metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by CMFRI Digital Repository

Indian J. Fish., 56(3) : 223-226, 2009

Note

Mitochondrial DNA (Cytochrome c oxidase I) sequencing of Indian marine mussels

P. R. DIVYA, A. GOPALAKRISHNAN, LIJO JOHN, P. C. THOMAS[1], AND W. S. LAKRA[2]

National Bureau of Fish Genetic Resources , Cochin Unit, CMFRI Campus, Cochin - 682 018, India
[1]Marine Biotechnology Division, Central Marine Fisheries Research Institute, Cochin - 682 018, India
[2]National Bureau of Fish Genetic Resources, Lucknow - 226 002, India
e-mail: divyanbfgr@gmail.com

ABSTRACT

Two species of marine mussels, the green mussel *Perna viridis* (Linnaeus, 1758) and the brown mussel *Perna indica* (Kuriakose and Nair, 1976) are found along the Indian coast. It had been suggested that *P. indica*, which occurs only along the Indian coast, is a synonym of the globally distributed *Perna perna*. Along the south-west coast of India, where both *P. viridis* and *P. indica* co-exist, a third type referred to as parrot mussel, which has shell shape of brown mussel and color of green mussel (suspected to be their hybrid/morphotype) also occurs. The present investigation is a preliminary attempt for resolving the taxonomic ambiguity among Indian marine mussel species using the mitochondrial cytochrome oxidase I (COI) gene. *P. indica* revealed 95% sequence similarity to *P. perna*. The sequence divergence between *P. indica* and parrot mussel was negligibly low (< 2%). Green mussel *P. viridis* showed 20.87% of sequence divergence with brown mussel *P. indica* as well as with the parrot mussel.

Keywords : Brown mussel, Cytochrome oxidase I (COI) gene, Green mussel, *Perna indica, Perna virdis*

Aquaculture importance of different mussel species including those belonging to the genera *Mytilus* and *Perna* is globally known. The leading mussel producing countries of the world includes China, Spain, Italy, Thailand, New Zealand, France, Ireland, Netherlands, Canada, United States of America, South American countries, Korea and Japan (FAO, 2006). The major characteristic feature by which the adults of *Perna* and *Mytilus* can be distinguished is the pattern of the scars left at the area of muscle attachment on the shell (Siddall, 1980). The confusion over the taxonomy of *Perna* extends far back into the history of natural science. 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). Siddall (1980) gave the taxonomic clarification of the genus *Perna* with three currently recognized species *viz., Perna perna* (Linnaeus, 1758), *Perna viridis* (Linnaeus, 1758) and *Perna canaliculus* (Gmelin, 1791). An apparent exception to the taxonomic scheme presented by Siddall (1980) is a "brown mussel" found confined to the southern tip of India (Rao, 1974; Appukuttan and Nair, 1980; Kuriakose, 1980; Silas *et al.*, 1982), which Rao (1974) referred to as *Mytilus* sp., and was later described as *P. indica* (Kuriakose and Nair, 1976). Though no proof exists, there is some evidence that this species might actually be *P. perna* (Vakily, 1989). Given the difficulty of reliably distinguishing *P. viridis* from *P. perna* because of the great 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).

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*. Along the Kollam coast of Kerala, both *P. viridis* and *P. indica* 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 occurs, which has a shell shape of brown mussel, but with green shell colouration. Kripa *et al.* (2001) reported it as parrot mussel and suggested it as a possible hybrid

between the green mussel, *P. viridis* and brown mussel, *P. indica.* Padhi (1998) has also reported the existence of intermediate morphotypes of mussels from Indian coast.

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). In the present study, the mitochondinal DNA(Mt DNA) COI gene fragments from green mussel, brown mussel and the suspected hybrid/ morphotype (parrot mussel) distributed along the Indian coasts were sequenced and compared to resolve the taxonomic ambiguity among them. Proper taxonomic identification of Indian mussels will assist their management, including conservation and sustainable use of these resources. This study is a preliminary attempt to use COI sequences for resolving the taxonomic ambiguity existing among the Indian marine mussels.

The mantle tissues (1 g each) were dissected from the three types of mussels: the green mussel, brown mussel and parrot mussel and were preserved in 1.25 ml of 95% ethyl alcohol and stored at 4^0C until further analysis. Extracting DNA of sufficient purity was difficult at the initial stage of work when conventional phenol- chloroform method of extraction was attempted. This was due to the presence of large amount of polysaccharides in mussel's mantle tissue, which are usually isolated along with DNA. The polysaccharide can inhibit the activity of polymerases and ligases. Hence, genomic DNA was isolated following the modified phenol chloroform method of Sokolov *et al.* (2000). Saturated KCl was added to the clear lysate of digested tissue samples, mixed well and incubated in ice for 5 min. At this stage, most of the polysaccharides and proteins are precipitated along with the insoluble sodium dodecyl sulphate.

