Development Of Molecular Markers For The Identification Of Bivalve Molluscan Larvae For Mariculture Applications

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CERTIFICATE

This is to certify that the PhD thesis entitled "Development of Molecular Markers for the Identification of Bivalve Molluscan Larvae for Mariculture Applications" submitted by Mr. Ranjith Kumar R (Reg.No. MU/EXB/Ph.D./CR.52/Bio.Sc./2009-10/E.13) to Mangalore University for the award of the degree of Doctor of Philosophy is a bonafide record of research work carried out by him under my supervision. The contents of this thesis, in full or in parts, have not been submitted to any other University for the award of any degree.

Dr. P.C. Thomas
Principal Scientist (Rtd)

DECLARATION

I, Ranjith Kumar R, a registered PhD student of Mangalore University would like to declare that this thesis is a presentation of my original research work entitled "**Development of Molecular Markers for the Identification of Bivalve Molluscan Larvae for Mariculture Applications**". Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research. The work was done under the guidance of Dr. P.C. Thomas, Princiapl Scientist (Rtd), Central Marine Fisheries Research Institute (CMFRI), Kochi. The contents of this thesis, in full or in parts, have not been submitted to any other University for the award of any degree.

Ranjith Kumar R

To The Loving Memory of...

Sujith Kalarikkal Unni

The successful completion of any task would be incomplete without mentioning the people who made it possible. I take privilege to express a few words of gratitude and respect to all those who helped me in completion of this work.

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ABSTRACT

Mariculture of *Perna viridis* (green mussel), Perna indica (brown mussel) and Crassostrea madrasensis (Indian backwater oyster) is getting popular in India but the productivity of this sector is affected by inadequate supply of spats. The natural spat resources are the only viable option available to the bivalve farmers to meet the seed requirements. Early detection of the larvae of the three target species in the plankton samples collected from the coastal waters will be helpful to predict the time of spat settlement which is crucial for the successful spat collection using cultch materials. The present work deals with the development of DNA markers in the form of Species Specific PCR (SSPCR) and Species Specific *nested* PCR (SS*n*PCR) for the specific and sensitive detection of the larvae of three target bivalves from unsorted plankton samples. Analysis of the experimental plankton samples using SS*n*PCR proved that the method can detect even a single veliger larva from a plankton biomass of 40mg. Similarly, SSPCR could be used to detect a minimum of 20 numbers of veliger larve from a plankton biomass of 40 mg. The SSPCR could also be used to assess the numerical density of the target bivalve larvae in coastal water and it was possible to detect approximately 106 larvae in 1000 liters of water. The utility of SSPCR and SS*n*PCR was also evaluated in the plankton samples collected from the coastal waters. The spat collectors placed in the study area were observed to have rich settlement of the target bivalve larvae showing a positive correlation with the lab results obtained. Screening of the plankton samples have proved that these methods can be effectively employed to detect the presence of the target bivalve larvae in the coastal waters and it can be used as a 'spat-fall prediction tool' to manage the spat collection process more effectively by the bivalve farmers.

AFLP	:	Amplified Fragment Length Polymorphism
3'	:	three prime
5'	:	five prime
°C	:	degrees Celsius
µg	:	Microgram(s)
µl	:	Microlitre(s)
µM	:	Micromolar
mM	:	Milli Molar
BLAST	:	Basic local alignment search tool
bp	:	Base pairs
cm	:	Centimetre(s)
CO1	:	Cytochrome Oxidase Sub Unit 1
DNA	:	Deoxy ribonucleic acid
dNTP	:	Deoxy ribonucleotide
DUI	:	Doubly Uniparental Inheritance
EDTA	:	Ethylene diamine tetra acetic acid
et al.	:	And others
g	:	Gram(s)
hpf	:	hours post fertilization
INDEL	:	Insertion and Deletion
ITS	:	Internal Transcribed Spacer
L	:	Litre(s)
MEGA	:	Molecular Evolutioary Genetics Analysis
min	:	Minute(s)
mg	:	Milligram(s)
ml	:	Millilitre(s)
mm	:	Millimetre(s)
mM	:	milli Molar
mRNA	:	messenger RNA
NaCl	:	Sodium Chloride
NaOAc	:	Sodium Acetate

NCBI	:	National Centre for Biotechnology Information
ng	:	nano gram(s)
PCR	:	Polymerase chain reaction
RAPD	:	Randomly Amplified Fragment Length Polymorphism
RFLP	:	Restriction Fragment Length Polymorphism
RNA	:	Ribonucleic acid
rRNA	:	ribosomal RNA
rpm	:	Revolutions per minute
SDS	:	Sodium Dodecyl Sulphate
sec	:	Second(s)
SSCP	:	Single Strand Conformational Polymorphism
SSPCR	:	Species Specific PCR
SS*n*PCR	:	Species Specific *nested* PCR
Taq Polymerase	:	*Thermus aquaticus* DNA polymerase
TBE	:	Tris-borate-EDTA
TE	:	Tris-EDTA
Tris HCl	:	Tris Hydro Chloric Acid

CHAPTER 1

INTRODUCTION

The organisms such as oysters and mussels have remarkable position in the day-to-day life of human being from the ancient time. Today, it has diverse utilities either in the forms of tasty meat, ornaments, nutraceuticals, building materials and bio-indicators of pollution. Bivalves are always in great demand owing to high nutritional value and excellent taste. These animals are mostly collected from the natural waters for consumption. The bivalves are always considered to be ideal for aquaculture as they do have higher market demand, minimal investment requirement on the culture infrastructure, minimum culture space requirement and negligible expenditure on the feed. Increased demand and restricted availability of the bivalves from natural waters have prompted its culture in the back waters, creeks, estuaries and even in the Sea which are now collectively known as Mariculture. Molluscs contribute about 23% of the global aquaculture production, of which major share is contributed by bivalves such as mussels and oysters (FAO, 2014). Forecasts by the International Food Policy Research Institute (IFPRI) suggest that India's aquaculture production could double by 2020 (FAO, 2004). In the mean time, the increasing popularity and demand for these organisms both in the domestic and international markets would greatly increase in the coming years.

