

MOLECULAR PHYLOGENETIC ANALYSIS OF TWO CLOSELY RELATED MARINE INDIAN MUSSELS OF GENUS *PERNA* (PHILIPSSON, 1788) BASED ON MITOCHONDRIAL (COI) AND NUCLEAR (ITS) GENES

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Abstract: Two species of marine mussels, the green mussel, *Perna viridis* (Linnaeus, 1758) and the brown mussel, *Perna indica* (Kuriakose & Nair, 1976) are found along the Indian coast. A molecular phylogeny is presented for marine Indian mussels based on nuclear internal transcribed spacer (ITS) and mitochondrial cytochrome oxidase I (COI) gene sequence data correlated to other *Perna* species from GenBank (NCBI). A range of phylogenetic analyses were used to investigate the current taxonomic assignments and evolutionary relationships of the *Perna* genus. The present investigation is an attempt for resolving the taxonomic ambiguity among Indian marine mussel species using the mitochondrial (COI) and nuclear (ITS) genes. The indication is that *P. indica* is a distinct species and not to be relegated as a synonym of *P. perna*, which clustered within the *P. perna* clade is not regarded as a separate species and hence is found the most closely related of the five *Perna* species. *P. indica* and *P. viridis* are closely related to *P. canaliculus* showing less sequence divergence. It was proved that the within-species divergence values are greater than 2%.

Key words: Brown mussel, Green mussel, *Perna indica*, *Perna viridis*, ITS gene, mitochondrial cytochrome oxidase I (COI) gene

INTRODUCTION

Mussels of the genus *Perna* (Philipsson, 1788) belong to the family Mytilidae or true mussels (Mollusca; Bivalvia; Lamellibranchia; Mytiloida; Mytilidae). This genus includes green and brown mussels from tropical, subtropical, warm temperate and cold temperate regions, mostly from the southern hemisphere, but also from northern Africa and the northern coasts of South America (Gosling, 2003; Siddall, 1980). Aquaculture importance of different mussel species including those belonging to the genera *Mytilus* and *Perna* is globally known. 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, *P. viridis* (Linnaeus, 1758) and

the brown mussel *P. indica* (Kuriakose and Nair, 1976) with affinity to *P. perna*. These species are cultured and/or harvested from wild populations in countries such as India, the Philippines, Thailand, China, Venezuela and New Zealand (Appukuttan and Nair, 1980; Hickman, 1991; Jeffs *et al.*, 1999; Narasimham, 1980; Parulekar *et al.*, 1982; Vakily, 1989).

As with other mytilid mussels, the genus *Perna* has a somewhat confused taxonomic history (Siddall, 1980; Vakily, 1989). The Roman author Pliny is reported to have used the common Latin name “*Perna*” (which means “ham”) to describe a species that is now known as *Pinna* (Cotte, 1944). The taxonomy, synonymies and geographical distribution of extant *Perna* species

were reviewed by Siddall (1980). In addition to the *Perna* Philipsson 1788 genus under consideration here, the name *Perna* has also been used to describe the genera *Modiolus* H & A Adams 1858, *Isognomon* Bruguiere 1792 and *Pinna* Linnaeus 1758 (Siddall, 1980; Vakily, 1989).

On the basis of morphological and chromosomal analyses the genus is thought to comprise three extant species (Siddall, 1980), although this was never been tested using molecular approaches. Siddall (1980) lists three species belonging to the genus *Perna*: *P. viridis* (Linnaeus, 1758), *P. canaliculus* (Gmelin, 1791) and *P. perna* (Linnaeus, 1758). He concedes that the three species can hardly be differentiated other than by their geographical distribution. Outer appearance of the shell is of little taxonomic value because “variations in shell coloration and patterns are considerable in all material” (Vakily, 1989). During the last two decades, mussels of the genus *Perna* have been introduced accidentally or intentionally to many parts of the world. While these introductions are both ecologically and economically important, they also present taxonomists with a problem because, in the absence of molecular identifications, morphological characters are a notoriously plastic and therefore unreliable means by which to accurately identify the different taxa (Siddall, 1980).

Karyotype studies of these taxa indicate that *P. canaliculus* and *P. viridis* each have 15 pairs of chromosomes and that *P. perna* has 14 pairs (Ahmed, 1974; Holland *et al.*, 1999; Jacobi *et al.*, 1990; Libertini *et al.*, 1996). *P. picta* born 1780 is reported from the North Atlantic and Mediterranean coasts of North Africa, including Mauritania, Algeria and Morocco (Shafee, 1989, 1992). An apparent exception to the taxonomic scheme presented by Siddall (1980) is the “brown mussel” found confined to a limited area around the southern tip of India (Rao, 1974; Kuriakose, 1980; Appukuttan and Nair, 1980; Silas *et al.*, 1982), which Rao (1974) referred to simply as *Mytilus* sp., and was later described as *Perna indica*

(Kuriakose, 1980). Though no proof exists, there is some evidence that this species might actually be *Perna perna*. Given the difficulty of reliably distinguishing *P. viridis* from *P. perna* because of the lack of distinct morphological variation within the genus *Perna* (Siddall, 1980), it is interesting to note that the major characteristics listed by Kuriakose (1980) to differentiate *P. indica* from *P. viridis* are identical with those given by Siddall (1980) to distinguish *P. perna* from *P. viridis*. However, *P. indica* was considered to be a synonym of *P. perna* by Hicks *et al.* (2001). A further indication that *P. indica* might actually be *P. perna* is its restricted distribution along south Indian coast and close geographical proximity to Sri Lanka where *P. perna* is reported to occur naturally (Sadacharan, 1982).

