ISSN 2321–340X

Journal of Aquatic Biology & Fisheries | Vol. 7 | 2019 | pp. 24 - 30

IDENTIFICATION OF EGGS, LARVA AND ADULTS OF *SCYLLA SERRATA* (FORSSKÅL, 1775) USING DNA BARCODES

Ashiq Ur Rahman, M.,*[1,2] Prasanna Kumar, C.,[2] Mohanchander, P.,[2,3] Manikantan, G.,[2] Ajmal Khan, S.[2] and Lyla, P.S.[2]

[1]Department of Zoology, Hajee Karutha Rowther Howdia College, Uthamapalayam - 625 533, Tamil Nadu, India
[2]Centre of Advanced Study in Marine biology, Faculty of Marine Sciences, Annamalai University, Parangipettai - 608 502, Tamil Nadu, India
[3]Department of Zoology, Madras Christian College, Tambaram, Chennai - 600 059, Tamil Nadu, India
**Email:ashique.md@gmail.com*

Abstract: DNA barcoding of eggs, larvae and adult of *Scylla serrata* (Forsskål, 1775) from a hatchery facility was made. The average genetic distance of the selected stages of *S. serrata* was low at 0.012% with a minimum of 0.00% and maximum of 0.021%. However the distances with other species of the same genus were also tested 13.5% for *Scylla olivacea* (Herbst, 1796). Overall, the sequences were heavily AT biased with 64.5%. The COI region in all the samples was in the range of 641-659bp. BLAST analysis revealed that COI sequences of the various stages of *S. serrata* of the present study had more similarity (average=99.11%) with previously sequenced adult *S. serrata* which showed that barcode gene variations are comparatively less within a species than between species (88% for *S. olivacea*). The similarity of the gene was relatively low in the congener and a confamilial, *Callinectes sapidus* (Rathbun, 1896). Phylogram drawn using the above sequences belonging to various stages of *Scylla serrata* clustered all the stages of *S. serrata* in the same clade, proving the taxonomic ability of the COI gene. DNA barcoding of planktonic eggs and larvae will greatly enhance our understanding of their dynamics that way the stock position and of immense help in evolving conservation strategies.

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

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 taxonomic resolution level is often restricted to genera, families or even orders, at least in the intricate groups (Gaston and O'Neill, 2004). The shortage of taxonomists and demand for precise species identification is increasing worldwide (Wilson, 1994; Lai *et al.,* 2010; Puillandre *et al.,* 2011; Kumar *et al.,* 2012). Therefore marine faunal inventories employed in biodiversity assessment fail to identify about one-third of specimens to the species level (Schander and Willassen, 2005) and the existence of cryptic species in the marine ecosystem (Knowlton, 2000; Etter *et al.,* 1999; Bezeng and Bank, 2019) further compounds the assessments. Hebert *et al.*(2004) proposed DNA Barcoding in

which a short nucleotide sequence of 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. Barcoding has been used only in few larval identification studies (Barber and Boyce, 2006; Pegg *et al.*,2006; Victor *et al.*,2009; Hubert *et al.*,2010; Brandão *et al.*, 2016; Wibowo *et al.*, 2016; Collet *et al.*, 2018; Lona *et al.*, 2019). But the scenarios of egg identification using DNA barcodes are rare. One great advantage of DNA barcoding method is that it can accurately identify organisms in any stage (larvae, seeds) of development (as the mitochondrial genome and its sequence orders in the animals remain stable duringits entire life stages) that are difficult to identify by traditional methods. Of the 26,000 crustacean species 53% of them pass through an indirect life cycle involving more than three larval stages (Pandian, 1994). We hypothesize that partial mitochondrial cytochrome oxidase *c* subunit I gene (COI) sequences of egg and larvae of *Scylla serrata* (Forsskål, 1775) will share relatively minimal variations with their adult counterparts compared to other species in *Scylla* genus and thereby facilitate the precise identification of eggs and larvae by DNA barcodes. Previous reports (Raupach and Radulovici, 2015) have been reported for various organisms and the present study was undertaken to test the hypothesis in crustaceans for the first time in India.