1.1. Bivalve mariculture in India

Though, India had a long history of the fishery and utilization of bivalves and other molluscs, the 1970s mark the beginning of the commercial scale production of bivalves through various projects initiated by the Central Marine Fisheries Research Institute (CMFRI). During this period CMFRI started experiments on the culture procedures and trials on the commercial scale culture systems of Brown mussel, Green mussel, Pearl oyster and Edible oyster (Appukuttan, 2001). CMFRI started a project on pearl oyster culture during 1972 at Tuticorin Research Centre on the South-East coast, and by the middle of 1990s the technology for culture production and hatchery production of the species flourished very well in India. During 1973 and 1974 the culture technologies for brown mussel and green mussel were developed successfully, and demonstrated at Vizhinjam and Calicut along the South-West coast of India. By the end of 1990s the hatchery technology for green mussel was standardized, and the intensification of farming was achieved in the succeeding years by promoting the group farming activities among the local farmers. Farming systems for edible oysters were developed in 1977 by CMFRI, and the standardization of hatchery production of the oyster spat was achieved in 1982. The commercialization and transfer of the technology of the species were possible through a project funded by NABARD during 1992-1995. The technology was successfully adopted by the fishermen and farming communities through the financial support extended by Brackishwater Fish Farmers Development Authority (BFFDA) during the late 1990s. Farming technology and seed production technology for the clams *Anadora granosa*, *Paphia malabarica* and *Meritrix casta* were developed between the years 1978 and 2000, and the farming demonstrations

were conducted at selected locations in Kerala. Thereafter, mariculture of these bivalve species were carried out at various coastal regions of India especially along the South-West coast, and there was continuous production of the same. According to the production statistics of CMFRI, the total bivalve production of India has reached 1.13 lakh tones during 2014, which includes 294 tones of oysters, 6243 tones of mussels and 1.07 lakh tones of clams (Gopalakrishnan, 2014). The major portion of the clam production was supported by clam fishery whereas mariculture supports the major portion of mussel and oyster production.

1.2. Potential for bivalve mariculture in India

Tropical climate, diverse and vast water resources and better primary productivity are the characteristic features of Indian coastal regions which promise a higher production potential for the mariculture of bivalve species. Timely intervention from the stake holders on the mariculture activities either in the forms of research input, sustainable resource management, financial inclusion by the major credit agencies and crop insurance has raised the Indian mariculture platform to a productive commercial industry which can keep the international standards. Achieving the **EcoLabel** certification from the Marine Stewardship Council (MSC) for *Paphia malabarica* fisheries and its culture in the Ashtamudi Lake of Kerala is one example of such progressive movement showcasing the potential and drive of India in the culture and fishery production of the bivalves. Interventions like group farming, diversification of products, unique culinary preparations and short marketing chains have created wide acceptance for the bivalve delicacies especially along the Malabar region of the South-West coast of India. It is a successful model that can be replicated at any other suitable coastal belt of India so that a steady demand in the domestic market for the bivalve meat and the allied products may be maintained throughout the year. Being the largest commodity produced by mariculture in the world (FAO, 2014), molluscs especially the bivalves form a promising category of organisms that have a great potential in the future growth of the mariculture sector in India. However, growth of the entire bivalve mariculture sector is being hampered by the severe shortage in the availability of bivalve seeds for farming. Hence, the R&D efforts to overcome the issue of short supply of bivalve seeds would provide the much needed impetus to the efforts for increasing the productivity of the bivalve mariculture sector of the country.

1.3. Candidate species for bivalve mariculture

The most common bivalves used for consumption in India includes the green mussel (*Perna viridis*), brown mussel (*Perna indica*), Indian back water oyster (*Crassostrea madrasensis*), short neck clam / yellow foot clam (*Paphia malabarica*), yellow clam (*Meritrix casta*), marine clam (*Sunnetta scripta*), blood clam (*Anadora granosa*) and the black clam (*Villorita cyprinoides*). The popular bivalve species used for mariculture in India includes *Perna viridis*, *Perna indica*, *Crassostrea madrasensis*,

Meretrix casta and *Villorita cyprinoides* (the last two species are exclusively formed of capture based culture fisheries).

However, the bivalve mariculture is dominated by the mussels *P. viridis & P. indica*, and the edible oyster *C. madrasensis.* These species are suitable for the extensive and intensive mariculture activities, they have higher market demand and fetch better price. Hence, the current research work is focused on these three species.

The systematic position of the candidate species are as follows,

Perna viridis (Linnaeus, 1758)

Kingdom	:	Animalia
Phylum	:	Mollusca
Class	:	Bivalvia
SubClass	:	Pteriomorpha
Order	:	Mytiloida
Family	:	Mytiloidae
Genus	:	Perna
Species	:	viridis

Perna indica (Kuriakose & Nair, 1976)

Kingdom	:	Animalia
Phylum	:	Mollusca
Class	:	Bivalvia
SubClass	:	Pteriomorpha
Order	:	Mytiloida
Family	:	Mytiloidae
Genus	:	Perna
Species	:	indica

Crassostrea madrasensis (Preston)

Kingdom	:	Animalia
Phylum	:	Mollusca
Class	:	Bivalvia
SubClass	:	Pteriomorpha
Order	:	Ostreoida
Family	:	Ostreidae
Genus	:	Crassostrea
Species	:	madrasensis

1.4. Challenges in bivalve mariculture

Seed availability, discontinuous market supply, siltation of backwaters etc., are the major constraints that slow down the growth of the bivalve farming sector in India. Seed availability is one of the major limiting factors for the expansion of bivalve farming, as farmers depend entirely on the spats (bivalve seeds) collected from the wild (Helm, 2004, Lovatelli, 1990 and Rao, 1976). Though the

mariculture of bivalves is being carried out to meet the growing demand, there is no commercialized hatchery set up in India for the continuous supply of the spats to fulfill the culture requirements. CMFRI has standardized the hatchery technology for green mussel, brown mussel and back water oyster, but the technology is yet to be taken up on a commercial scale. The bivalves take long periods of shell formation in order to attain a minimum size of a seed (25mm), and this makes the hatchery operation highly expensive. Therefore, the bivalve farmers still resort on the natural spat resources, as it is the only viable option available right now in India. The availability of spats of mussel and oyster from the natural resources is restricted due to the limited availability of suitable substrata for the attachment and growth of the spats. Hence, the farmers depend on the artificial substrata commonly known as the 'Cultch' in order to get the required quantities of spats for the culture requirements.