DNA markers are currently being used widely to detect differences among species, populations or individuals. Because of the fast sequence evolution and maternal non-recombining nature of inheritance in animals, mitochondrial genes are being widely used as a powerful tool in phylogenetic studies and species identification. Moreover, COI sequence is reasonably well conserved within species and is therefore now being widely used in invertebrate taxonomy (Baldwin, 1996; Yu *et al.*, 2003; An *et al.*, 2005). In fact, COI appears to possess a greater range of phylogenetic signal than any other mitochondrial gene and the evolution of this gene is rapid enough to allow discrimination of closely allied species (Hebert *et al.*, 2003). Sequence comparison of the ITS region is widely used in taxonomy and molecular phylogeny because it is (due to the high copy number of rRNA genes) easy to amplify even from small quantities of DNA, and it has a high degree of variation even between closely related species. This can be explained by the relatively low evolutionary pressure acting on such non-functional sequences. ITS has proven especially useful for elucidating relationships among congeneric species and closely related genera (Baldwin, 1992)

This study is a preliminary attempt to use both COI and ITS sequences for resolving the taxonomic ambiguity existing among the Indian marine mussels. Proper taxonomic identification of Indian mussels will assist in their management, including conservation and sustainable use of these resources. Molecular techniques and phylogenetic tools are being applied in the present study to address longstanding problems within the genus *Perna*. First to clarify the specific designations and to test the status of *P. indica*, *P. viridis*, *P. perna*, *P. picta* and *P. canaliculus*. Second, to determine the evolutionary relationships or sequence divergence between species of genus *Perna*.

MATERIALS AND METHODS

Sample Description and Collection

In the southern part of India, brown and green mussels have a wider distribution occurring all along the Indian coasts wherever submarine or intertidal rocky stretches are present. Along the Tamil Nadu Coast the brown mussel occurs from Colachel to Cape Comorin. Some of the submarine rocks are quite extensive up to about 800 meters in extent, as wide as 70 meters occurring at a depth of about 7 meters. The Colachel to Muttom area has the most productive mussel beds in this region. The green mussels are found in stray numbers. The brown mussels are locally known as “Thodu”, “Kallika chippi” or “Muthuva Chippi”. They are often grown in clumps, attaching themselves to rocks or to each other by means of sticky filaments, sometimes referred to as a “beard”.

The Green mussel (*Perna viridis*) and the Brown mussel (*Perna indica*) were collected from the rocky coastal areas of Kanyakumari district in Tamil Nadu state of Southern India. *P. canaliculus*, *P. perna* and *P. picta* (putative) sequences obtained from GenBank were also included in the analyses.

Total DNA can be prepared from almost any tissue of the mussel, but for consistency, the

mantle tissue just inside the rim of the shell is recommended. The mantle tissues (1 g each) were dissected from the two types of mussels: the brown mussel and green mussel and were preserved in 1.25 ml of 95% ethyl alcohol and stored at 4°C until further analysis.

DNA Extraction and PCR Amplification

Total DNA was extracted using proteinase K extraction protocol (Rawson and Hilbish, 1995). The purity of DNA was checked by reading the absorbance ratio A260/A280. The quantification of DNA was done using Nanodrop 2000 (USA). Electrophoresis of extracted DNA along with a marker (1 KB DNA ladder, Fermentas, U.S.A) was performed using 1% agarose gel, followed by staining with ethidium bromide in 1x TBE buffer for 30 min. The gel was visualized under UV transilluminator and documented using gel documentation system (Biorad, US). The primers LCO1490, 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO2198, 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' of Folmer *et al.* (1994) were used to amplify approximately 700 bp (base pairs) of the cytochrome oxidase c subunit I (COI) mitochondrial gene. Approximately 900 bp of the nuclear ribosomal RNA coding region spanning 18S, ITS1, 5.8S, ITS2 and 28S rRNA were amplified using (internal transcribed spacer) primers ITS5, 5'-GGA AGT AAA AGT CGT AAC AAG G-3' (White *et al.*, 1990) and ITS28, 5'-CGC CGT TAC TAG GGG AAT CCT TGT AAG-3' (Wagstaff and Garnock-Jones, 1998).

PCR were performed in a total reaction mix of 50µl of the isolated genomic DNA from 2 samples (Brown Mussel and Green Mussel) to amplify the COI gene and ITS region. The PCR was performed with all the above mentioned primer pairs. The PCR mixture contained 1µl of isolated genomic DNA (50 ng) from each sample, 1 µl of each primer (10 pmol/µl), 2 µl 10 mM dNTPs, 5 µl 10X PCR buffer containing MgCl₂ and 1µl of 5U/µl Taq DNA Polymerase (Applied Biosystems, USA). The PCR reaction

was conducted with the initial denaturation at 94°C for 2 minutes followed by denaturation at 94°C for 45 seconds, annealing at 62°C for 60 seconds and elongation at 72°C for 2 min. These cycles were then followed by 34 cycles of denaturation, annealing and elongation followed by an extended final elongation step at 72°C for 10 min in ABI GeneAmp 9700 PCR thermal cycler.

Electrophoresis of PCR products along with a marker (1 KB DNA ladder, Fermentas, U.S.A) was performed using 1% agarose gel, followed by staining with ethidium bromide in 1x TBE buffer for 30 min. The gel was visualized under UV transilluminator and documented using gel documentation system (Bio rad, U.S). The 1 kb amplicon was excised from the gel and the DNA was eluted from the gel slice by using GeneJet Gel extraction kit (Fermentas) according to the manufacturer's specifications.

DNA Sequencing and Analysis

Cycle sequencing was performed directly on purified PCR products using BigDye Terminator chemistry versions 3.1 (Applied Biosystems) and the PCR primers. All samples were sequenced in both directions. Precipitated cycle sequencing products were electrophoresed for capillary separation on an ABI 3730 XL Genetic Analyzer (Applied Biosystems) following the manufacturer's instructions. The obtained forward and reverse sequences were analyzed to get the contig sequence using Sequencher software v 4.10.1 and then subjected to further analysis by using various bioinformatics tools.

Sequence Alignment

The contig sequences obtained from sequencing were used for similarity search using BLAST (Basic Local Alignment Tool) of NCBI (National Centre for Biotechnology Information) (<http://www.ncbi.nlm.nih.gov/blast>). From among the BLAST hits, few sequences with reported accession IDs within (intra specific) and outside (inter specific) the given species are chosen.

