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Molecular identification of heat shock protein 70 (Hsp70) gene in the Indian edible oyster *Crassostrea madrasensis* (Preston) and Indian brown mussel *Perna indica* Kuriakose & Nair, 1976

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ABSTRACT

Bivalves are constantly exposed to different kinds of stressors as they live in a habitat with frequent changes in environmental parameters. The xenobiotic pollutants also contribute to the stressful routine of bivalves. Studies on the genes which mediate and contribute to the physiological plasticity of bivalves in stressful situations, induced by natural and anthropogenic agents are gaining importance. Among the stress related genes, HSP family genes play an important role in managing stress induced by various factors. Recent reports underline the role of heat shock proteins in thermo tolerance, host defense and even in aging. Here we report the molecular expression and detection of heat shock protein genes (Hsp70) from the Indian edible oyster *Crassostrea madrasensis* and the Indian brown mussel *Perna indica* with unique distribution in Indian waters. The c-DNA reverse transcribed from the total RNA of gill was used as template in Polymerase Chain Reaction (PCR) to amplify Hsp70 gene segments with primers designed from the conserved nucleotide sequences of *Crassostrea gigas* and *Perna viridis*. PCR products were sequenced, and the similarity search in NCBI-BLAST confirmed the molecular identity of targeted genes. Phylogenetic analysis of the Hsp gene sequence data reveals the unique position of the Indian edible oyster and Indian brown mussel among the other counterparts inhabiting rest of the world. This stands out as the first report on the expression and PCR amplification of stress related genes from Indian bivalves.

Keywords: *Crassostree madrasensis,* Heat shock protein 70 gene, Indian edible oyster, Indian brown mussel, *Perna indica*

Introduction

Marine bivalves are regularly exposed to varying physico-chemical conditions on a day to day or seasonal scale. Among these bivalves, the intertidal molluscs represented by oysters and mussels are regularly undergoing regimes of immersion as well as emersion and thereby exposed to abiotic stresses (Fabbri *et al*., 2008). Stress can be defined as a condition which disturb the dynamic equilibrium or homeostasis of an organism by the action of intrinsic or extrinsic forces usually referred as stressors (Wendelaar Bonga, 1997). A series of anomalies or abnormalities are induced within the cell on exposure to stress factors which include protein synthesis inhibition, structural and functional alterations of proteins, *etc*. which are detrimental to the animals. However, these stressors can trigger various cellular responses mediated by heat shock protein family of genes (Hsp 70), metallothioneins, antioxidant enzymes, *etc*. The success of these cell responses determines the survival or death of the organism. Among the stress related genes, members of Hsp family of genes are much studied across different flora and fauna (Boutet *et al*., 2003; Gourdon *et al*., 2000). They also represent the evolutionarily conserved molecular chaperones with prominent roles in managing all sorts of abiotic and biotic stress. The presence of both constitutive and induced isoforms indicate the importance of these proteins in bivalve life. Heat shock protein family members help the intertidal bivalves in making themselves 'prepared for stress' amidst the much challenging environment. The recent reports positively correlate the presence of Hsp 70 and the immune status of oysters, which emphasises the role of these proteins in host defense (Yan Li *et al*., 2007). The role of these multigene family members in thermotolerence is well documented among bivalves (Clegg *et al*., 1998). Latest report of Hsp gene sequence divergence and synonymous single nucleotide polymorphism (SNPs) suggest the potential use of these genes in population genomics as well (Narum and Campbell, 2010). The studies correlating the presence of Hsp and aging in bivalves has opened up a new way of assessing the process of aging (Ivanina *et al*., 2008). The Indian edible oyster (*Crassostrea madrasensis*) and Indian brown mussel (*Perna indica*) are two promising species in bivalve mariculture and therefore studies focusing the stress related genes like Hsp are essential in these bivalve species. Based on the background information gathered from the works in European oysters and mussels (Boutet *et al*., 2003; Franzellitti and Fabbri, 2005), an attempt was initiated to detect the molecular expression and identify the Hsp70 gene in *C. madrasensis*

and *P. indica* which mediate the cellular responses against the stress factors.