An internal fragment of the COI gene was amplified using a pair of metazoan invertebrate COI primer *viz*., LCOI1490/HCOI2198: 5'-GGTCAACAAATCATAAAGATATTGG-3'/ 5'-TAAACTTCAGGGTGACCAAAAAATCA -3' (Folmer *et al.,* 1994). The PCR amplifications were performed in 25 µl assay volume containing 2.5 µl 10x assay buffer (100 mM Tris, 500 mM KCl, 0.1% gelatin, pH 9.0) with 1.5 mM $MgCl_2$ (Genei, Bangalore, India), 5 p moles of each primer, 200 µM dNTPs (Genei, Bangalore, India), and 1.5 U Taq DNA polymerase, 18 ml de-ionized water and 20 ng of template DNA. The reaction mixture was initially denatured at 95 °C for 5 min followed by 29 cycles (94 °C for 45 s, 58 °C for 30 s and 72 °C for 45 s). The reaction was then subjected to a final extension at 72 °C for 5 min.

Electrophoresis of 3 ml of the PCR product along with a marker (100 bp DNA ladder, Genei, Bangalore, India) 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 Image Master VDS (Pharmacia Biotech, USA). All PCR products were purified using the QIA-quick PCR purification kit (Qiagen) and directly sequenced following the protocol outlined in the ABI Prism BigDye sequencing kit (PE Applied Biosystems) using the light strand primers on an ABI 3730 automated DNA sequencer following the manufacturer's instructions.

The DNA sequences were aligned and edited using the Clustal W software in the BIOEDIT sequence alignment editor version 7.0.5.2 (Hall, 1999). Exploratory data analysis including the phylogenetic analyses of sequences were performed using MEGA Version 3.1 (Kumar *et al.,* 2004) and a final adjustment was done manually. Estimates of mean pair wise sequence diversity (± SE) were made using 1000 bootstraps of the data. MegAlign was used to create phylogenetic trees based on two phylogenetic methods, Neighbor Joining- NJ (Saitou and Nei, 1987) and Maximum Parsimony-MP (Eck and DayHoff, 1966) using estimated pair-wise genetic distances (based on Kimuras' 2- parameter model).

A total of 477 bp of aligned sequence of COI gene from two individuals each of green, brown and parrot mussel were used for the comparative study. The sequences obtained in this study have been deposited in GenBank (NCBI) with accession numbers EU543992- EU543997. COI sequences of 3 haplotypes detected among the 6 specimens, along with published sequences of *P. perna* (NCBI Accession: DQ 917617, DQ 917618), *P. canaliculus* (DQ 917613, DQ 917609) and *Mytilus edulis* (DQ 917606) reported by Wood *et al*. (2007) were aligned. In the present study, *P. viridis* and parrot mussel revealed only single haplotype each, while *P. indica* had 2 haplotypes. There were no insertions and deletions in the sequence. Of the 477 bp unambiguous sequence resolved, 431 bases were constant and 46 bases (10%) exhibited variation. The transition: transversion substitution ratio was 1.4.

The pair-wise sequence divergence and mean genetic distances computed among the three Indian mussel haplotypes with the published sequences for *P. perna, P. canaliculus* and *M. edulis* (Wood *et al*., 2007) are given in Table 1. The sequence divergence between *P. indica* and parrot mussel was negligibly low (< 2%). *P. viridis* (green mussel) showed 20.87% sequence divergence with *P. indica* (brown mussel) as well as parrot mussel. *M. edulis* formed

Table 1. Pair wise sequence divergence and mean genetic distances computed among the three Indian mussel haplotypes with the published sequences for *P. perna, P. canaliculus* and *M. edulis* (Wood *et al*., 2007)

**	1	2	3	4	5	6	7	8	9	10	11
1											
2	0.0000										
3	0.2087	0.2087									
4	0.2087	0.2087	0.0000								
5	0.2117	0.2117	0.0021	0.0021							
6	0.2087	0.2087	0.0000	0.0000	0.0021						
7	0.2233	0.2233	0.0552	0.0552	0.0576	0.0552					
8	0.2203	0.2203	0.0552	0.0552	0.0576	0.0552	0.0042				
9	0.2447	0.2447	0.1953	0.1953	0.1982	0.1953	0.2044	0.2044			
10	0.2447	0.2447	0.1953	0.1953	0.1982	0.1953	0.2163	0.2163	0.0106		
11	0.3967	0.3967	0.4161	0.4161	0.4121	0.4161	0.4317	0.4276	0.4300	0.4340	

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

the out-group with 39% divergence from green mussel, 41% divergence from both brown mussel and parrot mussel. The sequence divergence of Indian brown mussel *P. indica* and *P. perna* was 5%. The haplotypes of brown and parrot mussels showed negligibly low divergence. This is also supported by the similarity in morphology between these two types of mussels. The difference between them was limited only to their colour.

