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Stock structuring in Asian green mussel *Perna viridis* population along the Indian coast based on shell morphometrics and RAPD markers

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ABSTRACT

In this study, shell morphometrics and random amplified polymorphic DNA (RAPD) techniques were used to evaluate the genetic variability in 4 wild populations of *Perna viridis* (H≈2500 km apart) along the Indian coast. The phenotypic stocks of mussels were separated using canonical discriminant function analysis. Scatter plots developed with CDA depicted overlapping clusters, indicative of the morphological uniformity of the species along the Indian coast, while Andaman population was forming a separate cluster. RAPD profiles generated from green mussels using 6 primers amplified a total of 43 different fragments ranging from <300 bp to 2500 bp, of which 30 were polymorphic. High genetic variability and moderate genetic differentiation (0.126) was noted among the populations. Percentage of polymorphism among green mussels ranged from 30.2% (Dona Paula) to 79.1% (Andamans). The observed genetic homogeneity among the green mussels of Indian coast indicated their high level of genetic mixing and it may be attributed to the pelagic larval dispersal along with coastal currents.

Key words: Indian coast, Mussel, Polymorphism, RAPD

Bivalves, one of the most important groups of molluscs in the tropics, constitute an inexpensive, high nutritional source of animal protein. Among bivalves, mussels are important source of several bioactive substances with medicinal and humoral properties. The Indian mussels include Asian green mussel *Perna viridis* and brown mussel *P. indica*. *P. viridis* has wider distribution extending from Gujarat on the West coast of India to West Bengal on the East coast, as well as along the isolated islands of Andamans, while *P. indica* is confined to the southern tip of the Indian coast, extending from Kanyakumari to Tiruchendur.

Of the two species reported from Indian coast, green mussel (*P. viridis)* is a shellfish species used for mariculture. In some areas along the Indian coast (Kanyakumari-Vizhinjam and Calicut-Tellicherry zones of South India), mussel seeds 10–20 mm along with the adults are indiscriminately exploited during the peak fishing season leading to depletion of the stock in the natural mussel beds (Shanmugam 2002). In this case, the mussel population becomes increasingly susceptible to changes in the environmental conditions and may become more prone to extinction. In India, CMFRI, Cochin has initiated the hatchery development, rearing and relaying of mussel seeds to enhance the fishery resources along the Southern Indian coast (CMFRI Annual Report 2009). To implement management strategies for a species, it is important to investigate its genetic diversity and geographical partitioning throughout its natural range (Mukunda *et al.* 2009). Hence in this study, a holistic approach—combining genotypic (RAPD) and phenotypic (CDA) methods was used to evaluate the genetic variability in four wild populations of *P. viridis* along the Indian coast. The data generated through this study will be used in planning the rehabilitation programmes of *P.viridis* initiated by CMFRI.

MATERIALS AND METHODS

Perna viridis were collected from 4 widely separated locations (H≈2500 km apart), viz. Dona Paula, Goa (15° 27′ N, 73° 48′ E); Kollam, Kerala (8° 53′ N, 6° 34′ E); Chennai, Tamil Nadu (17° 41′ N, 83° 17′ E) and Port Blair, Andamans (11° 38′ N, 92° 43′ E). Expert divers hand-picked 60 samples from these locations were used for morphometric analysis from each location. For RAPD analysis, the mantle tissue (1 g each) was dissected and preserved in 1.25 ml of 95% ethyl alcohol and stored at 4°C until further analysis.

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Table 1. Shell morphometric measurements made for *P.viridis*.

	Abbreviations
Pbrs	Posterior byssal retractor muscle scar
Pal	Distance between pallial line and ventral shell margin midway along shell
Padv	Distance between ventral edge of posterior adductor muscle scar and ventral shell margin
Padp	Distance between anterior edge of posterior adductor muscle scar and posterior shell margin
Lig	Length of ligament
Ht	Height of shell
Width	Width of shell
Thick	Thickness of shell

For morphometric analysis, 8 phenotypic variables (Table 1) were measured, following the methods of Toro *et al*. (2004). Each character was standardized using $\log_{10}$ and divided by $\log_{10}$ of shell length. The CDA was used to derive a canonical function that separated the four mussel populations, using systat V5.1 (Wilkinson 1991). The shell morphology variation analysis was carried out to check whether distinct phenotypic stocks exist among the green mussel populations distributed along the Indian coast.