Materials and methods

Live oyster (*C. madrasensis*) and mussels (*P. indica*) were collected from the Satar Island located near Kochi and Thankassery Bay located in southern Kerala near Kollam respectively. The gill tissues of the oysters and mussels were stored in RNA later (SIGMA) and 70% ethanol to isolate RNA and DNA respectively. The total RNA of both oyster and mussel was isolated from gill tissue using the RNA isolation Kit (MACHEREY NAGEL) and used to reverse transcribe through RT PCR using c DNA kit (Fermentas). Genomic DNA of *C. madrasensis*, isolated from ethanol preserved gills using standard phenol-chloroform method, was used as template for PCR amplification of Hsp 70 segments with the primers reported for *C. gigas* (Isaballae *et al.,* 2003). The amplified PCR product with a size of 700 bp consisting of both intron and exon sequences were gel eluted and sequenced. The primers targeting the coding region of the gene in *C. madrasensis* were designed from the exon sequences using the Primer premier software. These primers were: Cm Forward 5' GCTGTTGCTTATGGAGCAGCTGT -3 and Cm5' TCGACCTCCTCAATGGTGGGTCC 3'.

In case of *P. indica*, the primers targeting the coding region of Hsp 70 was designed from the published exon sequences of *Perna viridis* Hsp 70 gene (DQ988328) using the same software. These primers were: Pi forward 5' AAGGCTCTGAGAGATGCCAA 3' and Pi reverse 5' TCGACCTCCTCAATGGTGGGTCC 3'. The c DNA synthesised from both the species were used to amplify the Hsp 70 gene segments using the respective primers designed and custom synthesised. The standardised PCR parameters used consisted of an initial denaturation at 94 ^{0}C for 3 min. followed by 30 cycles of denaturation at 94 ^{0}C for 30 seconds, annealing at 58 ^{0}C for 30 seconds and extension at 72 ^{0}C for 1 min, and a final extension at 72 ^{0}C for 7 min. The PCR products were electrophoretically separated in a 1.5% agarose gel containing ethidium bromide along with known molecular weight marker. The specific PCR products were gel eluted using Qiagen gel extraction system and sequenced in both directions. The sequences (Figs. 1 and 2) were used for BLAST search in NCBI database and identity of the gene was confirmed. The cmHsp70 sequence representing the coding region of Hsp 70 gene of *C. madrasensis* was edited and deposited in NCBI (FJ 707369). Similarly, the sequence representing the coding region of *P. indica* Hsp 70 gene was also deposited in NCBI (GU391233).

Sequence divergence with related bivalves was estimated through multiple sequence alignment with

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1   GTCCAGGCTGCCATTTTGTCTGGTGACAAGTCTGAGGAGGTGCAAGATTTACTCTTGTTGGACGTCACCC
71  CCCTGTCCTTGGGTATCGAGACGGCTGGAGGAGTGATGACCAACCTTATCAAGAGGAACACCACCATTCC
141 AACAAAACAGACCCAGACCTTCACCACCTACTCGGACAACCAGCCAGGTGTGCTGATTCAGGTGTACGAG
211 GGAGAGCGAGCCATGACCAAAGACAACAACTTGCTCGGAAAGTTTGAGCTGACAGGAATTCCACCAGCAC
281 CCAGGGGTGTGCCCCAGATTGAGGTCACATTTGACATTGATGCCAACGGTATCCTGAATG
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Fig. 1. Nucloetide sequences of Hsp 70 gene segment of *C. madrasensis* (NCBI Acc: FJ 707369)