The published reports on Indian marine mussels' phylogeny and systematics are limited, except few (Kuriakose and Nair, 1976; Siddall, 1980). In our study, the indication is that *P. indica* is a distinct species and not to be relegated as a synonym of *P. perna.* According to Ward *et al*. (2008)*,* majority of within-species divergence values show less than 2%. The COI sequence divergence between *P. indica* and parrot mussel was also <2%. Parrot mussel

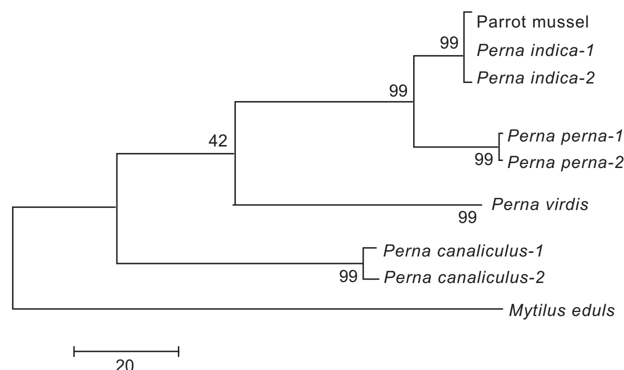

Fig. 1. Consensus Maximum Parsimony tree based on parsimony analysis of the haplotypes detected in this study, along with those sequences of *Perna perna*, *Perna canaliculus* and *Mytilus edulis* obtained through the study of Wood *et al*. (2007).

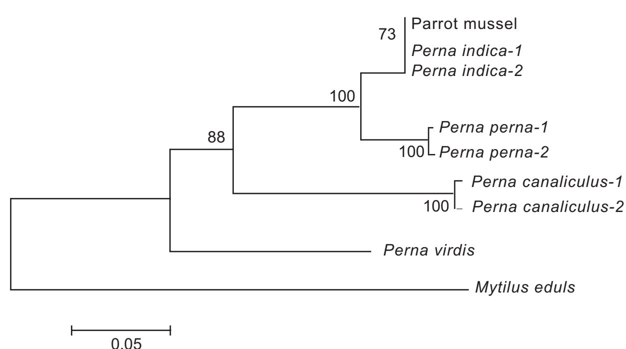

Fig. 2. Consensus Neighbor-joining tree based on the analysis of the haplotypes detected in this study, along with those sequences of *Perna perna*, *Perna canaliculus* and *Mytilus edulis* obtained through the study of Wood *et al*. (2007)

Consensus phylogenetic tree of the three Indian mussels as well as *P. perna, P. canaliculus* and *M. edulis* based on Parsimony analysis and Neighbor Joining method using the sequence data resolved in this study and the COI sequences published by Wood *et al*. (2007) are given in Fig. 1 and 2 respectively. The brown mussel and parrot mussel formed a single group (clade), while the green mussel formed a distinct clade. *P. indica* revealed 95% 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.*

may be a morphotype of *P. indica* and it is suggested that inclusion of nuclear genes in the study can confirm the genetic identity of parrot mussel. The phylogenetic results of the present study are based on data from a single mitochondrial gene. Inclusion of other markers, both nuclear and mitochondrial, is also required to resolve phylogenetic ambiguities. Further studies involving more number of individuals and more number of species-specific regions in the nuclear and mt DNA are essential, to confirm the taxonomic identity of *P. indica* and parrot mussel.

Acknowledgements

The authors thank the Director NBFGR, Lucknow for providing necessary facilities for completing the work. The first author also acknowledges CIFE/ICAR for the financial assistance received during the tenure of this work.

References

An, H. S., Jee, Y. J., Min, K. S., Kim, B. L. and Han, S. J. 2005. Phylogentic analysis of six species of Pacific abalone (Haliotidae) based on DNA sequences of 16s rRNA and Cytochrome *c* oxidase subunit É mitochondrial genes. *Mar. Biol.,* 7: 373-380.