The life cycle of bivalves are composed of the planktonic larval stages namely Trochophore, Veliger, Umbo and Pediveliger followed by the sedentary Spat. The free floating larvae develop pedal organ (foot) during the Pediveliger stage and settle down to the bottom substrata. This activity of the larvae is known as 'spat-fall' and the bottom settled pediveliger larvae later transforms into spat (Vakily, 1989; Alagarswami, 1980; Bayne, 1976). Spat collection using the cultch material requires the timely installation of the same exactly during the spat-fall as it is very crucial for getting the rich settlement of the larvae on the substratum being provided. Hence, accurate forecasting of spat-fall (Velayudhan, 2005) is inevitable for the successful spat collection since the duration of spat-fall is very short.

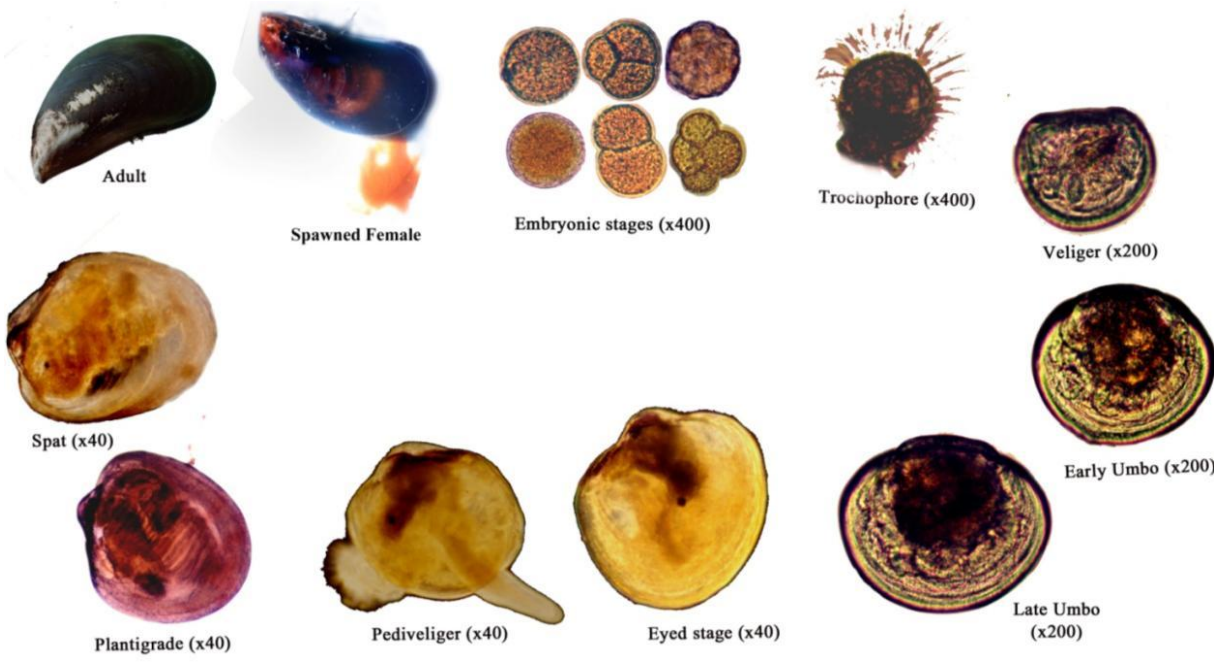


Fig 1: Life cycle of *Perna viridis*.

Courtesy: Manoj (2001)

1.5. Relevance of the study

Very often, farmers are unable to set the cultch materials in the right place at the right time to collect sufficient spats of the cultured species in spite of their close watch on the spat-fall (Silas, 1980). Untimely installation of cultch materials reduces the efficiency of spat collection. Keeping spat collectors before the initiation of spat-fall results in the settlement of fouling organisms, and the same, after spat-fall will result in few spats. Both cases can be avoided, and the spat collection strategy can be improved significantly, if the presence of larvae of the desirable bivalve species in the coastal waters can be accurately identified in time, and used for predicting the time of spat-fall.

Accurate identification of the candidate larval species is crucial for the assessment of larval abundance in the water body and the subsequent spat collection. Field level identification of the larval stages of desired species of bivalve is extremely difficult using the conventional tools based on morphology since the larval stages of different bivalve species shows close resemblance to each other (Hendriks *et al.,* 2005; Fraschetti *et al.,* 2002; Garland and Zimmer 2002 and Frischer *et al.,* 2002). Also the process is time consuming, requires highly skilled and trained operatives and often the identification of the species could be inaccurate. Similarly, the phenotypic plasticity can cause troubles with morphology based discrimination as the environmental conditions such as food concentration and water temperature can determine morphological characters (Patil *et al.,* 2005; Flores-Vergara *et al.* 2004; Strathman 1992 and Boidron-Metairon 1988). Therefore, an accurate identification system for the early detection of bivalve larvae in plankton samples is imperative for the prediction of spat-fall and the successful spat collection.

Since DNA, the genetic material, is unique for every eukaryotes organism, the larval identification based on DNA markers will be highly specific. Molecular detection techniques such as the Polymerase Chain Reaction (PCR) based species specific DNA markers will be useful for the specific and accurate detection of the veliger larvae found in the plankton, and therefore, it can be used as a tool to predict the spat-fall. The DNA based PCR technique is highly specific and sensitive, demands only minute quantities of genetic material and therefore, it will work well with the environmental samples containing complex of DNA from variety of organisms. This can bypass the need of prior sorting of plankton samples, and it provides high level of specificity, sensitivity and accuracy (Pan *et al.,* 2008, Santaclara *et al.,* 2007). Thus, the seed collection strategy can be managed more effectively this in turn, helps to reduce the indiscriminative exploitation of natural bivalve spat resources. Thus, this technique can be effectively used as a tool for the responsible management of natural spat resources.

