



†A molecular approach to reveal the genetic identity of parrot mussel and other sympatric mussel species distributed along the Kerala coast

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Abstract

Two commercially important mussel species are recorded from the Indian coast: green mussel *Perna viridis* (Linnaeus, 1758) and brown mussel *P. indica* (Kuriakose and Nair, 1976). Apart from this, a third type referred to as parrot mussel, which has shell shape of brown mussel, but with green shell colouration and suspected to be the hybrid of the above two species has also been reported from Kollam coast of Kerala, where both the species co-occur. In the present work, genetic identity of parrot and sympatric mussel species was determined using protein and genomic DNA markers. Protein markers viz. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and allozymes and the genomic DNA marker Random Amplified Polymorphic DNA (RAPD) were used for determining genetic identity of the three mussel groups. The green and brown mussels could be clearly differentiated using SDS PAGE. The parrot mussel protein pattern was similar to that of brown mussel, except for an additional band of molecular weight 48.7 Kda which is unique to brown mussel. Genus specific protein bands for *Perna* viz. 66 Kda, 43 Kda and 14.3 Kda, were detected in this study. Allozyme electrophoresis also followed a similar pattern. Of the 10 allozyme loci studied, seven revealed species-specific diagnostic differences between *P. viridis* and *P. indica*. They were *AAT-1** (Aspartate Amino Transferase-1*), *AAT-2**, *ME* (Malic Enzyme)*, *PGM-2** (Phospho Gluco Mutase-2*), *EST-1** (Esterase-1*), *EST-2**, *IcDH** (Isocitrate Dehydrogenase)*. Parrot mussel shared all the alleles of brown mussel, and no hybrid pattern was observed. Species-specific alleles clearly differentiated green mussel from both brown and parrot mussel. The genetic distance of green mussel from brown mussel, estimated from allozyme data was 1.1145 and with parrot mussel it was 1.105. The genetic distance between parrot mussel and brown mussel was negligibly low (0.0005). Using allozyme and RAPD data, the Nei's Unbiased Measures of genetic distance were calculated and the dendograms prepared based on these values clearly depicted the separation of parrot mussel from green mussel as well as the close resemblance of parrot mussel with brown mussel. The higher gene flow (1.1539) determined using RAPD marker also hints that brown and parrot mussel may be acting as single interbreeding population. Hence this study using molecular tools to test the genetic identity of parrot mussel has helped to conclude that parrot mussel is only a morphotype of brown mussel and not a true hybrid of the two.

Keywords: Mussel, SDS PAGE, allozymes, RAPD, hybrid

Introduction

Along the Indian coasts, two species of marine mussels are reported which are of economic importance and with great potential for shellfish

mariculture viz., the green mussel, *Perna viridis* (Linnaeus, 1758) and the brown mussel *P. indica* (Kuriakose and Nair, 1976). While the green mussel has a wider distribution all along the Indian coast,

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the brown mussel has very restricted distribution along the southern tip of India, extending from Kollam (Kerala) to Tiruchendur (Tamil Nadu). Along the Kollam coast, the two species co-occur. Due to the construction of breakwaters in this region, natural water currents in this area are limited. In this locality, a third morphotype of mussel occur, which has shell shape of brown mussel, but with green shell colouration was observed. Kripa *et al.* (2001) reported it as parrot mussel and suggested it as a possible hybrid between the green mussel and brown mussel. Mussel seed resources survey (Kripa *et al.*, 2001) had indicated the availability of the parrot mussel seed only along with brown mussels during the post-monsoon period along the southern regions of Kerala. Padhi (1998) also reported the existence of intermediate morphotypes of mussels from Indian coast.

The present study with the help of molecular tools examine whether the parrot mussel is colour morph of any of these species or a hybrid of the two or whether it is an entirely different species. Mussels usually inter breed, wherever they coexist (Skibinski *et al.*, 1983; Koehn, 1991). Hence hybridisation among *Perna* species is also expected. In this study a comparison of the parrot mussel with green and brown mussels has been made using SDS PAGE, allozymes and RAPD.

Material and Methods

Live green mussel, brown mussel and parrot mussel were collected by divers off Kollam, (southwest coast of India). For each mussel type, 42 samples were collected, packed in soaked gunny bags and transported live to the laboratory for further

processing. Mantle tissues (approximately 20 g) from each live specimen were removed and packed in aluminium foils for the allozyme studies. These samples were stored at -80°C until use. For DNA extraction, mantle tissue (1 g each) was taken from 20 samples of each mussel type and preserved in 1.25 ml of 95% ethyl alcohol. These samples were stored at 4°C until further analysis.