The above obtained sequences were aligned using the multiple sequence alignment option of ClustalW v.2.1 (Thompson *et al.*, 1994) from EBI and rechecked using ClustalX v.1.81 (Thompson *et al.*, 1997) with default parameters, by first aligning all ingroup taxa and then adding the outgroup taxa using the profile alignment option. Each alignment obtained above is checked for its statistics like Transition/Substitution ratios, Constants and Variables using the tool SeaView (Gouy, 2010), which is a multiplatform, graphical user interface for multiple sequence alignment and molecular phylogeny.

Distance Matrix and Molecular Phylogeny

The distance matrix for each alignment was calculated using PHYLIP (Phylogeny Inference Package), a package of programs for inferring phylogenies from Felsenstein lab (Retief, 2000). Estimates of mean pair wise sequence diversity (\pm SE) were made using 1000 bootstraps of the data. SeaView tool was used to construct phylogenetic trees based on three phylogenetic methods, Neighbor-Joining (NJ) (Saitou and Nei, 1987), Maximum-Parsimony (MP) (Eck and DayHoff, 1966) and Maximum-Likelihood (ML) (Cavalli-Sforza and Edwards, 1967; Felsenstein, 1981) with estimated pair-wise genetic distances (based on Kimura's 2- parameter model). The Bayesian estimation method was generated MrBayes software v3.1.2 (Ronquist and Huelsenbeck, 2005). Phylogenetic analyses were performed with PAUP*4.0b10 (Swofford, 2002) using the Maximum-Likelihood (ML) method. MODELTEST 3.07 (Posada and Crandall, 1998) was used to choose the best model for use in the ML analysis by Hierarchical Likelihood Ratio Tests (HLRTs). The trees were visualized using TreeView1.6.6 (Page, 1996). Likelihood settings and optimized parameters from best-fit model (HKY+G) selected by hLRT in Modeltest 3.7 for Nuclear ITS gene of Brown Mussel (BITS) are Lset Base=(0.2401 0.2426 0.2297) Nst=2 TRatio=1.2015 Rates=gamma Shape=1.8443 Pinvar=0. Likelihood settings

and optimized parameters from best-fit model (HKY+I) selected by hLRT in Modeltest 3.7 for Mitochondrial COI gene of Brown Mussel (BMT) are Lset Base=(0.3092 0.1747 0.1846) Nst=2 TRatio=2.6241 Rates=equal Pinvar=0.1333. Likelihood settings and optimized parameters from best-fit model (K80+G) selected by hLRT in Modeltest 3.7 for Nuclear ITS gene of Green Mussel (GITS) are Lset Base=equal Nst=2 TRatio=1.5151 Rates=gamma Shape=0.2162 Pinvar=0. Likelihood settings and optimized parameters from best-fit model (HKY+G) selected by hLRT in Modeltest 3.7 for Mitochondrial COI gene of Green Mussel (GMT) are Lset Base=(0.3075 0.1773 0.1842) Nst=2 TRatio=3.4234 Rates=gamma Shape=1.1192 Pinvar=0.

RESULTS AND DISCUSSION

Sequence Alignment and Analysis

A total contig of 739 bp Inter transcribed space (ITS) gene from *Perna indica* (Brown Mussel), 685 bp Mitochondrial (CO1) gene from *Perna indica* (Brown Mussel), 743 bp Inter transcribed space (ITS) gene from *Perna viridis* (Green Mussel) and 719 bp Mitochondrial sequence (COI) gene from *Perna viridis* (Green Mussel) were obtained by sequencing analysis and alignment. The sequences obtained in this study have been deposited in GenBank (NCBI) under accession numbers JQ622200, JQ622199, JQ622201 and JQ622198.

ITS and COI sequences of *P. indica* and *P. viridis* along with published sequences of *P. perna* (NCBI Accession: DQ924543, DQ924546, DQ917594 DQ917592), *P. canaliculus* (DQ924556, DQ924553, DQ917607) and *P. picta* (putative) DQ924548, DQ917597 reported by Wood *et al.* (2007) were used for the comparative study and alignment.

In the present study, for the Nuclear ITS gene of Brown Mussel (BITS), homology search using BLASTn showed more than 99% identity with a 98% query coverage without any error value to 18s rRNA gene of *Perna perna* voucher which is a

brown mussel. Of the 739 bp contig sequences resolved, 523 bases were constants and 274 bases (37%) exhibited variation. The Transition Substitution Ratio was 1.133. The percentage of similarity was 99% with *P. perna* and *P. picta*. The percentage of dissimilarity was 8% and 12% with *P. canaliculus* and *P. viridis* respectively.

For the Mitochondrial COI gene of Brown Mussel (BMT), homology search using BLASTn showed more than 100% identity with a 96% query coverage without any error value to cytochrome oxidase c subunit 1 gene of *Perna indica* which was a brown mussel. Of the 685 bp Contig Sequences resolved, 538 bases were constants and 277 bases (40%) exhibited variation. The Transition Substitution Ratio was 1.389. The percentage of similarity was 94% with *P. viridis*, *P. perna* and *P. picta*. The percentage of dissimilarity was 3% with *P. canaliculus*.

For the Nuclear ITS gene of Green Mussel (GITS), homology search using BLASTn showed more than 99% identity with a 100% query coverage without any error value to 18s rRNA gene of *Perna viridis* voucher which was a green mussel. Of the 743 bp contig sequences resolved, 680 bases were constants and 111 bases (15%) exhibited variation. The Transition Substitution Ratio was 1.225. The percentage of similarity was 81% with *P. perna* and *P. picta*. The percentage of dissimilarity was 13% with *P. canaliculus*.

For the Mitochondrial COI gene of Green Mussel (GMT), homology search of *Perna viridis* of Indian marine origin using BLAST showed more than 100% identity with a 91% query coverage without any error value to cytochrome oxidase c subunit 1 gene of *Perna indica*. Of the 719 bp contig sequences resolved, 531 bases were constants and 275 bases (38%) exhibited variation. The Transition Substitution Ratio was 2.038. The percentage of similarity was 100%, 95% and 94% with *P. indica*, *P. perna*, *P. viridis* and *P. picta*. The percentage of dissimilarity was 3% with *P. canaliculus*.