Therefore, the present research work entitled "Development of Molecular Markers for the Identification of Bivalve Molluscan Larvae for Mariculture Applications" was undertaken with the following objectives:

- To develop PCR based DNA markers for the precise identification of the larvae of *P. viridis, P.indica* and *C. madrasensis* in the plankton samples collected from coastal waters.
- To evaluate the specificity and sensitivity of these markers, and to validate their efficiency.
- To test the species specific DNA markers of target bivalve species for its field level application through the screening of plankton samples collected from coastal waters for the presence of the larval stages of respective bivalves.
- To predict the spat-fall, and to validate its accuracy through practical spat collection.

This novel approach for predicting the spat-fall is envisaged to help the growth of the bivalve mariculture sector of India by the increased production of bivalve seeds, and thereby, augmenting the culture production.

CHAPTER 2

REVIEW OF LITERATURE

2.1. A Brief History of Bivalve Mariculture

Molluscs had a valuable position in human life from the time immemorial. Numerous citations could be noted in the classics and myths of India mentioning the usage of the molluscs like sacred chank and pearl oysters. The study of the works by Aristotle, Hippocrates and many other famous philosophers of Greece showed that out of the 35 exploited marine invertebrates recorded in the texts, 20 were molluscs, among which 11 were bivalve names (Voultsiadou *et al.,* 2009). Bivalves are the second most diverse group of molluscs behind the gastropods. In the western society, bivalves form the second most important invertebrate food item after the decapods. Hence, there are considerable number of research works published with regard to the stock availability and genetics of bivalves (Thorpe *et al.,* 2000).

One-third of the world's farmed food fish harvested in 2010 consisted of the bivalves and filter-feeding carps i.e., the organisms grown without any particular feed input (FAO, 2012). The flat oyster *Ostrea edulis*, a native of Europe, has been a part of the human diet for many centuries. The Romans built ponds to stock and sort oysters. A primitive way of collecting oyster spats and deploying them in the salt marshes of France was in practice during 17th century, and it could be considered as a kind of beginning of the practice of bivalve culture. Increased fishing efforts led to the over-exploitation of natural bivalve resources during the 18th and 19th centuries. Serious decline in the bivalve seed recruitment and the destruction of European bivalve natural beds happened due to extreme cold winter. The increased demand for the bivalve seeds prompted the usage of artificial spat collectors and their systematic use facilitated the growth of the sector. Wooden spat collectors were initially used followed by the strings of oyster shells and slates. The liming tile technique (roof tiles coated with a mixture of lime and fine sand), and wooden boxes to grow juveniles were developed in south-western France during 1865 (Goulletquer, 2004). On the Mediterranean coast, off-bottom culture was initiated in 1900, using oysters cemented onto steel poles. The culture of oysters and clams through on-bottom method of culture flourished during the 1970s in USA (Milne, 1979). Culture of *Mytilus edulis*, has attained a high degree of mechanization in Holland. During the 1970s, Rack method of bivalve culture was put into practice in different countries like Japan, France, Australia and Philippines (Fujiya, 1970; Koganezawa, 1979; Milne, 1979). The Raft method of bivalve culture has become popular in Spain, Japan and Philippines by the end of 1970s (Hurlburt and Hurlburt, 1980, Guerrero, 1983). Spat collection using mussel and oyster shells has become common in the 20th century. Major diseases in bivalve mariculture were also noticed during this period. The practice of spat collection using the tubular nets filled with mussel shell was proved to be more cost effective. It was in common use since the 1980s along the southern Brittany, France. More recently, hatcheries have begun to produce cultchless flat oyster spat.

2.2. Current Scenario of Bivalve Mariculture

At the present pace of growth, it is estimated that, the global fish supply shall continue to increase to cross 180 million tons by the year 2030. In a publication by the World Bank, 'FISH TO 2030 Prospects to Fisheries and Aquaculture', it is proposed that half of this projected fishery production would be contributed by global aquaculture. South-Asian countries would be the second largest suppliers to this projected demand and supply. Global bivalve production during 2012 was estimated as 13.4 million tones. Molluscs contribute about 23% of the global aquaculture production, of which bivalves such as mussels and oysters form a major share (FAO, 2014). The bivalve mariculture need to be promoted in the developing countries as they are one of the cheap and best protein source, demands less capital investment mainly because of its non-fed type of culture practice.

Owing to the great production potential, India has become one of the leading countries in fishery production and aquaculture. The nation ranks second in terms of production from capture fisheries and culture fisheries among the global leaders. Out of the total fishery production of 9.19 million tons the share of aquaculture production is 49.5 percent (FAO, 2015). Major portion of the annual fish production of the country is contributed by fresh water aquaculture while the potential for mariculture production remains largely untapped (FAO, 2014).

Bivalve farming has emerged as one of the important mariculture activity along the maritime states of Indian subcontinent (Modayil *et al.*, 2007). According to the recent estimates of CMFRI, the clams formed 94.3% of the annual bivalve production, followed by mussels (5.5%) and oysters (0.3%) (Gopalakrishnan, 2014). Capture fisheries form the major portion of clam production whereas the culture fisheries or the mariculture contributes the major portion of mussels and oyster production. Capture fisheries needs well planned management strategies in order to avail a sustainable production. Mariculture is one of the alternative strategies to address the issues of dwindling fishery resources. The capture based culture fisheries of clams form only a meager portion of the total production of bivalves. In a global perspective, oysters are most in demand species among the maricultured molluscan groups. *Crassostrea gigas* is the oyster species with highest global production by quantity (FAO 2014). Owing to its high nutritional value and the peculiar culinary preferences the oysters are consumed raw in many of the European countries. India can become a leader in this sector, if right effort is put on the culture and value addition of the bivalves, as large quantities of production is expected during this period. Clean and depurated farmed molluscan shellfish must be made an integral and mandatory part of Indian shellfish industry as the global demand for live shellfish is expanding.