DNA was isolated using the phenol-chloroform method, following the modifications according to Sokolov *et al.* (2000). Selection of the RAPD primers for the study was made from the initial PCR screening of 40 random decamer primers using DNA from five mussel samples. The primers which were used for the initial screening included OPAH (1–20) series and OPA (1–20) series. Random primed PCR can often produce non-reproducible amplification product. Only primers which generated good number of reproducible amplicons and visualized as sharp bands on electrophoresis gel were selected for the RAPD analysis. These included OPAH-01, 04, 15, 19, OPA-06 and OPA-13 and the primer sequences are given in Table 2. To check for DNA contamination, a negative control was set up omitting the template DNA from the reaction mixture. PCR amplifications were performed using PTC 200 gradient thermal cycler in 25µl reactions containing 1x reaction buffer with 1.5 mM $MgCl_2$, 7.5 pmoles of primer (random primers), 200 mM dNTPs, 2 U Taq DNA polymerase and 50 ng of template DNA. The reaction mixture was pre-heated at 95°C for 3 min followed by 40 cycles (94 °C for 1 min, 40°C for 1 min and 72 °C for 90 sec). The reaction was then subjected to a final extension at 72 °C for 10 min. PCR products were run through 1.5% agarose gels, stained with ethidium bromide (5µg/ml) in 1x TBE buffer (pH 8.0) and were visualized under UV transilluminator and documented using Image Master VDS. The size of RAPD bands was determined by comparison with λ DNA digested with *Eco*RI/*Hin*dIII molecular weight marker. Bands observed in each lane were compared with all the other lanes of the same gel and reproducible bands scored as present (1) or absent (0). The molecular weight band was estimated using the standard molecular marker with image master 1D Elite Ver. 3.01. Parameters such as the number of polymorphic loci, percentage polymorphism (P), gene diversity (h), Nei's coefficient of genetic differentiation (G_{ST}) - overall and pairwise G_{ST} and gene flow ($Nm = 0.5\ (1\text{-}G_{ST})/G_{ST})$), average pairwise similarity index (SI) and genetic distance (GD) were estimated using POPGENE Software (Yeh *et al.* 2000) and TFPGA Software (Miller 1997). Shannon diversity that provides a relative estimate of the degree of variation within each population was also calculated for each population. A dendrogram was constructed based on the genetic distances using the unweighted pair-group method with arithmetic averages (UPGMA) modified from NEIGHBOR procedure of PHYLIP Version 3.6a. To test the confidence level of each branch of UPGMA based program, the binary matrix was bootstrapped 10000 times, using Winboot programme.

Table 2. Primer sequences used in the study

Primer	Primer sequence (5′→3′)
OPAH–01	TCCGCAACCA
OPAH-04	CTCCCCAGAC
OPAH-15	CTACAGCGAG
OPAH-19	GGCAGTTCTC
OPA-06	GGTCCCTGAC
OPA-13	TCGGCGATAG

RESULTS AND DISCUSSION

Multivariate analysis of shell morphology: *Canonical discriminant function analysis*

Scatter plots developed with CDA (Fig.1) indicated that the Andaman population was phenotypically distinct among the four mussel population studied. In CDA, first discriminant function explained 60.6% of the variation and together with second discriminant function it explained 86.4% of the variation. Three discriminant functions together explained 100% of the variation. Variables including pbrs (posterior