In our study based on nuclear internal transcribed spacer (ITS) gene sequence data, Indian brown (BITS) and green (GITS) mussels revealed 99% and 81% sequence similarity to *P. perna* and *P. picta*. They showed 8% and 13% dissimilarity to *P. canaliculus*. Based on mitochondrial cytochrome oxidase I (COI) gene sequence data, Indian Brown (BMT) and Green (GMT) Mussels revealed 94% and 95% sequence similarity to *P. perna* and *P. picta*. They showed 3% dissimilarity to *P. canaliculus*. The indication is that *P. indica* is a distinct species and not to be relegated as a synonym of *P. perna*.

Phylogenetic Inference

In our study, the distance matrix values supported the phylogenies inferred using Maximum Likelihood (ML) and Bayesian approaches which produced almost similar, well supported topologies and verified the monophyly of the genus with respect to five *Perna* species. Based on nuclear internal transcribed spacer (ITS) gene sequence data, Indian Brown Mussel (BITS) is most closely related to *P. perna* and *P. picta* representing <1% sequence divergence. Based on mitochondrial cytochrome oxidase I (COI) gene sequence data, Indian Brown (BMT) and Green (GMT) Mussels are most closely related to *P. canaliculus*.

P. viridis (GITS, GMT) and *P. indica* (BMT) each formed distinct clades, confirming their specific status. *P. perna* (Atlantic), *P. canaliculus* (New Zealand) and putative *P. picta* (North Africa) clustered within the *P. indica* and *P. viridis* clades were less divergent and closely related to each other. *P. picta* clustered within the *P. perna* clade was not regarded as a separate species and hence was found the most closely related of the five species. *P. indica* and *P. viridis* were closely related to *P. canaliculus* showing less sequence divergence. It was proved that the within-species divergence values was greater than 2%.

The values obtained in distance matrix for each alignment with estimated pair-wise genetic

distances based on Kimura's 2-parameter model represented the evolutionary distance (number of substitutions per nucleotide) or sequence divergence between species. The distance matrix was obtained by Kimura's two parameter model (1980) using PHYLIP tool. The evolutionary distance (number of substitutions per nucleotide) using the sequence data resolved in this study and the ITS and COI sequences published by Wood *et al.* (2007) are given in Tables 1-4 respectively. All the values in the distance matrix are multiplied by 100 to calculate the percentages.

The evolutionary distance (number of substitutions per nucleotide) between Brown Mussel (BITS) and *P. viridis* (Green Mussel) equals 0.166422. All the values in the distance matrix are multiplied by 100 (Table 1). Therefore it can be interpreted that Brown Mussel (BITS) showed 16.64% sequence divergence from *P. viridis* and 9% divergence from *P. canaliculus*. *P. perna* and *P. picta* showed negligibly low (< 1%) sequence divergence with Brown Mussel (BITS). *M. edulis* formed the out-group with 54% divergence from *P. viridis*, *P. canaliculus*, *P. perna*, *P. picta* and 56% divergence from Brown Mussel (BITS). The *P. indica* species showed significantly high sequence divergence with all the other *Perna* sps. From the distance matrix, it was evident that the Mitochondrial (COI) sequence of Brown Mussel (BMT) was more closely related to *Perna indica* and showed significant divergence with the other sequences of *Perna* species.

The evolutionary distance between Green Mussel (GITS) and *P. viridis* was <1% and hence it showed negligibly low sequence divergence. GITS showed 17.5% sequence divergence from *P. picta*, 17.7% divergence from *P. perna* and 18.4% divergence from *P. canaliculus*. The distance matrix showed that GITS is more closely related to *Perna viridis*. From the distance matrix it was evident that the Mitochondrial COI sequence of Green Mussel (GMT) was more closely related to *Perna viridis* and showed significant divergence with the other sequences of *Perna* species.

Table 1. The pair wise sequence divergence and mean evolutionary genetic distances computed among the published sequences of *P. viridis*, *P. canaliculus*, *P. perna*, *P. picta*, *P. indica* and *M. edulis* (Wood *et al.*, 2007) with the Nuclear ITS gene of Brown Mussel (BITS) using PHYLIP software

Distance Matrix: (Lower triangular distance matrix based on Kimura's model) using PHYLIP tool

**	1	2	3	4	5	6	7	8	9	10	11
1gb DQ924555.1											
2gb DQ924550.1	0.000000										
3gb DQ924541.1	0.000000	0.000000									
4gb DQ924556.1	0.169985	0.169985	0.169985								
5gb DQ924553.1	0.169985	0.169985	0.169985	0.000000							
6gb DQ924543.1	0.163050	0.163050	0.163050	0.098527	0.098527						
7gb DQ924548.1	0.160925	0.160925	0.160925	0.100137	0.100137	0.001359					
8gb DQ924546.1	0.163050	0.163050	0.163050	0.098527	0.098527	0.000000	0.001359				
9Sample_BITS	0.166422	0.166422	0.166422	0.099405	0.099405	0.000000	0.001372	0.000000			
10gb FJ428753	1.208618	1.208618	1.208618	1.214489	1.214489	1.191836	1.195889	1.191836	1.195889		
11gb FJ428754	1.195875	1.195875	1.195875	1.201818	1.201818	1.179213	1.183264	1.179213	1.183264	0.000000	
12gb AY695798	0.539864	0.539864	0.539864	0.543532	0.543532	0.545972	0.546299	0.545972	0.564772	1.573218	1.551465

** Number indicates: (1,2&3) *P. vindis*, (4&5) *P. canaliculatus*, (6&8) *P. perna*, (7) *P. picta*, (9) BITS, (10&11) *P. indica*, (12) *M. edulis*