2.3. Edible Bivalves of Mariculture Importance

The highest farm produced mussel species through bivalve mariculture in India is *Perna viridis* (Harikumar and Rajendran, 2007), and its culture production has increased from 1600 tones in 2002 to 18432 tones in 2009 (Kripa *et al.,* 2009). *P. viridis* occurs naturally and is widely distributed along the intertidal coasts of India (Jones and Alagarswami, 1973). According to Siddall (1980), *P. viridis* is broadly distributed in the Indo-Pacific region where it ranges west from the Persian Gulf and east to New Guinea and Japan. *Perna indica* is another mussel species with high potential for culture production. This species shows better growth rate in typical marine conditions rather than in the backwaters and estuaries. Though the mariculture prospect of this species was established in the early 1970s (Appukuttan, 2001) the farming industry has not flourished well up to the mark. The culture activities of *P. indica* in the marine habitat were at stake particularly during the monsoon season due to the typical climate and prevalence of rough weather (James, 1988; Silas, 1980). Now, the mariculture scenario of India has changed with the improvements in the technologies and methods employed, and with the introduction of new ventures such as cage culture activities. Hence, the opportunities for the improvisations of *P. indica* mariculture shall be taken up wisely. The farming technologies for *Crassostrea madrasensis* developed by CMFRI during the late 1970s were adopted by the aqua-farmers at a very slow pace mainly because of the lack of demand during that period. Post harvest handling and the limited markets were the major reasons for this. Oyster farmers were tempted to turn towards the mussel farming because of the lesser difficulties in handling. Recently, this trend is being reversed due to better market price and the realization that the oysters are more euryhaline than mussels and are more conducive for culture in an estuarine environment.

2.4. Impediments to Bivalve Mariculture

The mussel or oyster beds found in Europe are extensive and in most cases they are dominant with a single species. For such reasons the spat-fall always been found to be abundant in those locations (Silas, 1980). Hence, hatchery systems to produce the bivalve spats were not so essential in those countries. However, the faster growth in the sector demanded the need of huge quantities of bivalve spats and thus the hatchery techniques for various bivalves were formulated (Breese and Malouf, 1975; Dupuy *et al.* 1977; Robert and Gerard, 1999; Helm and Bourne, 2004). Though, considerable improvement have been made in the grow-out systems of bivalve mariculture of India to meet the growing demand for the commodity in the domestic and international markets, the most important input deficit which set-back the growth of the entire bivalve mariculture industry in India is the short supply of bivalve spats. The increased thrust for mussel mariculture in the late 1990s demanded plenty of mussel spats along the South-West coast of India. But, the mussel beds on the Indian coast are limited and scattered, and rocky surface area for spatfall is also restricted. The natural seed resource on the beds cannot support mussel

culture industry of some magnitude (Rajagopal *et al.,* 1998a; Rajagopal *et al.,* 1998b; Silas, 1980). These factors make it imperative to develop techniques for seed production. The technologies for spawning and larval rearing of *P. viridis*, *P. indica* and *C. madrasensis* have been already standardized (Sreenivasan *et al.,* 1988; Appukuttan *et al.,* 1987; Alagarswami, 1980). Even then, there are no commercialized hatchery set ups in India for the continuous supply of seeds to meet the culture requirements. The prospects of the hatchery produced spats are hardly realized as the hatchery process up to the marketable seed size is not often economically feasible. Hence, the commercial hatchery facilities for the production of the spats of mussels and oysters are not yet available in India and the farmers depend entirely on the spats collected from the wild.

Successful spat collection requires accurate prediction of the spatfall (Velayudhan, 2005). Spatfall prediction depends on the timely detection of the target bivalve larvae in the coastal water. Conventional method of bivalve larval detection is mainly based on microscopic examination of the plankton samples collected from the coastal waters during the spawning season of the corresponding bivalve. Field level identification of the bivalve larval species using microscope is cumbersome and time consuming (Chanley and Andrews, 1971; LePennec, 1980). Resemblance of the larvae of different bivalve species (Hendriks *et al.* 2005; Garland and Zimmer, 2002; Fraschetti *et al.,* 2002; Frischer *et al.,* 2002) and the phenotypic plasticity (Strathman, 1992, Boidron-Metairon, 1988; Flores-Vergara *et al.* 2004; Patil *et al.* 2005) makes it intricate to discriminate the species. Also, the process requires highly skilled and trained manpower and often the identification of species is incorrect. Hence, an advanced, faster and precise technology is very much required to overcome this problem.

2.5. Molecular Techniques to Identify Bivalve Larvae

DNA based molecular detection techniques using species specific markers will be useful for the specific detection of the veliger larvae found in the plankton. Other molecular techniques such as those based on proteins and immunology are comparatively less specific and needs isolation of complex protein molecules. PCR technique is particularly suitable to test the environmental samples as this can function well with DNA samples having compromised quality (Santaclara *et al.,* 2007). Also, this technique can offer high specificity, sensitivity and accuracy. High degree of automation is possible with this and therefore large number of samples can be processed at a time. There are numerous research works on the identification of the bivalve species based on various molecular techniques which has application in various studies related to larval distribution, population structure, species invasion, fisheries and aquaculture.

2.5.1. Dominant Markers

Use of protein and DNA based dominant and co-dominant markers for the species identification and evaluation of the genetic variation among the bivalves and other related mollusks have been studied by many investigators. Randomly Amplified Polymorphic DNA (RAPD) is a technique which amplifies various DNA fragments of different molecular sizes which can serve as dominant marker. The RAPD pattern characteristic to the oyster species namely *C. belcheri, C. iredalei* and *Saccostrea cucullata* have been reported by Klinbunga *et al.*(2000). Development of species specific RAPD markers that can be used to identify the genetic diversity of five different oyster species, *C. belcheri*, *C. iredalei*, *Saccostrea cucullata, S. forskali* and *Striostrea mytiloides* have been reported by Klinbunga *et al.* (2001). RAPD markers were developed by Rego *et al.*(2002) to identify *Mytilus galloprovincialis*. The genetic structuring in the nineteen subsets of greenshell mussel, *Perna canaliculus* was evaluated by Star *et al.*(2003) using RAPD markers, and genetic discontinuity could be observed between the Northern and Southern populations at New Zealand.