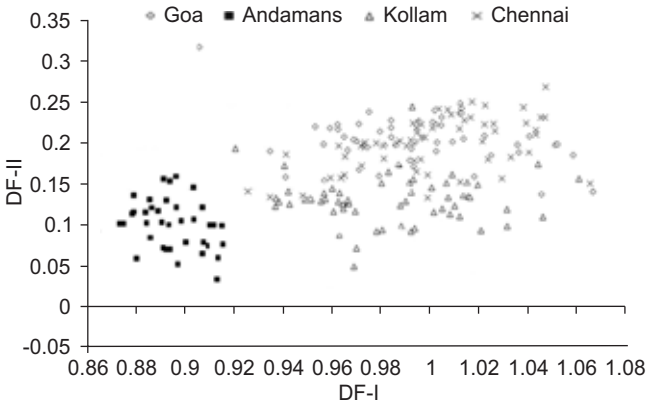

Fig. 1. Scatter plot to differentiate green mussels stocks using canonical discriminant function analysis.

byssal retractor muscle scar) and shell height have maximum positive loadings in representing the morphometric variations of green mussel stock. Morphometric approach for stock identification is based on the assumption that the genotype of the organism interacts with their environment leading to phenotypic variation. Since the environmental factors has an impact on shell morphology, a holistic approach using at least one phenotypic and one genotypic method together can help in resolving the discrepancies implied by each method and many investigators in the stock identification studies are following this combined approach (Toro *et al*. 2004). The CDA of morphometric variation indicates that most individuals from Dona Paula, Chennai and Kollam, do provide overlapping clusters, while Andaman population formed seperate cluster, indicating that the shell morphology of this population is most distinct among the populations analysed.

Genetic characterization of green mussels using RAPD Marker

RAPD profiling from green mussels using 6 primers generated 43 fragments ranging from <300 bp to 2500 bp, of which 30 were polymorphic. Thirteen fragments, viz.1290 bp, 947 bp, 376 bp produced by primer OPAH-01 (Fig. 2); 1744 bp and 1140 bp by OPAH-04; 1584 bp, 1375 bp and 947 bp by OPAH-15; 1247bp, 1076 bp and 831 bp by OPAH-19; and 710 bp and 564 bp by OPA-13 were shared by all individuals belonging to the 4 populations. Primer OPA-06 produced only polymorphic bands. No site-specific amplicons were discovered with any of these primers. The overall estimate of gene diversity (h), Shannon information index, number of polymorphic loci and the percentage polymorphism in the four green mussel populations are given in Table 3. No site-specific amplicons were discovered with any of these primers. The Shannon index determined the intra-specific diversity within green mussels and the value ranged from 0.1795 ± 0.2914 (Dona Paula) to 0.5003 ± 0.2743 (Andamans), indicating that among the four green mussel populations, Andaman samples showed relatively more genetic variation, and this may be due to the lesser exploitation pressure undergone by the species in the remote Andaman islands. Pair-wise SI, GD values and G_{ST} values

Table 3. The overall estimate of genetic parameters in the green mussel populations using RAPD

Parameter	Dona Paula	Andamans	Chennai	Kollam
Gene diversity (h)	0.1253	0.3512	0.2142	0.2022
Shannon Information index (I)	0.1795	0.5003	0.309	0.2870
No. of polymorphic loci	13	34	22	19
Polymorphism %	30.23%	79.07%	51.16%	44.19%

Table 4. Data showing pair-wise comparison of similarity index (above diagonal) and genetic distance (below diagonal) of green mussels based on Nei (1978), calculated for 6 primers

Sites	Dona Paula	Andamans	Chennai	Kollam
Dona Paula	*******	0.8490	0.9659	0.9234
Andamans	0.1637	*******	0.8755	0.8630
Chennai	0.0347	0.1330	*******	0.9228
Kollam	0.0797	0.1473	0.0803	*******

Table 5. Pair-wise G_{ST} calculated for the four green mussel populations using TFPGA software

S.no	Dona Paula	Andamans	Chennai	Kollam
Dona Paula	0			
Kollam	0.2115	0		
Chennai	0.0905	0.1486	0	
Andamans	0.1736	0.1642	0.1369	0

estimated for the populations are given in Tables 4 and 5, respectively. Fig. 3 depicts the UPGMA dendrogram of green mussel populations based on the genetic distance, where Andaman population forms a separate node.