MATERIALS AND METHODS

Sampling and DNA barcoding

Adult, larvae 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, larva (zoea) and adults of *S. serrata* were preserved in 95% ethanol and transported to the laboratory. Change in color of the preserved ethanol indicated its DNA damaging capability and therefore the medium was immediately replaced by fresh 95% ethanol. Samples were maintained at 4°C until DNA isolation. DNA extraction from adult specimens was carried out as per standard protocols of Ward *et al.* (2005). Amplification was made using the forward primer LCO1490: 5'-GGTCAACAA ATCATAAAGATATTGG-3' and reverse primer HCO2198: 5'-TAAACTTCAGGGTGACCAA AAAATCA-3' (Folmer *et al.*, 1994). The PCR was conditioned as hot start at 94°C for 1 minute followed by 5 cycles of 94°C for 30 seconds, annealing at 45°C for 40 seconds, and extension at 72°C for 1 minute and 35 cycles of 94°C for 30 seconds, 51°C for 40 seconds, and extension 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 egg, larvae and adult 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 Bio Edit (Hall, 1999) and the final sequence length of >640bps was considered as near full length sequences and are taken for further sequence analysis.

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 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.

Table 1. Egg, larvae and adult samples of *Scylla serrata* and its code

Life stage		Code used	Number of samples
Egg	female1	NAT1	5
	female2	NAT2	5
	female3	NAT3	5
Larvae	zoea1	NAT4	5
	zoea3	NAT5	5
	zoea5	NAT6	5
Adult	adult1	NAT7	5
	adult2	NAT8	5
	adult3	NAT9	5

RESULTS

The COI sequences of eggs, larvae and adults were submitted in the Gen Bank (NCBI) and the accession numbers are JN085428 (NAT1), JN085429 (NAT2), JN085430 (NAT3), JN085431 (NAT4), JN085432 (NAT5), JN085433 (NAT6), JN085434 (NAT7), JN085435 (NAT8) and JN085436 (NAT9). Of which NAT1 – NAT3 were eggs, NAT4 – NAT6 were zoea and NAT7 – NAT9 were adults. The COI region in almost all the samples was in the range of 641-659 bps.

The average Kimura 2 Parameter distance of the egg, zoea and adult stages of *S. serrata* was 0.01%with a minimum of 0.00% and maximum of 0.02% (Table 2).This indicated that COI variations among different stages of the same species will be low. *S. serrata* appeared to be closer to its sibling *S. olivacea* with the pair wise distance of 13.5% whereas species belonging to other genus (*Callinectes sapidus*) showed higher distance of 18.36% with that of *S. serrata* proving that the distance within the members of the genus is lower than with the members belonging to various genera (Fig. 1). Average Adenine and Thymine (AT) content of the selected stages of *S. serrata* was 64.48% and Guanine and Cytosine (GC) content 35.52% (Table 3). The maximum AT content was 65.14% found in NAT1 (egg) and the minimum was 63.49% in 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, which was attributed to the higher average AT content of 64.48%.

In the phylogram constructed using Neighbour Joining (N-J) method, the COI sequences of eggs, larvae and adult of *S. serrata* were in one big head clade (Fig. 2). COI sequence of *S. olivacea* (FJ827760) used as an outgroup was clearly away from the above large clade proving that COI sequences of egg and larvae of *S. serrata* will share relatively minimal variation with their adult counter parts compared to other species in *Scylla* genus thereby facilitating the precise identification of eggs and larvae by DNA barcodes.This was further proved with the BLAST analysis which also showed that COI sequences of eggs, larvae and adults of *S. serrata* (NAT1 to NAT9) obtained presently shared more similarity (average=99.11%) with COI sequences of adult *S. serrata* previously sequenced from Thailand (Table 4). The average similarity levels with the COI sequences of the adults of *Scylla* such as *S. serrata* and *S. olivacea* were 99.11% and 88% respectively suggesting that barcode gene variations are comparatively less within a species than between species.

DISCUSSION

The DNA barcoding has successfully identified the eggs and larvae of *S. serrata*. It has also clearly demarcated other species belonging the same genus. Hence this study has demonstrated the usefulness of identifying a species from its egg and larval stages. About 75% (n=2000) of larvae collected from the Antarctic region (Heimeier *et al.*, 2010) and 50% larvae collected from Indo West-Pacific region (Barber and Boyce, 2006) could not be identified up to species level with the current DNA database. Thanks to DNA barcoding all these can be linked to their respective adults. However, there is a need to expand the COI data in NCBI or BOLD by barcoding all the species (adults).