Uses of other markers like Allozymes, Microsatellite loci, Amplifed fragment length polymorphism (AFLP) etc have been reported. Allozymes and microsatellite loci were used to characterise the levels and patterns of genetic variation in four successive mass selection lines of Tasmanian hatchery produced stocks of Pacific oysters, *Crassostrea gigas* (Appleyard and Ward, 2006). Shell colour inheritance of the bay scallop *Argopecten irradians irradians* was studied using Amplifed Fragment Length Polymorphism (AFLP) markers and it was believed that it can be used in breeding program where desired colour pattern was expected in the bay scallop (Qin *et al.,* 2006). A mix of genetic markers such as AFLP, nuclear microsatellites and the mitochondrial CO1 gene were used to evaluate the connectivity among nine populations of California red abalone, *Haliotis rufescens*. While the CO1 gene sequence and microsatellite markers could not differentiate the populations, the AFLP marker was effective to serve the purpose (Gruenthal *et al.,* 2007).

2.5.2. Species Identification and Genetic Characterization Using RFLP markers

Randomly Amplified Fragment Length Polymorphism (RFLP) is a molecular marker used to identify a species or to characterize a subset of a population. In this, the restriction enzymes are used to digest the amplified PCR products so that different PCR fragments characteristic to a species may be obtained. Geller *et al.*(1994) used RFLP of 16SrRNA as a marker to monitor the larval transportation and accidental introduction of *Mytilus trossulus* and *Mytilus galloprovincialis* in North American waters. RFLP of the mitochondrial CO1 gene was used as a diagnostic marker for differentiating the invasive Zebra mussel, *Dreissena polymorpha* (Baldwin *et al.*, 1996; Claxton *et al.,* 1997). Boudry *et al.,* (1998) used RFLP of CO1 and 16sRNA genes to differentiate the Portuguese oyster *C. angulata* and Pacific oyster *C. gigas*. Klimbunga *et al.* (2003) used RFLP markers to successfully identify different abalone

species. The genetic diversity between *Crassostrea belcheri, C. iredalei, S. cucullata, S. forskali* and *Striostrea mytiloides* were studied by Klimbunga *et al.*(2005)using RFLP technique with CO1, 16S and 18SrRNA genes. Santaclara *et al* (2006) reported that molecular identification of cooked bivalve meat containing *Perna viridis* could be successfully identified using RFLP of 18SrRNA gene. RFLP with the 18SrRNA genes could be successfully employed to detect the invasive alien bivalve species, *Xenostrobus securis* in Spanish waters (Santaclara *et al.*, 2007). RFLP of non coding region of the mitochondrial DNA was used to differentiate different culture populations of *C. gigas* (Okimoto *et al.*, 2008). In a comparative study, RFLP of the CO1 gene region could reveal significant percentage of post settlement survival of the less important bivalve species *Aulocomya atra maoriana* in the New Zealand waters (Phillips *et al.*, 2008).

2.5.3. Sex Identification

Male and female individuals in the mytilid mussels could be identified using DNA markers. Sex segregation in the mytilid mussels based on the phenomenon Doubly Uniparental Inheritance (DUI) was studied. In all animals the mitochondrial DNA is maternally transmitted. However, in the case of most of the mytilid mussel groups the mitochondrial DNA was also paternally transmitted during the cytoplasmic division of meiosis. This causes individuals with two types of mitochondrial DNA i.e., heterozygous condition could be observed with considerable size difference in the mitochondrial genomes. Such heterozygous individuals become male and the rest remains female. DUI was proved in *Mytilus edulis, M. trossulus, M. galloprovincialis* and also in the hybrid of *M. edulis* × *M. galloprocincialis* (Skibinski *et al.*, 1994; Rawson *et al.*, 1995; Kenchington *et al.*, 2002; Cao *et al.*, 2004; Mizi *et al.*, 2005; Breton *et al.*, 2006).

2.5.4. Species Identification and Genetic Characterization Using Ribosomal RNA markers

Characterization of the ribosomal RNA genes was useful to identify, distinguish and differentiate different bivalve species of aquaculture and fishery importance. 18SrRNA gene was employed by Naganuma *et al.* (1998) to distinguish two different closely related species of abalones through sequence characterization. 16SrRNA gene was used to differentiate the tissues of three oyster species namely *C. gigas, C. sikamea* and *C. ariakensis*. This was useful in locating the distribution of these species and their larvae in the Ariake Sea. These results emphasize the value of molecular markers for discriminating these morphologically plastic species both in the field of ecology and aquaculture (Hedgecock *et al.*, 1999). Genetic identification of *Ostrea edulis* was done with 16SrRNA and CO1 gene (Morton *et al.*, 2003). A specific multiplex polymerase chain reaction (PCR) was developed for the identification of *Crassostrea angulata, C. gigas, Ostrea edulis,* and *O. stentina* oyster species. Six pairs of species specific primers were developed in the non transcribed spacer region were used to identify four species in a multiplex PCR

test (Cross *et al.*, 2006). 16SrRNA sequence variation was used to differentiate seven different populations of Moon scallop, *Amusium pleuronectes* in Thailand (Mahidol *et al.*, 2008).