For SDS PAGE, homogenates of mantle tissue were prepared in the ratio 1:1.5 (w/v). SDS PAGE carried out in the present study was on the lines of Laemmeli *et al.* (1970), using stacking gel (6%) and separating gel (11.5%). SDS protein molecular weight marker (Genei, Bangalore, India) was also run along with the sample. A constant voltage of 60 was applied until the dye front crossed the stacking gel and it was later increased to 140 volts.

Samples for allozyme markers were prepared as in the case of SDS PAGE. Initial screening was carried out for sixteen enzymes. Seven of these enzymes, which were found to give scorable activity, were selected for extensive screening of the three mussel types. The name of the selected enzyme, enzyme loci, enzyme commission numbers and quaternary structure are given in Table 1. Electrophoretic analysis of the tissue samples was carried out using Poly Acrylamide Gel Electrophoresis (PAGE) at gel concentration of 7.25%. Electrophoresis was carried out in a vertical gel apparatus (Hoefer - Pharmacia, LKB).

The buffer systems used for the study was TBE (560 mM Tris, 650 mM boric acid and 16 mM EDTA, pH 8.0) and the electrophoresis was carried out at constant voltage of 150 V. After completion

Table 1. Name of enzymes used in allozyme analysis with their enzyme commission (E.C.) number and quaternary structure

Enzymes	Abbreviation	Enzyme Commission number	Quaternary Structure
Aspartate amino transferase	<i>AAT</i>	2.6.1.1	Dimer
Esterase	<i>EST</i>	3.1.1.1	Monomer
Glucose phosphate isomerase	<i>GPI</i>	5.3.1.9	Dimer
Isocitrate dehydrogenase	<i>ICDH</i>	1.1.1.42	Dimer
Malic enzyme	<i>ME</i>	1.1.1.40	Tetramer
Phospho-glucomutase	<i>PGM</i>	5.4.2.2	Monomer
Superoxide dismutase	<i>SOD</i>	1.15.1.1	Dimer

of each run the gel was stained for a specific allozyme using standardized protocols. The staining recipe used for the allozyme detection was modified from that of Shaw and Prasad (1970) and Shaklee *et al.* (1990). After staining, the gels were documented in Bio-vis gel documentation system.

For RAPD analysis, genomic DNA was isolated using the phenol chloroform method, following the modifications suggested by Sokolov *et al.* (2000). Selection of the primers for the study was made from the initial PCR screening of 40 random decamer primers using mussel DNA. The primers which were used for the initial screening included OPAH (1-20) series and OPA (1-20) series. Those 7 primers which generated reproducible amplicons that could be visualized as sharp bands on electrophoresis were selected for the RAPD analysis. Details of those selected primers are given in Table 2. PCR amplifications were carried out in a PTC 200 gradient thermal cycler (M.J. Research, Inc., Watertown, Massachusetts, USA). PCR amplifications were performed in 25 μ l reactions containing 1x reaction buffer with 1.5 mM MgCl₂ (Genei, Bangalore, India), 7.5 pmoles of primer (random primers), 200 mM dNTPs, 2 U Taq DNA polymerase (Genei, Bangalore, India) and 50 ng of template DNA. The reaction mixture was pre-heated at 95°C for 3 minutes followed by 40 cycles (94°C for 1 minute, 40°C for 1 minute and 72°C for 1.50 minutes). The reaction was then subjected to a final extension at 72°C for 10 minutes. Random primed PCR can often produce non-reproducible amplification product. Reactions were therefore, performed following strict protocol with standardized conditions. PCR products were electrophoresed using 1.5% agarose gels, stained with ethidium bromide (5 μ g/ml) in 1x TBE buffer (pH 8.0) and were visualized under UV transilluminator and documented using Image Master VDS (Pharmacia Biotech, USA). The size of RAPD bands was determined by comparison with λ DNA digested with EcoRI / HindIII molecular weight marker. RAPD data was analyzed using POPGENE 1.31 (Yeh *et al.*, 1999). Dendrogram based on genetic distances using the unweighted pair-group method with arithmetic averages (UPGMA) modified from NEIGHBOR procedure of PHYLIP Version 3.5c was constructed. The robustness of the dendrogram

was tested by boot strap analysis using 1000 pseudoreplications.