** Number indicates: (1,2&3) *P. vindis*, (4&5) *P. canaliculatus*, (6&8) *P. perna*, (7) *P. picta*, (9) BITS, (10&11) *P. indica*, (12) *M. edulis*

Table 2. Pair wise sequence divergence and mean genetic distances computed among the published sequences from NCBI for *P. indica*, *P. viridis*, *P. picta*, *P. perna* and *P. canaliculus* (Wood *et al.*, 2007) with the Mitochondrial COI sequence of Brown Mussel (BMT) using PHYLIP software

**	1	2	3	4	5	6	7	8	9	10	11	12
1gb HM545311.1												
2gb HM545309.1	0.001564											
3gb HM545310.1	0.001564	0.003130										
4gb HM545308.1	0.001564	0.003130	0.000000									
5gb FJ428754.1	0.001606	0.001614	0.001614	0.001614								
6gb FJ428755.1	0.004797	0.004821	0.004821	0.004821	0.003059							
7gb FJ428753.1	0.001579	0.001586	0.001586	0.001586	0.000000	0.003073						
8Sample_BMT	1.327200	1.318788	1.312169	1.312169	1.334640	1.318064	1.330669					
9gb DQ917587.1	0.060150	0.060462	0.060462	0.060462	0.056910	0.053462	0.056910	1.400246				
10gb DQ917597.1	0.067282	0.067632	0.067632	0.067632	0.063843	0.060370	0.063843	1.393065	0.022980			
11gb DQ917594.1	0.069073	0.069433	0.069433	0.069433	0.065585	0.062105	0.065585	1.400246	0.024644	0.001622		
12gb DQ917592.1	0.069073	0.069433	0.069433	0.069433	0.065585	0.062105	0.065585	1.400246	0.024644	0.001622	0.000000	
13gb DQ917607.1	0.187931	0.189021	0.189021	0.189021	0.187268	0.183130	0.187268	1.607796	0.191178	0.193260	0.195346	0.195346

** Number indicates : (1-7) *P. indica*, (8) BMT, (9) *P. viridis*, (10) *P. picta*, (11&12) *P. perna*, (13) *P. canaliculus*

Molecular phylogenetic analysis of Perna

Table 3. Pair wise sequence divergence and mean genetic distances computed among the published sequences from NCBI for *P. viridis*, *P. picta*, *P. perna*, and *P. canaliculus* (Wood *et al.*, 2007) with the Nuclear ITS gene of Green Mussel (GITS) using PHYLIP software

**	1	2	3	4	5	6	7	8
1gb DQ924539								
2gb DQ924550	0.000000							
3gb DQ924541	0.000000	0.000000						
4gb DQ924555	0.000000	0.000000	0.000000					
5gb DQ924544	0.000000	0.000000	0.000000	0.000000				
6Sample_GITS	0.009698	0.009691	0.009691	0.009691	0.008296			
7gb DQ924548	0.161410	0.161273	0.161273	0.161273	0.161013	0.175157		
8gb DQ924559	0.163007	0.162869	0.162869	0.162869	0.162869	0.177050	0.001358	
9gb DQ924556	0.168001	0.169603	0.169603	0.169603	0.169603	0.183848	0.092372	0.090650

** Number indicates : (1-5) *P. viridis*, (6) GITS, (7) *P. picta*, (8) *P. perna*, (9) *P. canaliculus*

Table 4. Pair wise sequence divergence and mean genetic distances computed among the published sequences from NCBI for *P. viridis*, *P. picta*, *P. perna*, *P. indica*, and *et al.*, *P. canaliculus* (Wood *et al.*, 2007) with the Mitochondrial COI sequence of Green Mussel (GMT) using PHYLIP software

**	1	2	3	4	5	6	7	8	9	10	11
1gb DQ917616.1											
2gb DQ917617.1	0.001622										
3gb DQ917618.1	0.001622	0.003247									
4gb DQ917603.1	0.027973	0.029645	0.029645								
5gb DQ917597.1	0.027973	0.029645	0.029645	0.000000							
6gb DQ917587.1	0.024638	0.026304	0.026304	0.022975	0.022975						
7gb FJ428753.1	0.055152	0.056875	0.056875	0.063800	0.063800	0.056875					
8gb HM545311.1	0.058339	0.060112	0.060112	0.067234	0.067234	0.060112	0.001579				
9gb FJ428754.1	0.055152	0.056875	0.056875	0.063800	0.063800	0.056875	0.000000	0.001607			
10gb DQ917608.1	0.198602	0.200687	0.200687	0.196522	0.196522	0.194445	0.186414	0.187074	0.186414		
11gb DQ917607.1	0.194687	0.196767	0.196767	0.192613	0.192613	0.190542	0.186645	0.187312	0.186645	0.004880	
12Sample_GMT	1.238887	1.233064	1.233064	1.238887	1.238887	1.256402	1.318408	1.284688	1.309511	1.349214	1.354730

** Number indicates: (1-3) *P. perna*, (4&5) *P. picta*, (6) *P. viridis*, (7-9) *P. indica*, (10&11) *P. canaliculus*, (12) GMT

The consensus phylogenetic trees of the two Indian mussels, *P. indica* and *P. viridis* as well as *P. perna*, *P. picta* and *P. canaliculus* based on Maximum Likelihood (ML) method and Bayesian Estimation method using the sequence data resolved in this study and the ITS and COI sequences published by Wood *et al.* (2007) are shown from Fig. 1 to Fig. 8 respectively.

The Kimura's two parameter model corrects for multiple hits, taking into account transitional and transversional substitution rates, while assuming that the four nucleotide frequencies are the same and that rates of substitution do not vary among sites. While we want to find the best point estimates of parameter values in maximum likelihood, the goal in Bayesian phylogeny is instead to find a full probability distribution over all possible parameter

values. Model test using Modeltest3.7 executed in PAUP*4.0b10 interphase generated parameters used to construct Maximum Likelihood trees. These trees were visualized using the software TreeView1.6.6. The results obtained were similar to the trees generated based on the Maximum-likelihood Kimura's two parameter model.