2.5.5. Species Identification and Genetic Characterization Using Mitochondrial CO1 and Nuclear ITS Markers

The mitochondrial gene, Cytochrome Oxidase C subunit 1 (CO1) have been reported to be an ideal gene for studying the population genetics. Mitochondrial CO1 gene sequence was used by Zardi *et al.*(2007)to reveal the population genetic structure of *Perna perna* and *Mytilus galloprovincialis* along the East and West coasts of South Africa. The populations established along the coasts due to the larval dispersal through differential oceanic currents were distinguished using this method. The nuclear non-coding DNA, Internal Transcribed Spacers (ITS) that are placed in between the RNA genes are also found to be a potential marker that can be used to distinguish an invertebrate species. They are being used extensively in molecular taxonomy. Phylogenetic analysis of the nucleotide sequences of the mitochondrial CO1 gene helped to resolve taxonomic confusions between different species of the family Pectinidae (Matsumoto and Hayami, 2000). Six species of pearl oysters were differentiated using the sequence characterisation of the ITS2 region (He *et al.*, 2005). Amplification of ITS1 region followed by the characterization of its size polymorphism and differential pattern obtained on restriction enzyme digestion were used to identify different mussel species belong to the genera *Mytilus, Perna, Aulacomya, Semimytilus, Brachidontes, Choromytilus*, and *Perumytilus* from the processed food materials. This marker was found to be useful as a diagnostic or forensic tool to screen mussel meat in the processed food items (Santaclara *et al.*, 2006). Phylogenetic analysis of Pectinidae was conducted with the structural arrangement of ITS1 region. Organisms with highly divergent sequences are found to be ideal for differentiation using this method (Wang *et al.*, 2007). Phylogenetic analaysis of the ITS and CO1 nucleotide sequence helped to resolve the confusion in identifying the putative samples of *C. ariakensis* and other species of cupped oysters from across Asia. Phylogenetic trees generated based on the two independent molecular datasets were highly congruent, and indicate that many oysters collected for this study as *C. ariakensis* were originally misidentified. Results also indicate that *C. ariakensis, C. hongkongensis* and *C. nippona* are distinct, but closely related species. There is strong support in both analyses for a close relationship between *C. gigas* and *C. sikamea*, as well as between *C. belcheri* and *C. gryphoides*, and between *C. iredalei* and *C. madrasensis* (Reece *et al.*, 2008). The species of *Perna* namely *P. perna, P. canaliculus, P. viridis* and a putative species *P. picta* were analysed for their phylogenetic relationship using the molecular markers based on ITS1&2 and mitochondrial CO1 gene. The study could differentiate each species comparing with the specimens collected from different countries. The putative *P. picta* was identified as originally *P. perna* only (Wood *et al.*, 2008).

2.5.6. Detection of Bivalves and Other Molluscs in the Plankton Samples

Taxonomic identification and genetic analysis of marine invertebrate larvae have been vexing problems. Plankton sample analysis using molecular markers for identifying the species of different constituent organisms is gaining importance. Hu *et al.* (1992) reported that the polyacrylamide gel electrophoresis technique provides an economical tool for large scale taxonomic, ecologic, and genetic studies of meroplanktonic stages of various species. A polyacrylamide mini-gel electrophoresis technique was used by him for resolving proteins from individual bivalve larva and applied this technique to three species of laboratory-cultured oyster larva, *Ostrea edulis, Crassostrea gigas* and *C. virginica* reared during summer 1989. Electrophoretic patterns of proteins clearly discriminated among the three species and allow genetic analysis of a polymorphic allozyme locus (Pgi) in field-collected larvae and juveniles of *C. virginica*.

Nucleotide sequence variation in the 16SrRNA gene sequence was used to distinguish *C. gigas* and *C. sikamea sp;* Sequence-specific PCR primers, dot-blot hybridization, and restriction digests were used as alternate techniques for rapid diagnosis of *Crassostrea* oyster larvae from the plankton samples (Banks *et al.,* 1993). Molecular hybridization techniques were employed to detect the presence of the invertebrate larvae in the plankton samples. Goffredi *et al.*(2006) used rRNA targeted signal probes to detect the barnacle larvae with a specificity up to the levels of Order and Species.

2.5.7. Bivalve Larval Detection Using Specific PCR Markers

PCR based molecular techniques have been reported to be particularly useful for the identification of the bivalve and other related molluscan larvae. Larsen *et al.*(2005) successfully used single step nested multiplex PCR to differentiate six different bivalve larvae, *Cerastoderma edule, Macoma balthica, Mytilus edulis, Spisula subtruncata, Ensis americanus* and members of the order *Myoida*. Mitochondrial and nuclear genes such as 16SrRNA and 18SrRNA are used for species identification of bivalves and Scallops. Multiplex species specific primers on 16SrRNA and 18SrRNA were designed and tested for the species specificity in the cases of *Mytilus* sps. and the King scallop *Pecten maximus*. 16SrRNA primers were found to be more specific. The random plankton samples containing 10 numbers of Mytilus larvae could be detected by using this method (Bendezu *et al.,* 2005). The adult tissue specimens of three different *Perna* species, *P. viridis, P. perna* and *P. canaliculus* were distinguished using specific primers designed based on the mitochondrial gene, NADH4 and the intergenic spacers. But, the application of these molecular markers on the field collected plankton samples have not been reported (Blair *et al.*, 2006). Patil *et al.* (2005) have developed species specific PCR primers based on the mitochondrial CO1 gene in order to detect the invasive Pacific Cup Oysters, *C. gigas* from the ballast water plankton samples collected from Tasmania, Australia. Nested PCR systems were developed for the sensitive identification of the larvae from the plankton samples. The larvae of golden mussel *Limnoperna fortunei* were

identified in the plankton samples using the species specific PCR primers by Pie *et al.* (2006). The species is native to the continental China, but is emerging as an important invasive species. It was first recorded in South America in 1991 in the estuary of the Rio de La Plata and dispersed into the largest rivers systems of the Plata basin (Rio de la Plata, Rio Parana, Rio Uruguay and Rio Paraguay), travelling inland at an estimated rate of 240 km per year. The specific primers were designed based on the mitochondrial CO1 gene sequence. Single step nested multiplex PCR designed based on the 18SrRNA gene was used to identify the bivalve larvae from plankton samples that belonging to six different groups *Mytilus edulis, Ensis spp*., the common cockle (Cardiidae family), members of the *Abra* and *Macoma* genera of the Tellinoidae superfamily and members of the surf clam genera, *Spisula* spp (Larsen *et al.*, 2007). A pair of Species specific PCR primers was designed for the specific identification of *P. viridis, P. perna* and *P. canaliculus* based on the NAD4 and CO1 mitochondrial genes. The pair of primers could specifically identify the target DNA and distinguish them with different sized PCR products. This primer pair could also be used in real-time PCR system and the same was used for detecting the invasive *P. viridis* in Australian waters (Dias *et al.*, 2013). Trials with the field collected plankton samples were not mentioned in the work. The abundance of the bivalve larvae in the natural water body is mainly dependent on the quantity of food material available and their optimal temperature requirement. Philippart *et al.* (2014) analysed the seasonal match between environmental conditions and larval presence of six coastal bivalve species over eight consecutive years (2006–2013) in the western Wadden Sea. They used the species specific primers designed based on the mitochondrial CO1 gene in order to identify the target bivalve larvae such a *Crassostrea gigas, Mytilus edulis, Macoma balthica, Cerastoderma edule, Mya arenaria* and *Ensis directus* in the plankton samples.