Appukuttan, K. K. and Nair, T. P. 1980. Fishery and biology of the brown mussel, *Perna indica* Kuriakose & Nair. *Bull. Cent. Mar. Fish. Res. Inst.,* 29: 5-9

Baldwin, B. S., Black, M., Sanjur, O., Gustafson, R., Lutz, R. A. and Vrijenhoek, C. 1996. A diagnostic molecular marker for zebra mussels *(Dreissena polymorpha)* and potentially co-occurring bivalves mitochondrial COI. *Mol. Mar. Biol. Biotechnol.,* 5(1): 9-14.

Eck, R. V. and Dayhoff, M. O. 1966. *Atlas of protein sequence and structure,* . National Biomedical Research Foundation, Silver Spring, Maryland.

Folmer, O., Black, M., Hoeh, W., Lutz, R. and Vrijenhoek, R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotech*., 3(5): 294–299.

Hall, T. A. 1999. Bioedit: A user friendly biological sequence alignment editor and analysis program for Windows 95/98 NT. *Nucleic Acids Symp. Ser.,* 41: 95-98.

Hicks, D. W., Tunnel Jr, J. W., Robert, F. and Mc Mahon 2001. Population dynamics of the non-indigenous brown mussel *Perna perna* in the Gulf of Mexico compared to other worldwide populations. *Mar. Ecol. Prog. Ser.,* 211: 181-192.

Kripa, V., Muhamed, K. S., Velayudhan, T. S., Laxmilatha, P., Radhakrishnan, P., Joseph, M., Alloycious, P. S., Sharma, J. and Appukuttan, K. K. 2001. Recent developments in edible bivalve farming in India. *Aquacult. Asia, 40-43.*

Kumar, S., Tamura, K. and Nei, M. 2004. MEGA 3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinform.,* 5: 150-163.

Kuriakose, P. S. 1980. Mussels (Mytilidae: Genus *Perna*) of the Indian coast. *Bull. Cent. Mar. Fish. Res. Inst*., 29: 1-4.

Kuriakose, P. S. and Nair, N. B. 1976. The genus *Perna* along the coasts of India with the description of a new species *Perna indica*. *Aquat. Biol*., 1: 25-36

Padi, A. 1998. *Electrophoretic profile of the general proteins in the green (Perna viridis, Linnaeus) and the brown (P. indica Kuriakose and Nair) mussels.* M. F. Sc. Dissertation, Central Institute of Fisheries Education, Deemed University, Mumbai.

Rao, K. S. 1974. Edible bivalves: mussels and oysters. *Bull. Cent. Mar. Fish. Res. Inst*., 25: 4-39.

Sadacharan, D. H. 1982. Country port: Sri Lanka, In : Day, F. B. and Graham M. (Eds.), *Bivalve culture in Asia and the Pacific*: *Proceedings of a workshop held in Singapore,* 16-19 February. International Development Research Centre, Ottawa, Canada, 72 pp.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.,* 4 (4): 406-425.

Siddall, S. E. 1980. A clarification of the genus *Perna (Mytilidae). Bull. Mar. Sci.,* 30(4): 858-870.

Silas, E. G., Alagarswami, K., Narasimham, K. A., Appukutan, K. K. and Muthiah, P. 1982. Country report: India. In : Davy, F. B. and Graham M. (Eds.), *Bivalve culture in Asia and the Pacific*: Proceedings of a *workshop held in Singapore,* 16-19 February. International Development Research Centre, Ottawa, Canada p. 34-43.

Sneath, P. H. A. and Sokal, R. R. 1973. *Numerical taxonomy*. W. H. Freeman and Company, San Francisco.

Sokolov, E. P. 2000. An improved method for DNA isolation from mucopolysaccharide -rich molluscan tissues. *J. Moll. Stud.,* 66: 573-575.

Vakily, J. M. 1989. The biology and culture of mussels of the genus *Perna*. ICLARM *Stud. Rev.,* 17: 1-63.

Ward, R. D., Holmes, B. H., White W. T. and Last, P. R. 2008. DNA barcoding Australasian chondrichthyans: results and potential uses in conservation. *Mar. Freshwat. Res.,* 59: 57–71.

Wood, A. R., Apte, S., MacAvoy, E. S. and Gardner, J. P. A. 2007. Molecular phylogeny of the marine mussel genus *Perna* (Bivalvia: Mytilidae) based on nuclear ITS (1&2) and mitochondrial (COI) DNA sequences. *Mol. Phylogenet. Evol.,* 44 (2): 685-698.

Yu J., Zi Niu, Yu, K. X., Suo, Z. L., Guo, X. M. and Hai, X. J. 2003. Taxonomic status of four *Crassostrea* oysters from China as inferred from mitochondrial DNA sequences. *J. Shellfish Res.,* 22(1): 31-38.

Date of Receipt : 02/08/08
Date of Acceptance : 23/03/09