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Note

Identification of brown mussel (*Perna indica*) larvae using molecular tool

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ABSTRACT

Species identification of larvae is crucial in understanding larval distribution and population ecology of bivalves. There is great prospects for brown mussel (*Perna indica*) mariculture and therefore the natural spat collection as a source of seed for culture gains importance. It is critical to precisely identify the larvae of the species in order to predict the spat fall for successful spat collection. The larvae of different bivalve species have similar morphology which makes it difficult to identify the species by microscopy. The partial coding sequences of the mitochondrial gene, cytochrome oxidase c subunit 1 (CO1) of *P. indica* and other related bivalve species were compared and a pair of species specific PCR (SSPCR) primers was designed that can specifically amplify the DNA of *P. indica*. The assay conducted to establish the specificity of SSPCR primers produced 291 bp PCR product, only with the target species. The SSPCR conducted with individual veliger larva sorted from the field collected plankton samples have confirmed the presence of *P. indica*. The SSPCR with the whole plankton samples showed presence of the species from the last week of May until the last week of June 2011.

Keywords: Larval distribution, *Perna indica,* Plankton, Spat fall, Veliger

The brown mussel *Perna indica* is the native mussel species of India distributed along the southern peninsula. Prominent brown mussel beds are present from Varkala near Kollam to Kanyakumari along the west coast and from Kanyakumari to Thiruchendur along the east coast (Kuriakose, 1980). Coexisting populations of *P. indica* and the green mussel *Perna viridis* can be seen at Kollam and the former species is completely absent towards the north of the place. Similarly, *P. viridis* populations are not distributed south of Kollam. The reason behind this restricted distribution is not yet clear. Studying the dynamics of brown mussel larval distribution in relation with the oceanographic parameters may provide key evidences to explain this phenomenon.

The prospects and constraints related to farming of *P. indica* were brought into light after the initiation of experimental farming and hatchery production of the species in 1970s (Appukuttan, 1988). Large scale commercial production of the species is not yet realised due to various reasons such as limited supply of spats (mussel seed) for farming, lower growth rate in brackishwater and difficulties in maintaining mussel rafts in the sea (Silas, 1980). There is scope for promoting culture of *P. indica* as an organic extractive component in integrated multitrophic aquaculture (IMTA) (Troell, 2009). Natural spat collection is the readily available and feasible option for sourcing seed for farming. Successful spat collection using cultch (artificial substrata used for spat settlement) requires the knowledge on specific timing of appearance and about density of the brown mussel larvae in the coastal waters. If the time and location of spat fall can be predicted, the cultch materials could be installed at appropriate site and time which will in turn help to reduce the settlement of bio-fouling organisms that adversely affect the spat settlement.

Accurate identification of the larval species is inevitable in understanding the larval distribution along the coastal waters as well as in prediction of spat fall of *P. indica*. This assumes importance as the larvae of different bivalves look similar in morphology and it is extremely difficult to differentiate the species under microscope. Therefore a specific and rapid technique to identify and differentiate brown mussel larvae from other commonly occurring organisms in plankton samples would be of great help. The present study deals with the development of a species specific DNA marker for the identification of brown mussel larvae among a wide variety of planktonic organisms.

Specimens of *P. indica* and the plankton samples were collected from Thankassery Bay (between $8^0$52'38" N, $76^0$34'24" E and $8^0$52'49" N, $76^0$34'41" E) in Kollam

District, Kerala, India. The plankton samples were collected during the spawning season (May to July) of the species (Appukuttan and Prabhakaran, 1983) from the bay using plankton collection net (80 µ) operated from a canoe. The adductor muscle tissues of *P. indica* and the plankton samples were immediately preserved in 95% ethanol.

DNA isolation from tissue samples was carried out following the phenol-chloroform method (Sambrook *et al*., 1989), while DNA isolation from the ethanol preserved plankton samples was done using the salting out protocol (Miller *et al.,* 1988). Veliger larvae were sorted from the plankton samples using a micro-capillary tube under a compound microscope. The DNA from individual veliger larva was extracted by cell disruption through differential thermal treatment. In this method, the individual veliger larva isolated was introduced into 15 µl Milli-Q water in a 200 µl snap cap microtube and incubated for about 10 min at 95^{0}C in a water bath and immediately kept at -20^{0}C until freezing. The sample was then slowly thawed and immediately used as template for PCR.Concentration of the isolated DNA was quantified and quality of the DNA was checked through electrophoresis in 0.8% agarose gel and the images were documented using a UV Gel Documentation system.