DNA barcoding, as found in the present study, has immense use in stock assessment studies. Quantification of standing stock of eggs and larvae are also useful in the forecasting of commercial fisheries. But these are all handicapped due to our inability to identify the species to which they belong. This is possible as DNA barcoding can identify a species in its many forms, from eggs through larvae to adults. It is proposed that 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

Table 2. Pairwise analysis table constructed using Kimura 2 Parameter method for *Scylla serrata* life stages

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					64.478	35.521

Table 4. Percentage of similarity of COI sequences of eggs, larvae and adult of *Scylla serrata* of the present study with the adults of *Scylla serrata*, *Scylla olivacea* and *Callinectes sapidus* (outgroup)

Stages of *S. serrata*	Percentage of similarity (%)		
	S. serrata **(FJ827758)**	***S. olivacea*** **(FJ827760)**	***C.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

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 barcode gap with the selected life stages is minimum (0.012%) compared to interspecies distance (13.5% for *S. olivacea*) suggesting no overlap in the distribution of divergence between the species. Similarly DNA barcoding addressed the taxonomic ambiguity of the Indian mud crabs of genus *Scylla* and clearly proved that only two species (*S. serrata* and *S. olivacea*) are present in Indian coastal waters (Mandal *et al.*, 2014) and hence these two species have been considered for the analysis and comparison apart from the outgroup (*C. sapidus*) in order to avoid misperception.

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 selected life stages of *S. serrata*. When applied in field for identification of egg and larvae, DNA barcoding will certainly expand our perception in

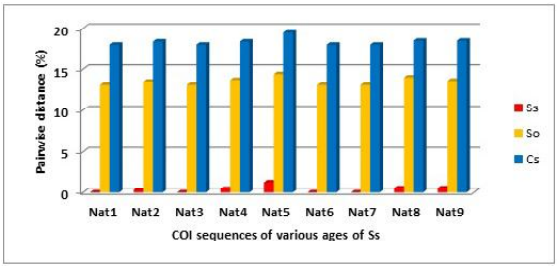

Fig. 1. Pairwise distance between COI sequences of all ages of *Scylla serrata* (Ss) and other member of the *Scylla* genus – *Scylla olivacea* (So) and *Callinectes sapidus*(Cs).

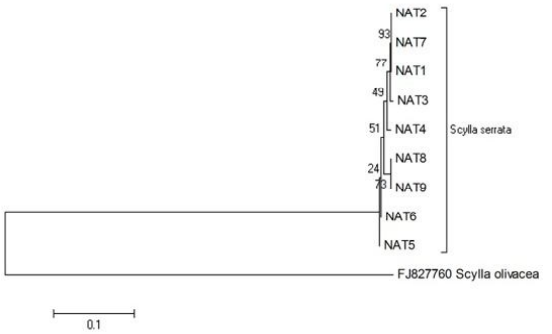

Fig. 2. Phylogram drawn using the sequences of eggs, larvae and adult of *Scylla serrata* with *Scylla olivacea* as outgroup.

biomonitoring, as the need for species-level identification in biomonitoring is litigious (Bailey *et al.*, 2001; Lenat and Resh, 2001). It could also guarantee consistent quality of taxonomic results in studies where the worth of taxonomic data might be negotiated by the inability to identify eggs, early instars, smashed specimens, or fragments of specimens (Stribling *et al.*,2003).

In ecosystems with no obvious physical barriers (e.g. between two mangrove ecosystem along the shoreline area), 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 mobility of eggs and larvae. However, community level processes such as competitive exclusion, assortative settlement and habitat selection may strongly influence species distribution and thereby, community connectivity (Loreau and Mouquet, 1999; Mouquet and Loreau, 2002; Webb *et al.*, 2002; Leibold *et al.*, 2004). Moreover, the improved taxonomic resolution delivered by DNA barcoding would afford more sensitive measures of the magnitudes and types of environmental impacts (Lenat and Resh, 2001).Thus the hypothesis tested was found positive and it is clearly reflected in the present study. Hence barcoding planktonic eggs and larvae will greatly enhance our perceptivity in devising and understanding conservation protocols. In a way, DNA taxonomy and barcoding make thus ecological research independent of the imponderability of future taxonomical developments and keep ecological studies comparable over space and time.

ACKNOWLEDGEMENTS

We thank Dean, Dr. K. Kathiresan, Faculty of Marine sciences, Annamalai University for his encouragement and support and the authorities of our university for the facilities.We also thank Mr. Y. C. Thampi Samraj, and Mr. S. Pandiarajan, RGCA, Sirkazhi for their immense help during the study.

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