, larvae and adults of *S. serrata* all form a single clade. On the contrary, *S. olivacea* form a distinct clade from the different life stages of *S. serrata* (Fig. 2). BLAST analysis further showed that the COI sequences of eggs, larvae and adults of *S. serrata* obtained during the present study shared a maximum similarity (mean = 99.11%) with that of a previously sequenced adult *S. serrata* (Table 4). While the percentage of similarity in COI sequences was high for the same species, it is relatively low in the congener, *S. olivacea* (88.00%) and confamilial, *C. sapidus* (84.77) (Table 4).

Table 1. Egg, larva and adult samples of *Scylla serrata* used for DNA barcoding

Life stage		Code used	GenBank Accession numbers	Number of samples
Egg	female1	NAT1	JN085428	5
	female2	NAT2	JN085429	5
	female3	NAT3	JN085430	5
Larvae	zoea1	NAT4	JN085431	5
	zoea3	NAT5	JN085432	5
	zoea5	NAT6	JN085433	5
Adult	adult1	NAT7	JN085434	5
	adult2	NAT8	JN085435	5
	adult3	NAT9	JN085436	5

Table 2. Matrix of percentage pairwise nucleotide divergence with K2P distance based COI sequences between different life stages of *Scylla serrata* (also including the outgroup *Scylla olivacea*)

Code	1	2	3	4	5	6	7	8	9	10
NAT5 (1)										
NAT6 (2)	0									
NAT2 (3)	0.008	0.008								
NAT7 (4)	0.008	0.008	0							
NAT1 (5)	0.008	0.008	0	0						
NAT3 (6)	0.013	0.013	0.005	0.005	0.005					
NAT4 (7)	0.018	0.018	0.01	0.01	0.01	0.013				
NAT8 (8)	0.013	0.013	0.021	0.021	0.021	0.018	0.011			
NAT9 (9)	0.013	0.013	0.021	0.021	0.021	0.018	0.011	0		
Scylla olivacea (10)	0.942	0.942	0.961	0.961	0.961	0.968	0.963	0.954	0.954	

Table 3. Percentage composition of nucleotides (A, T, G, C, AT and GC) in *Scylla serrata* strains

Code	A	T	G	C	AT	GC
NAT5	27.83	37.16	16.21	18.81	64.98	35.02
NAT6	27.96	36.87	16.28	18.89	64.82	35.18
NAT2	27.62	37.18	16.08	19.12	64.8	35.2
NAT7	27.66	37.23	16.11	19	64.89	35.11
NAT1	27.68	37.46	16.06	18.81	65.14	34.86
NAT3	27.55	36.84	16.41	19.2	64.4	35.6
NAT4	27.36	36.63	16.54	19.47	63.99	36.01
NAT8	27.3	36.5	16.72	19.48	63.8	36.2
NAT9	27.3	36.19	17	19.5	63.49	36.51
S. olivacea	29.29	35	13.86	21.86	64.29	35.71
Mean					64.48	35.52

Table 4. Percentage of similarity of COI sequences of eggs, larvae and adults of *Scylla serrata* obtained from the present study with the adults of *Scylla serrata*, *Scylla olivacea*, and *Callinectes sapidus*

Life stages of *Scylla serrata*	Percentage of similarity (%)		
	***Scylla serrata* (FJ827758)**	***Scylla olivacea* (FJ827760)**	***Callinectes sapidus* (AY682075)**
NAT1	100	88	85
NAT2	100	88	85
NAT3	99	88	85
NAT4	99	88	85
NAT5	99	88	85
NAT6	99	88	85
NAT7	100	88	85
NAT8	98	88	84
NAT9	98	88	84
Mean	99.11	88	84.77

4. Discussion

Our data (as deciphered from K2P distance, phylogram and BLAST analysis) strongly support the fact that the COI sequences of eggs and larvae of *S. serrata* will share a relatively minimal variation in relation to their adult counterparts when compared to that of a congener. DNA barcoding is, therefore, an effective method that can be employed successfully for the identification of eggs and larvae of an animal.