Table 2. Details of the operon random primers used in the study

Primer	Primer sequence (5→3')	Molecular weight (Da)
OPAH-01	TCCGCAACCA	2957
OPAH-03	GGTACTGCC	3019
OPAH-04	CTCCCCAGAC	2933
OPAH-05	TTGCAGGCAG	3068
OPAH-12	TCCAACGGCT	2988
OPAH-15	CTACAGCGAG	3037
OPAH-19	GGCAGTCTC	3019

Results and Discussion

SDS PAGE: SDS PAGE revealed both genus specific and species-specific protein bands (Fig. 1), which clearly differentiated the green and brown mussels. Seven protein bands were identified in the green mussel (*P. viridis*) and 5 in brown mussel. The genus specific bands observed for *Perna* were 66 KDa, 43 KDa and 14.3 KDa. The brown mussel protein pattern was similar to that of parrot mussel (66 KDa, 43 KDa, 14.3 and 5.5 Kda), except for an additional band of molecular weight 48.7 KDa unique to brown mussel. The bands specific to *P. viridis* were >100 KDa, 23.1 KDa, 6.5 KDa and 4.7 KDa (4 bands).

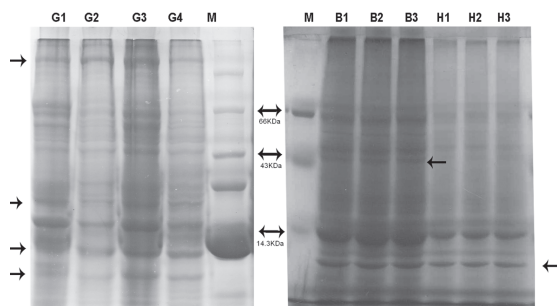


Fig. 1. SDS showing comparison of green mussel (G), brown mussel (B) and parrot mussel (H)

Allozyme electrophoresis: Allozyme marker is a useful tool to clarify taxonomic ambiguity in species as well as for hybrid identification (Menezes and Qasim, 1993). It was reported to differentiate to three sibling species: *M. edulis* Linnaeus (1758),

M. galloprovincialis Lamarck (1819), and *M. trossulus* Gould (1850) (McDonald *et al.*, 1991). Allozyme marker was used in hybrid identification of marine mussel *Mytilus* spp. by Gardner (1996). To discriminate *P. indica*, *P. viridis* and parrot mussel only loci yielding scorable activity in both species were considered. Thus, out of 16 enzyme screened, 10 loci expressed by 7 enzymes system were available for analysis. While four of the enzymes (*SOD*, *GPI*, *IcDH* and *ME*) were found to be with single locus, three enzymes were with two loci (*PGM*, *AAT* and *EST*). Four of the enzymes studied were monomorphic and three were found to be polymorphic (*PGM*, *AAT* and *EST*). An excess of homozygotes was observed at many loci in most of the individuals.

Species-specific diagnostic differences between *P. viridis* and *P. indica* were observed at 7 loci of 5 enzyme systems, viz. *AAT-1**, *AAT-2**, *ME**, *PGM-2**, *EST-1**, *EST-2**, *IcDH**. Details of results obtained for each enzyme system are given below:

a) Aspartate amino transferase (*AAT-1** and *AAT-2**): *AAT-1** and *AAT-2** showed species specific alleles. Alleles 72 (*AAT-1**) and allele 116 (*AAT-2**) were unique to *Perna viridis*, while the most common allele 100 (*AAT-2**) was unique to *Perna indica* and parrot mussel.

b) Malic enzyme (*ME**): Though a variable quaternary structure has been reported for *ME** in both vertebrates and invertebrates, in our studies this enzyme was apparently in homozygous condition determined by a single locus (*ME**). Two species-

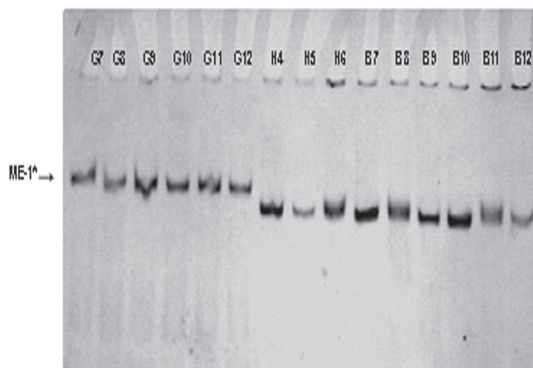


Fig. 2. ME showing comparison of green mussel (G), brown mussel (B) and parrot mussel (H)

specific alleles were noted (Fig. 2). While allele 73 was present in *P. viridis* and the most common allele 100 was noted in *P. indica* and parrot mussel.