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1   TTGTACTTGTTGGTGGATCTACTAGAATTCCAAAAATTCAGAAACTTCTGCAAGATTTCTTCAATGGAAA
71  GGATCTGAACAAATCCATTAACCCTGATGAAGCTGTTGCTTATGGAGCAGCTGTCCAGGCTGCCATCTTG
141 TCTGGAGACAAGTCTGAGGAAGTACAGGATTTGTTGCTGTTGGATGTTGCACCACTGTCCCTTGGTATTG
211 AGACTGCAGGTGGTGTGATGACCTCACTTATCAAACGTAACACAACCATTCCAACCAAACAGACCCAAAC
281 TTTCACCACCTACTCAGACAACCAGCCTGGTGTGTTGATCCAGGTTTATGAAGGAGAGCGTGCTATGACC
351 AAGGATAACAACTTACTTGGAAAGTTTGAGTTGACAGGAATCCCACCAGCACCAAGAGGTGTGCCACAAA
421 TTGAAGTCACCTTTGATATTGATGCAAACGGTATTCTCAATGTATCAGCTGTGGACAAGAGTACAGGCAA
491 AGAGAACAAGATTACTATCACTAATGACAAAGGACGACTAAGCAAAGAAGAAATTGAACGAATGGTCAAT
561 GATGCTGAGAAATATAAGGACGAAGATGAAAAACAGAAAGATAGAATCAGTGCTAAGAACTCTTTGGAGA
631 GCTATTCTTTCAACATGAAATCAACTGTTGAAGATGAGAAACTGAAGGATAAAATCAGTGAGGAGGACAA
701 AAAAGTAATCATGGACAAATGTGATGACGTCATCAAATGGTTAGATGCTAACACATTAGCTGAGAAGGAG
771 GAGTTTGAAGACAAACAGAAGGAGTTGGAAAAAGTATGTAACCCGATTATCACTAAATTGTACCAGGCTG
841 CTGGTGGTGCCCCTGGTGGAGCAGGAGGAATGCCCGGTGGTATGCCTAACTTCGGAGGTGCTGGTGGACC
911 AACAGGTGGT
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Fig. 2. Nucloetide sequences of Hsp 70 gene segment of *Perna indica* NCBI (GU391233)

CLUSTAL W Programme using the Bio Edit software. The phylogenetic analysis using the neighbour-joining method for both the species were carried out using MEGA 5 software version 5.05.

Results and discussion

The aim of the study was to detect the expression of heat shock protein genes and amplify the gene segments through PCR from the mussel and oyster of Indian origin, since such reports are totally lacking in these species, even though their mariculture is picking up momentum. This study was successful in establishing the presence of Hsp70 gene and its expression in both *C. madrasensis* and *P. indica*.

PCR with c DNA from *C. madrasensis* and *P. indica* using Hsp 70 primers designed from European oyster and mussel has successfully amplified the corresponding gene segments. The successful amplification using primers designed from the Hsp70 gene domains of related species indicate the presence of the evolutionarily conserved domains within the candidate gene in the mussels and oysters distributed in Indian waters as well as the robustness of the primers. Sequencing of PCR products followed by the BLAST search conducted in NCBI-BLAST confirmed its identity as HSp 70 gene as well as the sequences homology to related bivalves available with the database. The Hsp 70 gene segment of *C. madrasensis* have shown 91% identity with oyster species *C. ariakensis* and *C. gigas* and 90% with *Ostrea edulis*. The Hsp 70 gene

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                                10        20        30        40        50
                       ....|....| ....|....| ....|....| ....|....| ....|....| ....|
FJ707369 Crassostrea madrasensis GTCCAGGCTG CCATTTTGTC TGGTGACAAG TCTGAGGAGG TGCAAGATTT ACTCT
AY172024 Crassostrea ariakensis ........C. .....C.... G..A...... ..C....... .A..G..C.. G...C
AB122064 Crassostrea gigas      ........C. .....C.... ...A.....A ..C....... .A..G..C.. G...C
AJ318883 Ostrea edulis          ........C. .....C.... C..A.....A ..C....... .A..G..C.. G...C
AF144646 Crassostrea gigas      ........C. .....C.... C..A.....A ..C....... .A..G..C.. G...C
AJ318882 Crassostrea gigas      ........C. .....C.... C..A.....A ..C....... .A..G..C.. G...C
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Representative sequences of Hsp 70 gene of *C. madrasensis* showing divergence within different bivalves

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                                10        20        30
                       ....|....| ....|....| ....|....| ....|....|
GU391233.1 Perna indica         TTGTACTTGT TGGTGGATCT ACTAGAATTC CAAAAATTCA
DQ988328.1 Perna viridis        .......... ......T... ..A....... .C.....C..
AY861684.1 Mytilus galloprovincialis ....CT.G.. A........A ..C....... .......C..
AB122064.1 Crassostrea gigas    .C..C..G.. C..A.....C ..AC.T..C. ....G.....
AF144646.1 Crassostrea gigas    .C..C..G.. C..A.....C ..AC.T..C. ....G.....
AY172024.1 Crassostrea ariakensis .C..C..G.. C..A..G..C ..AC.T..C. ....G..C..
EF011061.1 Pinctada fucata      .C..C..G.. ...A..C... ..C....... .C.....C..
AJ318882.1 Crassostrea gigas    .C..C..G.. C..A.....C ..AC.T..C. ....G.....