The overall genetic differentiation (G_{ST}) value was 0.1260 among all the populations. The pairwise G_{ST} estimate indicated moderate genetic differentiation and is within the range (0.09–0.21) as reported in other molluscan species (DeWolf *et al.* 2003). They have also estimated the gene flow in periwinkle *Littorina littorea*, a planktonic developer, producing long-living (4–7 weeks) free-floating larvae, which are presumed to disperse over long distances. The high Nm value (3.4674) observed indicate high gene flow between

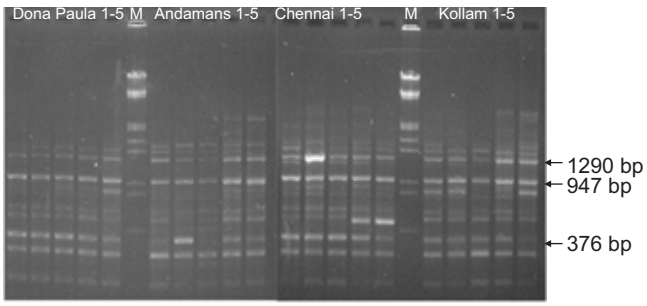

Fig. 2. RAPD pattern of *P. viridis* using primer OPAH -01

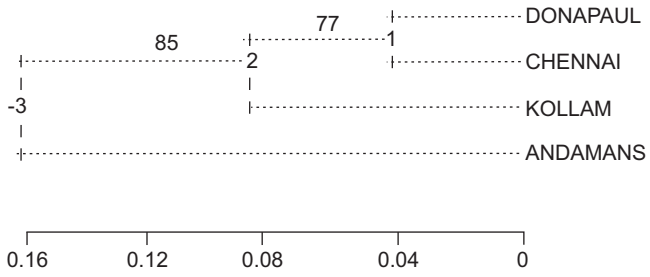

Fig. 3. UPGMA dendogram showing relationships in *P. viridis* from various locations using Popgene software.

the green mussel populations. Green mussel larvae are free-swimming and reported to have the potential for long-distance dispersal over hundreds of kilometers along the Indian coast, favoured by the ocean currents (Rajagopal *et al.* 2006). This might have promoted high gene flow sufficient to prevent differentiation among populations by genetic drift, as reported in other marine bivalves. The geographical separation of Andaman Islands from Indian waters might be playing a role in the genetic separation of Andamans stocks from others.

The coastal currents, which facilitate pelagic larval dispersal around Indian coast, are frequent and they change the direction with seasons. (Shankar 2002). The continuously changing coastal current pattern may result in the exchange of free-living larvae of mussels along the Indian coast, leading to the low genetic differentiation among stocks. Moreover, the reproductive strategy of green mussels is broadcast spawning with high fecundity. A detailed investigation is essential to prove that the pelagic larval dispersal happens in congruence with the distribution of coastal current. The Andaman population of green mussels was found to be more genetically distinct from rest of the populations. Wide geographical separations of the Andaman waters from Indian mainland waters (~2500km) may be the reason for relatively higher genetic differentiation of Andaman population from the other three coastal populations. The highest genetic distance (GD) value was (0.1637) found between samples from the farthest sites-Dona Paula and Andaman. The UPGMA dendrogram showed the lesser genetic distance between Kollam, Chennai and Dona Paula samples and the wider separation of Andaman samples. The result indicated that there is a similarity in population structure of green mussels along the Indian coast, with the possible exception of Andaman samples.

Although RAPD-PCR is sensitive to several factors, it is quite useful, when used with caution, for several applications in marine organisms. In the present investigation, we demonstrated the use of RAPD analysis to study the population structure of *P. viridis* along Indian Coast. *Perna* being a commercially important species, information on genetic stock structure can be useful to avoid the natural gene pool contaminated, while adopting the spat relaying programmes to enhance the fishery. Advanced molecular markers like microsatellites are now been developed in *P. viridis* and used for population study (Ong *et al.* 2009). These markers may be utilised in future to corroborate our findings. In terms of management of the green mussel fishery distributed along the Indian coast, the present study concluded that the wild populations of *P. viridis* from the mainland are genetically heterogeneous, but the Andaman stocks should be managed separately to maintain the genetic integrity and for avoiding any possibilities for the gene pool dilution.

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