c) Superoxide Dismutase (*SOD**): *SOD** loci was clearly monomorphic in all the individuals of *Perna* spp. studied. No species specificity was observed with this enzyme.

d) Esterase (*EST**): Esterase showed a fast moving and a slow moving zone in all the three groups. *EST-1** and *EST-2** loci showed species-specific alleles (Fig. 3). Alleles 100, 108, and 125 were present in *EST-1** and alleles 94, 100 were detected as species-specific alleles in *EST-2**. The most common allele 100 of brown mussel was shared with parrot mussel, while allele 94 was specific to green mussel alone.

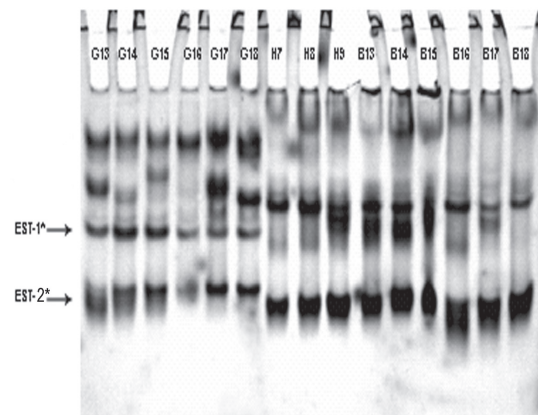


Fig. 3. EST showing comparison of green mussel (G), brown mussel (B) and parrot mussel (H)

e) Glucose phosphate isomerase (*GPI**): *GPI** was resolved to be with a single locus, with expected 1 and 3 band phenotypes. It was polymorphic in some individuals, but percentage of polymorphism was low.

f) Phospho-gluco mutase (*PGM**): *PGM* was resolved to be with two loci, *PGM-1** and *PGM-2**, and the genetic variants appeared as one and two band phenotypes as expected for this monomeric enzyme. While *PGM-1** was monomorphic in all the individuals of *Perna* species studied. *PGM-2** was polymorphic with two alleles, 83 and 100.

g) Isocitrate dehydrogenase (*IDH**): *IDH* was resolved with single locus, but with two different species-specific alleles, 100 and 126. Parrot and brown mussels shared allele 100, while allele 126 was unique to green mussel.

Data generated through allozyme electrophoresis was analysed using Genetix software. Nei's Unbiased genetic distances between pairs of the three types of mussels were estimated (Nei, 1978). Genetic distance value between green mussel and brown mussel was 1.1145 and that with parrot mussel was 1.105. The genetic distance values between parrot mussel and brown mussel were significantly low (0.0005) using allozyme marker. On the basis of calculated genetic distance values, the phylogenetic relationships between three mussel types were made through a dendrogram (Fig. 4) following the unweighted pair group method using arithmetic averages (UPGMA) method. Comparative studies of allozyme patterns of the three mussel types *P. viridis*, *P. indica* and the suspected hybrid/morphotype (parrot mussel) revealed that the parrot mussel has shared alleles mostly with the brown mussel. This indicates that the parrot mussel may be a colour morph of the brown mussel and not a real hybrid of the two.

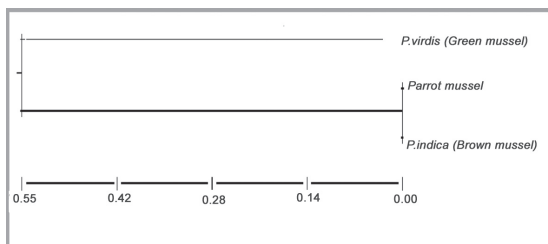


Fig. 4. UPGMA dendrogram of allozyme analysis showing comparison of *Perna* spp.