The partial coding region of the mitochondrial cytochrome oxidase c subunit-1 (COI) gene was amplified through a standard PCR protocol with the universal primers LCO1490: GGTCAACAAATCATA AAGATATTGG and HCO2198: TAAACTTCAGGGTGACC AAAAAATCA (Folmer *et al*., 1994). Reaction was carried out in a 25 µl reaction mix containing 1x NEB standard Taq buffer, 200 µM dNTPs, 0.2 µM of each primer, one unit Taq polymerase (New England Biolabs, Ipswich, England) and 20 to 50 ng of total DNA. The thermal cycling conditions comprised an initial denaturation at 94^{o}C for 3 min, followed by 35 cycles of denaturation at 94^{o}C for 30 sec, annealing at 52^{o}C for 30 sec, extension at 72^{o}C for 45 sec and a final extension at 72^{o}C for 5 min. All the PCR reactions were carried out in an S1000 Thermal Cycler (Bio-Rad, USA). The amplified PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Germany) and sequenced by Sangers method.

Amplification of the mitochondrial CO1 gene region with the universal primers resulted in approximately 700 bp PCR product. The CO1 sequence data generated for *P. indica* was edited using BioEdit 7.0 (Hall, 1999). BLAST search of these partial coding sequences showed more than 98% sequence similarity with that of *P. indica* depositions already available in the database. The sequences were deposited in NCBI GenBank, with the accession numbers from FJ428753 to FJ428755.

ClustalW multiple alignment method (Thompson *et al*., 1994) was used to compare the CO1 nucleotide sequences of different bivalve species in order to locate the sequence regions, where nucleotide polymorphisms were evident between *P. indica* and other related species. Two pairs of species specific PCR (SSPCR) primers were designed manually from such regions taking care to avoid the chances of formation of secondary structures with the help of the online tool OligoCalc (Kibbe, 2007). Specificity assay was conducted to select the most specific primer pair from the two sets of SSPCR primers designed. DNA samples from bivalves and also from other organisms whose larval stages are usually found in the habitat of *P. indica* were collected and used in the specificity assay. This comprised *P. viridis* (green mussel), *Sunetta scripta* (marine clam), *Paphia malabarica* (short-neck clam), *Meretrix casta* (yellow clam), *Villorita cyprinoides* (black clam), *Crassostrea madrasensis* (Indian backwater oyster), *Saccostrea cucullata* (Indian rock oyster), *Pinctada fucata* (pearl oyster), *Balanus* sp. (barnacles), *Patella* sp. (limpets) and *Parapenaeopsis stylifera* (kiddi shrimp). A positive control PCR using the universal 18SrRNA primers, NSF1179: AATTTGACTCAACACGGG and NSR1642: GCGACGGGCGGT GTGTAC (Wuyts *et al*., 2001) was also set along with the specificity assay. PCR was conducted in a 25 µl reaction mix having the same composition and thermal cycling conditions as mentioned previously except for the annealing temperature of 50^{0}C for 30 seconds in SSPCR specificity assay and 55^{0}C for 30 sec in positive control.

The primers PICO1F284 and PICO1R575 (Table 1) were found to be highly specific and it amplified only the target DNA (Fig. 1) and therefore this particular primer combination which produces PCR product of 291 bp size was selected for screening the plankton samples to detect the presence of *P. indica* larvae. DNA templates of 96 individuals of *P. indica* collected from locations such as Kollam, Vizhinjam and Kulachal were tested for PCR amplification with the selected SSPCR primers in order to confirm that there is no false negative amplification.

The veliger larvae sorted from the plankton samples were subjected to PCR screening using the selected SSPCR primers for the detection of the larvae of *P. indica*. All the sorted veliger larvae were subjected to SSPCR screening in order to verify the occurrence of the larvae of different species at the same time. The total

Table 1. Details of the SSPCR primer designed for *P. indica*

Primer name	Primer sequence	T_a	Product size
PICO1F284	CTCCTAATGCTCTCTATTTAT	50	291 bp
PICO1R575	AGAACAGGTACAGAAATAATC		