                                40        50        60        70
                       ....|....| ....|....| ....|....| ....|
GU391233.1 Perna indica         GAAACTTCTG CAAGATTTCT TCAATGGAAA GGATC
DQ988328.1 Perna viridis        .......... .....C.... .......... .....
AY861684.1 Mytilus galloprovincialis ...GT.A..T ..G..C..T. .TC.A..C.. A..AT
AB122064.1 Crassostrea gigas    ......A..T ..G..C.... ....C..C.. ...A.
AF144646.1 Crassostrea gigas    ......A..T ..G..C.... ....C..C.. ...A.
AY172024.1 Crassostrea ariakensis ......C..A ..G..C.... .T..C..CG. ...A.
EF011061.1 Pinctada fucata      A.....G... .......... ....C..C.. ...A.
AJ318882.1 Crassostrea gigas    ......A..T ..G..C.... ....C..C.. ...A.
```

Representative sequences of Hsp 70 gene of *P. indica* showing divergence within different bivalves

segment of *P. indica* have shown 91% and 83% identities with other mussel species *P. viridis* and *Mytilus galloprovincialis* respectively, and 80% similarity with oyster species *C. gigas*. These results show the uniqueness of both Indian edible oyster and Indian brown mussel among the other related species. The multiple sequence alignment with the CLUSTAL W program showed a general homology as well as unique diversity between sequences. Phylogenetic analysis of the Hsp 70 gene sequences of *C. madrasensis* using Neighbor-joining method with MEGA 5 software version 5.05 placed the species in a separate cluster with high bootstrap value (Fig. 3) indicating substantial sequence substitutions within their gene compared to the related bivalves. The ratios of the transitional and transversional pairs are found to be 1.47 for *C. madrasensis* and 1.18 for *P. indica* as detected by MEGA 5 software. Such findings are expected as the Indian edible oyster inhabits a region with different environmental parameters compared to European waters. This is in tune with the recent report on "the environment influenced sequence divergence" within Hsp 70 gene of Oncorhynchids (Shawn R. Narum *et al.*, 2010). This indicate the potential use of the functional gene Hsp 70 in population genomic studies of the Indian edible oyster. The phylogenetic analysis of *P. indica* Hsp gene70 shows the genetic relatedness with *P. viridis* and divergence from other bivalves (Fig. 4). Thus, the study has succeeded in identifying the heat shock protein 70 gene (Hsp70) and their expression in Indian edible oyster *C. madrasensis* and in Indian brown mussel *P. indica*. The study has also provided an insight into the genetic relatedness of the candidate species with other bivalves.

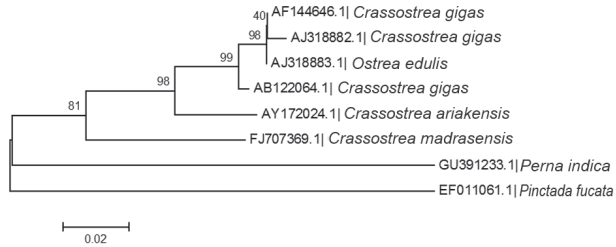

Fig. 3. Phylogentic analysis of Hsp 70 gene of *C. madrasensis* with other closely related species using NJ tree

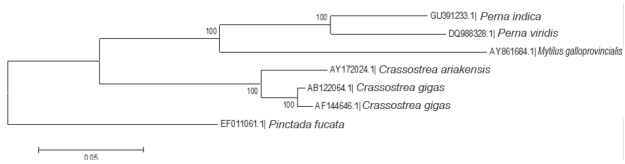

Fig. 4. Phylogentic analysis of Hsp 70 gene of *P. indica* with other closely related species using NJ tree

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