Maximum Likelihood method (Fig. 1) and Bayesian Estimation method (Fig. 2) confirmed that the Nuclear ITS gene of Brown Mussel (BITS) was more closely related to the reported sequences of *P. perna* and *P. picta* while *P. indica* showed a significant divergence.

From Maximum Likelihood method (Fig. 3) we confirmed that the Mitochondrial (COI) sequence of Brown Mussel (BMT) was less evolved from

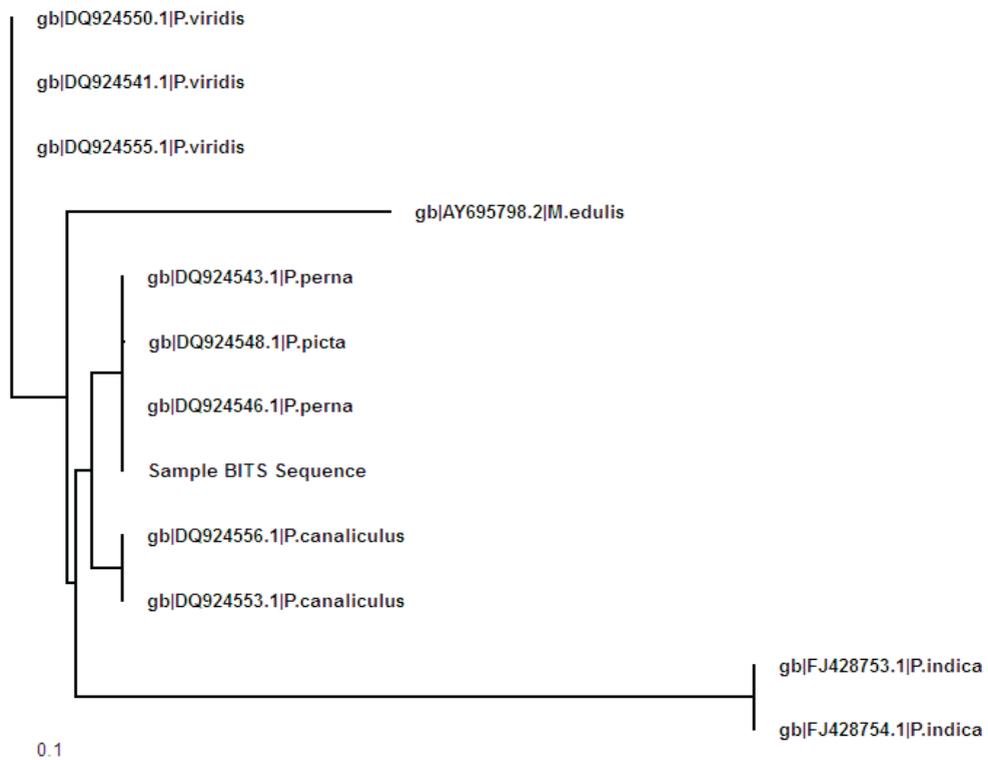


Fig. 1. Consensus Maximum Likelihood method tree obtained using the sequence data of Nuclear ITS gene of Brown Mussel (BITS) resolved in this study and sequences obtained through the study of Wood *et al.* (2007)

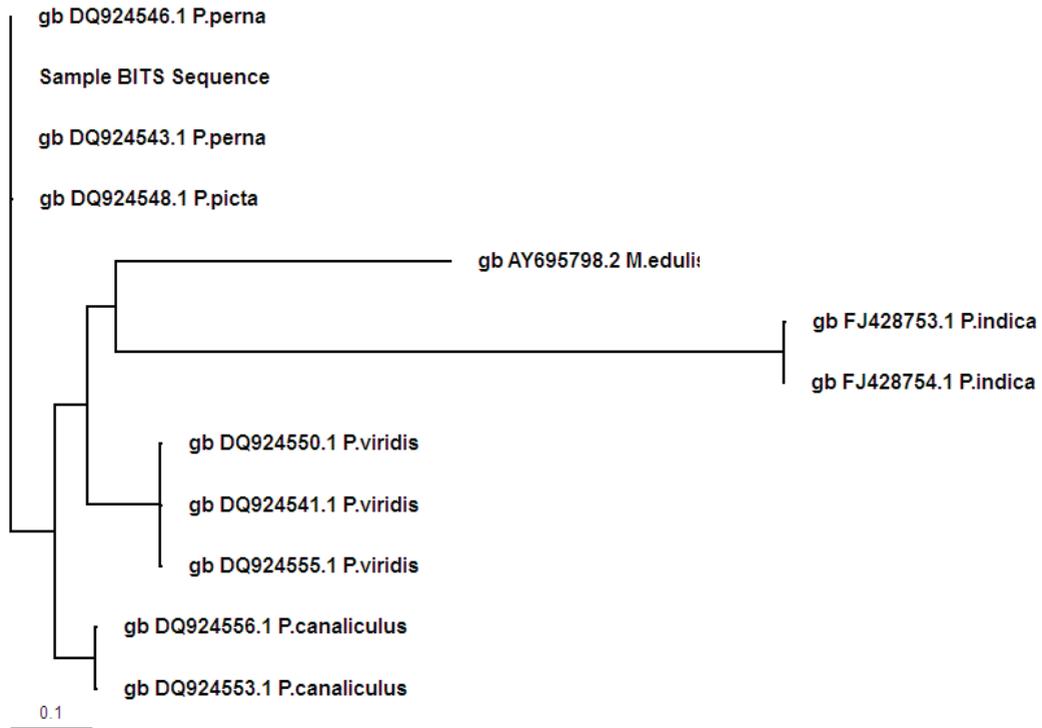


Fig. 3. Consensus Maximum Likelihood method tree obtained using the sequence data of Mitochondrial (COI) sequence of Brown Mussel (BMT) resolved in this study and sequences obtained through the study of Wood *et al.* (2007)

the sequence of *P. canaliculus*. Based on Kimura's two parameter model, the reported sequences for *Perna indica* and other *Perna* species formed a clade with Mitochondrial (COI) sequence of Brown Mussel (BMT) and kept a probabilistic distance of 50.001 from the main clade. From Bayesian Estimation method (Fig. 4) it is evident that Mitochondrial COI sequence of Brown Mussel (BMT) was more closely related to *P. canaliculus*, while it showed a significant divergence with the *P. indica* and *P. perna*.