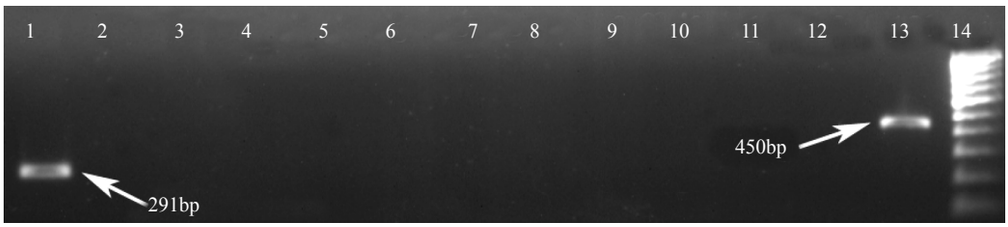

Fig. 1. PCR products of the specificity PCR assay conducted for the SSPCR primers PICO1F284 and PICO1R575.
Lane 1: *P. indica*, Lane 2: *P. viridis*, Lane 3: *S. scripta,* Lane 4: *P. malabarica,* Lane 5: *M. casta,* Lane 6: *V. cyprinoides,* Lane 7: *C. madrasensis,* Lane 8: *S. cucullata,* Lane 9: *P. fucata,* Lane 10: *Balanus* sp., Lane 11: *Patella* sp., Lane 12: *Parapenaeopsis stylifera,* Lane 13: positive control, Lane 14: 100 bp DNA ladder

DNA (20-50 ng μl^{-1}) isolated from the whole plankton samples collected during the spawning season of *P. indica* (Table 2) was subjected to SSPCR analysis. A positive control reaction (18SrRNA) was also included for assuring the quality of DNA used in the SSPCR analysis of individual veliger larva and also of the whole plankton samples. The reaction mix composition and thermal cycling conditions were same as that of SSPCR method described above.

Presence of *P. indica* larvae in the plankton samples could be successfully detected using the SSPCR primer developed. SSPCR conducted with the DNA of individual veliger larvae sorted from the plankton samples yielded 291 bp PCR product. In the case of larval samples 3 and 6 (Fig. 2), the SSPCR failed to produce a positive result. This could be due to the presence of the veliger larvae of other species in the same plankton sample such as that of *P. viridis*. Results of SSPCR analysis with the whole plankton samples (Table 2) collected during the spawning season of *P. indica* showed presence of the target species from the last week of May until the last week of June 2011. This is in line with the observation made by Appukuttan and Prabhakaran Nair (1983), that the spawning season of *P. indica* begins from April and lasts until September with yearly variations.

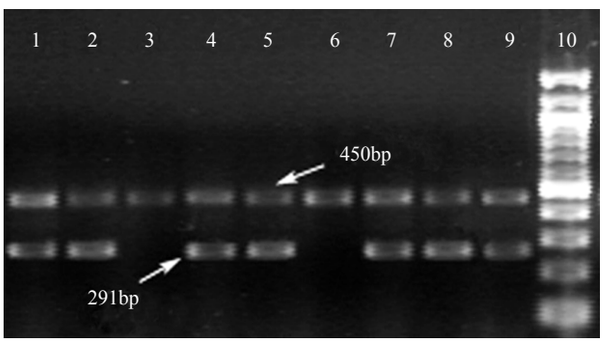

Fig. 2. PCR products of SSPCR conducted with the veliger larvae isolated from the plankton sample THA1104 collected from Thankassery Bay.
Lane 1 to 9: PCR amplicons of 18S rRNA positive control PCR (450 bp) and the PCR amplicons of SSPCR (291 bp) of the veliger larvae loaded in the same lane, Lane 10: 100 bp DNA ladder

Identification of *P. indica* larvae sorted from the plankton samples was successful by using the SSPCR method. The methodology developed will be a useful tool of assistance to detect the presence of larvae during spawning season which is an indication for the impending recruitment of the species at a specific location. This would provide vital information to the mussel farmers about when and where the cultch materials should be

Table 2. Details of the plankton samples subjected to SSPCR analysis

Plankton sample code	Season of collection (2011)	Wet weight of plankton (mg)	Dry weight of plankton (mg)	Positive control PCR (18SrRNA)	SSPCR	Result (presence/ absence of larvae)
THA1101	May	40.42	10.20	+	-	Absent
THA1102	May	37.21	09.32	+	-	Absent
THA1103	May	40.12	10.01	+	+	Present
THA1104	June	21.04	05.14	+	+	Present
THA1105	June	43.51	11.63	+	+	Present
THA1106	June	35.60	09.12	+	+	Present
THA1107	June	49.63	13.05	+	-	Absent
THA1108	July	39.55	10.80	+	-	Absent
THA1109	July	28.70	07.10	+	-	Absent
THA1110	July	40.65	10.44	+	-	Absent

placed in order to get sufficient spat settlement. Similarly this technique can be used to monitor larval transportation and distribution during different seasons and to correlate larval availability with oceanographic parameters, climatic factors and the establishment of mussel population. However, further analysis of the frequently collected whole plankton samples for the presence of *P. indica* larvae during different seasons and its quantification need to be done to achieve these targets.

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