RAPD: From OPA (1-20) and OPAH (1-20) series of primers screened among these three mussel types, seven primers that gave good amplification in all the types were selected for final screening viz. primers OPAH-1, 3, 4, 5, 12, 15 and OPAH-19. The utility of RAPDs in generating taxonomic specific markers has been demonstrated in a number of taxa (Hadrys *et al.*, 1992). Species in which such markers were used for taxonomic studies include the oyster genera *Crassostrea*, *Striostrea* and *Saccostrea*

(Klinbunga *et al.*, 2000) and the tropical abalones *Haliotis asinina*, *H. ovina* and *H. varia* (Klinbunga *et al.*, 2004). In this study, the amplification of DNA from 20 individuals with the above seven primers produced a total of 51 amplicons, which appeared as distinct bands on agarose gel electrophoresis. The parrot mussel shared the major bands generated by all the individuals of brown mussel. The bands shared by parrot mussel and the green mussel were rare. Fragment of 1584 bp produced by primers OPAH-01 and OPAH-04, 1480 bp fragment produced by primer OPAH-03, 1200 bp fragment produced by primers OPAH-05 and OPAH-12, 564, 764 and 890 bp produced by primer OPAH-15 are shared by the brown mussel as well as parrot mussel. The fragment of 947 bp produced by OPAH-03 primer was common to all the *Perna* spp. studied. The number of amplicons ranged from 4 (OPAH-15) to 9 (OPAH-1, OPAH-12 and OPAH-19) in all the three types.

Genetic diversity (G_{ST}) and gene flow (N_m) between parrot mussel to green mussel and brown mussel were estimated separately. G_{ST} values of green mussel with parrot mussel as well as brown mussel were similar (0.6964 and 0.6527 respectively). The gene flow value between brown mussel and parrot mussel was the highest (1.1539). Considerably high amount of gene flow between the parrot mussel and brown mussel indicates that these two may be acting as single interbreeding population, similar to the discussion made by Toro *et al.* (2004) in *Mytilus chilensis*. Genetic distance (GD) of parrot mussel with brown mussel was negligibly low (0.0898) and that with green mussel it was comparatively high (0.7460). This value (0.7460) was very close to the genetic distance of brown mussel with green mussel (0.7125). Therefore, the Nei's unbiased measures of genetic distance (Nei, 1978) indicate separation of parrot mussel from green mussel, and the similarity of the parrot mussel with brown mussel. The dendrogram pattern also clearly depicted the distinct species status of *P. viridis* and *P. indica* and the genetic similarity of parrot mussel with brown mussel (Fig. 5). An attempt to reveal the genetic identity of parrot mussel using mitochondrial DNA gene COI sequence also hints the close genetic similarity of parrot mussel with brown mussel (Divya *et al.*, 2009).

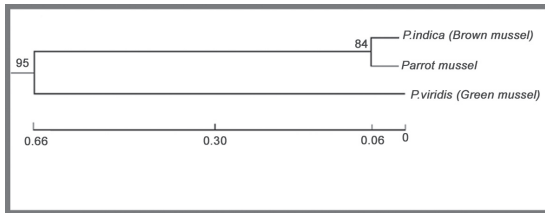


Fig. 5. UPGMA dendrogram of RAPD analysis showing comparison of *Perna* spp.

The parrot mussel reported in this study had similarity with brown mussel in its morphology and the differences were evident only in their colour. Marin *et al.* (2007) have reported a case similar to that of parrot mussel in abalones. They have expected hybridisation between the two species of abalones, Pacific abalone *Haliotis discus hannai* and Californian red abalone *H. rufescens*. Their study concluded that no true hybridisation had happened between Pacific abalone and Californian red abalone and the colour variation was due to some environmental adaptation and/or genetic polymorphism.

On the basis of concordant results in protein electrophoresis and RAPD markers, it may be concluded from this study that parrot mussel might be a colour variant of brown mussel and not a true hybrid. The colour and shell morphology of parrot mussel may be due to some environmental adaptations, rather than hybridisation as reported in abalones by Marin *et al.* (2007). However Winkler *et al.* (2001) reported that although some external coloration and pigmentation patterns in molluscan shells may be attributable to environmental factors; most variations in these phenotypic characters depend on uncomplicated genetic mechanisms. Genetic factors have been identified to be responsible for the variations in shell colour of a few bivalves (Adamkewicz and Castagna, 1988; Winkler *et al.*, 2001), abalones (Kobayashi *et al.*, 2004) and other gastropods (Boulding and Hay, 1993; Rolan *et al.*, 2004). Further detailed investigations are required to explain the exact genetic mechanism responsible for the shell colour variations in the brown mussel.

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