Based on Kimura's two parameter model Maximum Likelihood method (Fig. 5), the reported sequences of *Perna viridis* formed a clade with the ITS region of Green Mussel (GITS) while it showed more divergence to other *Perna*

sps. Based on Bayesian Estimation method the reported sequences of (Fig. 6) *Perna viridis* formed a clade with the ITS region of Green Mussel (GITS) while it showed more divergence to other *Perna* species.

From Maximum Likelihood method (Fig. 7), it was evident that the Mitochondrial COI sequence of Green Mussel (GMT) showed very less divergence with the *P. perna* and *P. viridis*. From Bayesian Estimation method (Fig. 8) it was evident that the Mitochondrial COI sequence of Green Mussel (GMT) was more closely related to *P. canaliculus* and showed very less divergence towards it. At the same time it showed significant divergence towards *P. viridis* and *P. indica*.



Fig. 2. Bayesian Estimation method (MrBayes software) tree obtained using the sequence data of Nuclear ITS gene of Brown Mussel (BITS) resolved in this study and sequences obtained through the study of Wood *et al.* (2007)

DISCUSSION

The Phylogenetic trees based on separate analyses of the COI and ITS datasets recovered almost similar well supported topologies and verified the monophyly of the genus *Perna*. COI and ITS showed higher resolutions within intra-species clades and inter-specific evolutionary relationships. *P. indica*, *P. viridis* (Indo-West Pacific), *P. perna* (Atlantic), and *P. canaliculus* (New Zealand) each formed distinct clades, confirming their specific status. Putative *P. picta* from North Africa clustered within the *P. perna* clade and is not regarded as a separate species.

The Indian marine brown mussel and green mussel formed distinct clades. *P. indica* revealed 94% similarity to *P. perna* compelling for a re-look of the suggestion of Vakily *et al.* (1989) that the former is only a variant of *P. perna*. It has also been suggested that *P. indica*, which occurs only along the Indian coast, is a synonym of the globally distributed *P. perna* (Hicks *et al.*, 2001). It has been confirmed in the present study that *P. indica* is a distinct species and not to be relegated as a synonym of *P. perna*. According to Wood *et al.* (2007), *P. perna* and *P. canaliculus* were the most closely related of the *Perna* species. In the present

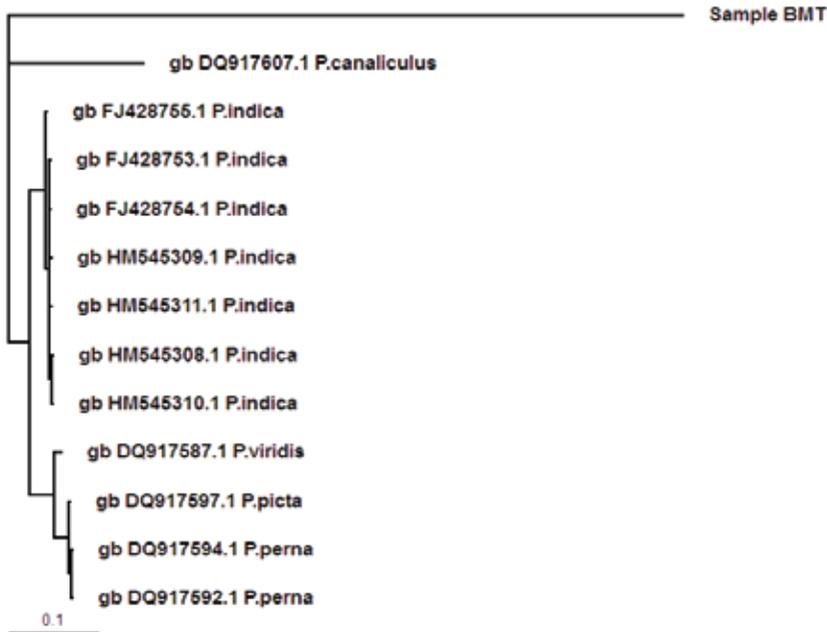


Fig. 4. Bayesian Estimation method (MrBayes software) tree obtained using the sequence data of Mitochondrial COI gene of Brown Mussel (BMT) resolved in this study and sequences obtained through the study of Wood *et al.* (2007)



Fig. 5. Consensus Maximum Likelihood method tree obtained using the sequence data of Inter transcribed space of Green Mussel (GITS) resolved in this study and sequences obtained through the study of Wood *et al.* (2007)