Quantification of standing stock of eggs and larvae is useful in the forecasting of commercial fisheries (Cadima, E.L., 2003). Quantifying stock of fishery resources is nevertheless impacted due to inaccuracy in species identification. In this situation, DNA barcoding can be employed successfully for stock assessment studies since the method can identify a species at any of its life stages. The efficacy of the barcoding approach relies on the existence of a 'barcoding gap' between intra- and inter-specific divergence (Meyer and Paulay, 2005). This gap is created by the fact that mutation is more frequent than speciation, therefore, lineages diversify more quickly between species than within species (Pons *et al.*, 2006). In the present study, the barcoding gap was minimum (0.012%) within the selected life stages of *S. serrata* while the interspecies distance was high (13.5% for *S. olivacea*). This suggests that no overlap in the distribution of divergence between the species. Recently, the presence of two mud crabs (*S. serrata* and *S. olivacea*) in Indian coastal waters was revealed with the help of DNA barcoding (Mandal *et al.*, 2014). That is why these two species along with *C. sapidus* were included in the pairwise distance analysis in order to confirm that all the species are distinct from each other with minimum variations in COI sequences within the species. In the phylogram drawn, the sequences of eggs, larvae and adults

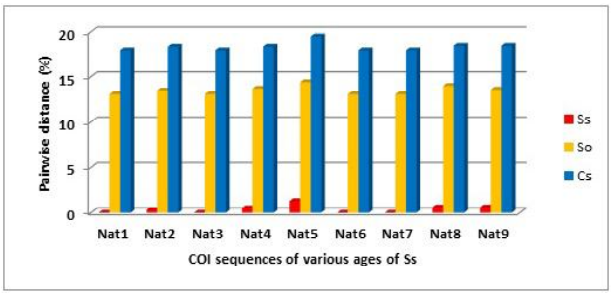

Fig. 1. Pairwise distance between COI sequences of all ages of *Scylla serrata* (Ss) and two other members of the family viz. *Scylla olivacea* (So) and *Callinectes sapidus* (Cs).

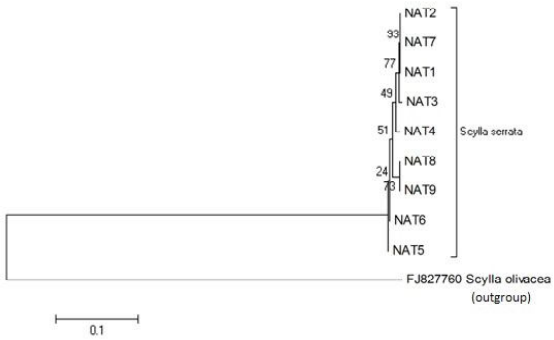

Fig. 2. Phylogram drawn using the sequences of eggs, larvae and adults of *Scylla serrata*, with *Scylla olivacea* as an outgroup.

of *S. serrata* formed an expected ingroup leaving *S. olivacea* in a distinct clade and justified its role as an outgroup.

The pattern of divergence (pairwise distance data) together with the topology of species' genealogies (phylogenetic analysis) supports that barcoding with COI is effective for the species-level identification of the selected life stages of *S. serrata*. When applied in the field for identification of eggs and larvae, DNA barcoding will certainly expand our perception in biomonitoring as the need for species-level identification in biomonitoring is litigious (Bailey *et al.*, 2001; Lenat and Resh, 2001). It can also guarantee a consistent quality of results in those studies in which the worth of taxonomic data might be negotiated by the inability to identify eggs or early instars or smashed specimens or fragments of specimens (Stribling *et al.*, 2003).

In the ecosystems with no obvious physical barriers (e.g., between two mangrove ecosystems along the shoreline), assessing the determinants of connectivity is a priority for conservation practices (Mora *et al.*, 2006; Claudet *et al.*, 2008). In marine systems, connectivity is widely assessed through analysis of gene flow (Jones *et al.*, 1999; Almany *et al.*, 2007), which may be due to the mobility of eggs and larvae. Community level processes (such as competitive exclusion, assortative settlement and habitat selection), however, may strongly influence the species distribution and thereby the community connectivity (Loreau and Mouquet, 1999; Mouquet and Loreau, 2002; Webb *et al.*, 2002; Leibold *et al.*, 2004). The improved taxonomic resolution delivered by DNA barcoding also would afford more sensitive measures of the magnitudes and types of environmental impacts (Lenat and Resh, 2001). The hypothesis tested herein was found to be positive, and it is clearly reflected in the present study. DNA barcoding of planktonic eggs and larvae will, therefore, greatly enhance our perceptivity in devising and understanding conservation protocols. In a way, DNA barcoding makes ecological research independent of future taxonomic changes of a species and keeps the ecological studies comparable over space and time. In the past, nearly 75% of the larvae collected from the Antarctic region (Heimeier *et al.*, 2010) and 50% of the larvae collected from the Indo-Pacific region (Barber and Boyce, 2006) could not be identified up to species rank. Thanks to DNA barcoding, all these can be now linked to their respective adults. There is, however, an urgent need to expand the COI sequence data for all the known species.

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