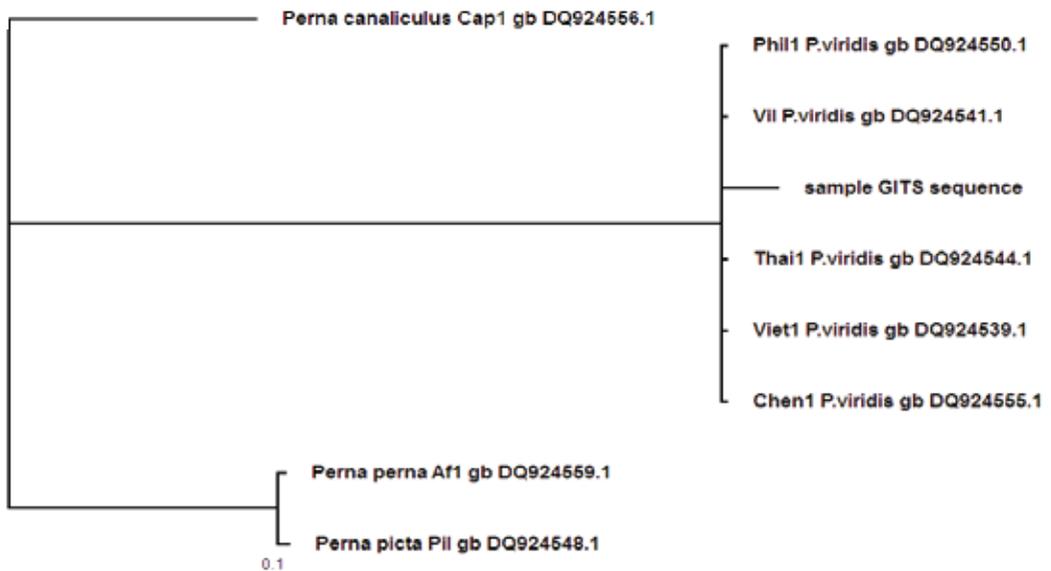


Fig. 6. Bayesian Estimation method (MrBayes software) tree obtained using the sequence data of Nuclear ITS gene of Green Mussel (GITS) resolved in this study and sequences obtained through the study of Wood *et al.* (2007)



Fig. 7. Consensus Maximum Likelihood method tree obtained using the sequence data of Mitochondrial sequence of Green Mussel (GMT) resolved in this study and sequences obtained through the study of Wood *et al.* (2007)

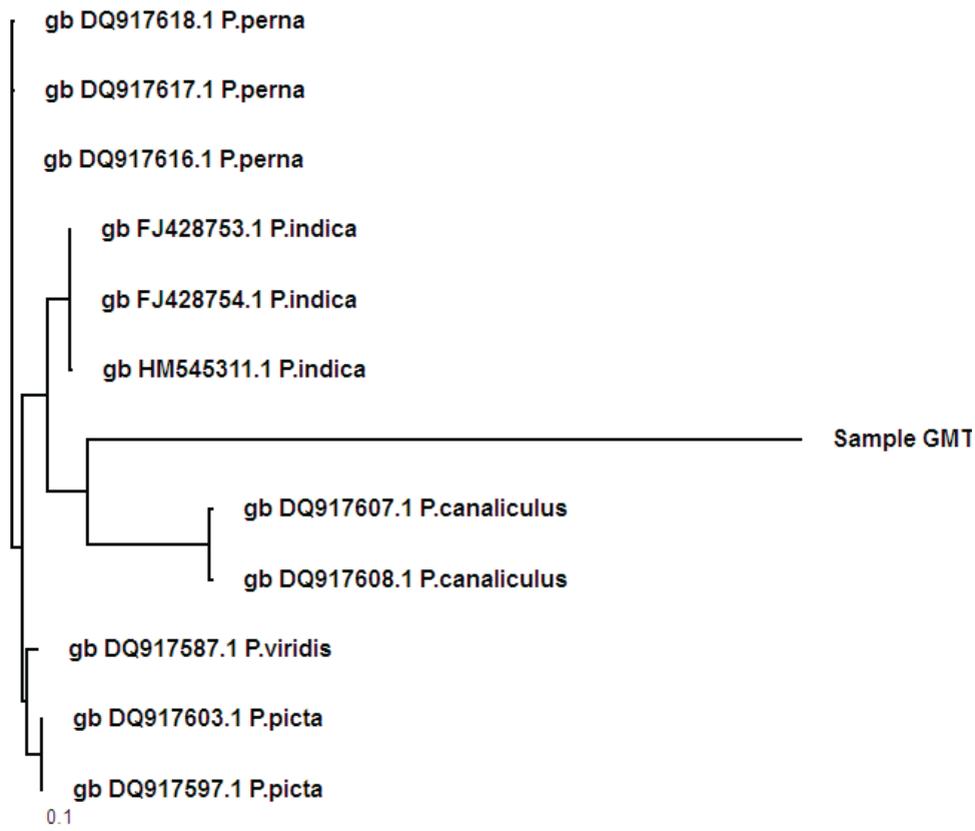


Fig. 8. Bayesian Estimation method (MrBayes software) tree obtained using the sequence data of Mitochondrial COI gene of Green Mussel (GMT) resolved in this study and sequences obtained through the study of Wood *et al.* (2007)

study, the *P. picta* clustered within the *P. perna* clade is not regarded as a separate species and hence is found the most closely related of the five species. *P. indica* and *P. viridis* are closely related to *P. canaliculus* showing less sequence divergence. According to Ward *et al.* (2008), majority of within-species divergence values show less than 2%. The COI sequence divergence between *P. indica* and a morphotype of *P. indica* was <2% (Divya *et al.*, 2009). In the present study, it was proved that the within-species divergence values are greater than 2%.

In the present study, the distance matrix values

supported the phylogenies inferred using Maximum Likelihood (ML) and Bayesian approaches which produced almost similar, well supported topologies and verified the monophyly of the genus with respect to five *Perna* species. Based on nuclear internal transcribed spacer (ITS) gene sequence data, Indian Brown Mussel (BITS) is most closely related to *P. perna* and *P. picta* representing <1% sequence divergence. Based on mitochondrial cytochrome oxidase I (COI) gene sequence data, Indian Brown (BMT) and Green (GMT) mussels are most closely related to *P. canaliculus*. *P. viridis* (GITS, GMT)

and *P. indica* (BMT) each formed distinct clades, confirming their specific status. *P. perna* (Atlantic), *P. canaliculus* (New Zealand) and putative *P. picta* (North Africa) clustered within the *P. indica* and *P. viridis* clades are less divergent and closely related to each other. 1000 Boot strap replications were applied to obtain the bootstrap value which gives the estimate of reliability of the tree topology. Bootstrap support for *P. indica*, *P. viridis* and *P. canaliculus* was 100% under MP but was lower for the *P. perna* and *P. picta* clade (74%).

The phylogenetic results of the present study are based on both nuclear and mitochondrial markers to resolve phylogenetic ambiguities. Further studies involving more number of individuals from different geographical locations and more number of species-specific regions in the nuclear and mitochondrial DNA are essential, to confirm the taxonomic identity of Indian marine mussels of genus *Perna*. Possible biogeographic explanations for the present *Perna* species distributions also need to be evaluated.

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