2.5.8. Prediction of Spat-Fall Based on DNA Markers

Prediction of spat-fall has become an essential component of the successful spat collection for the mariculture needs. Arbitrary fixing of the spat-collectors simply based on the general breeding season of bivalves may not be successful as the mass spawning of the bivalves are greatly influenced by the seasonal distribution of food and temperature (Rajagopal *et al.*, 1998a). Detection of the bivalve larvae within the plankton samples based on the species specific DNA markers shall be an appropriate method that can give an insight of the right time of spat settlement in the natural water body. Research works to detect the bivalve larvae in a spat collection perspective has also been taken up by a few researchers. Specific and multiplex PCR based on the mitochondrial 16S rRNA gene for detecting the DNA of *C. rhizophorae* and *C. brasiliana* from pool of plankton DNA was developed by Ludwig *et al.* (2011). The system developed could detect an equivalent quantity of DNA of a target bivalve larva.

The current research work is focussed on developing DNA based molecular markers for the specific detection of the mussels and the oyster of mariculture importance in India, namely, *P. viridis*, *P. indica* and *C. madrasensis*. The specific markers developed would be used to screen the seasonally collected plankton samples, and a procedure for spat-fall prediction would be developed for the three target bivalve species. Validation of the developed technology with the subsequent field observation and trials would also be conducted.

CHAPTER 3

MATERIALS & METHODS

The present research work focuses on the development of a molecular tool to specifically identify the larvae of economically important bivalves such as *Perna viridis* (green mussel), *Perna indica* (brown mussel) and *Crassostrea madrasensis* (edible oyster). Application of such a tool is expected to assist the bivalve mariculture sector of India by predicting the time of spat-fall to enhance the spat collection from the wild so that it may provide an effective solution to resolve the short supply of bivalve seeds.

3.1. Collection and Preservation of Bivalve Specimens

Mature specimens of *Perna viridis*, *Perna indica* and *Crassostrea madrasensis* were collected from the maritime states of India based on their distribution and availability. Due to the lack of suitable substratum, only intermittent patchy distribution these species could be observed all along the coastal regions of Indian peninsula. The specimens of *P. viridis* were collected from Maharashtra, Goa, Karnataka and Kerala in the West coast, and from Tamil Nadu, Andhra Pradesh and Orissa in the East coast. The *P. indica* has a restricted distribution, and so it could be collected from Kerala and Tamil Nadu only. The *C. madrasensis* specimens were collected from Karnataka, Kerala and Tamil Nadu. Other bivalve species used for the comparison studies such as *Villorita cyprinoides* (black clam), *Paphia malabarica* (short neck clam**)**, *Meritrix casta* (yellow clam), *Sunnetta scripta* (marine clam), *Saccostrea cucculata* (rock oyster) and *Isognomon ephippium* (Mangrove oyster) were collected from the coastal waters of Kerala, and the Indian pearl oyster, *Pinctada fucata* was collected from Tuticorin of Tamil Nadu. The scientists of Molluscan Fisheries Division, CMFRI have extended their help by bringing a few specimens of black pearl oyster *Pinctada margaritifera* from Andaman & Nicobar Islands.

A total of 495 *P. viridis* specimens, 105 *P. indica* specimens and 125 numbers of *C. madrasensis* specimens were collected from the sampling sites (Table 1). About 2 to 6 grams of the adductor muscle tissue was dissected from these bivalve specimens and preserved in 80% ethanol kept in 30 ml air tight screw capped polypropylene plastic containers. Care was taken to maintain a minimum ratio of 1:5 between the volumes of preserved tissue and preservative within the storage containers. The ethanol in the container was replaced twice in order to avoid dilution due to dehydration of tissues.

3.2. Collection and Preservation of Plankton Samples from the Study Areas

3.2.1. Description of the Study Areas

Thankassery Bay in Kollam district and Azhikode Estuary in Ernakulam district of Kerala (Fig. 4) were the locations selected for plankton collection since they are known for the natural beds of mussels and oysters. Seasonal distribution of larvae of the target bivalve species in these locations were studied using DNA markers developed in the present work in order to predict the spat-fall.

Table 1: Details of the Tissue Samples Collected and Preserved

Name of the Species	Sample Code	Place of Collection	Coordinates	No. of samples
Perna viridis	PVKOL	Kollam	08^{0}52^{l}34.53^{ll}N; 76^{0}34^{l}21.60^{ll}E	25
	PVERN	Ernakulam	09^{0}59^{l}47.46^{ll}N; 76^{0}13^{l}06.77^{ll}E	75
	PVTHIK	Calicut	11^{0}29^{l}38.85^{ll}N; 75^{0}36^{l}53.15^{ll}E	15
	PVMOO	Calicut	11^{0}28^{l}01.53^{ll}N; 75^{0}39^{l}03.20^{ll}E	05
	PVCHO	Calicut	11^{0}39^{l}38.84^{ll}N;75^{0}33^l 05.85^{ll}E	10
	PVELA	Calicut	11^{0}20^{l}53.47^{ll}N; 75^{0}44^{l}14.27^{ll}E	25
	PVQUI	Quilon	11^{0}26^{l}26.19^{ll}N; 75^{0}41^{l}15.71^{ll}E	10
	PVMAH	Mahi	11^{0}41^{l}46.04^{ll}N;75^{0}32^l 00.46^{ll}E	15
	PVTHA	Kannur	11^{0}45^{l}54.08^{ll}N;75^{0}28^{l}45.76^{ll}E	10
	PVDAR	Kannur	11^{0}46^{l}43.88^{ll}N; 75^{0}27^{l}39.47^{ll}E	10
	PVPAD	Kasargod	12^{0}10^{l}08.77^{ll}N; 75^{0}27^{l}39.47^{ll}E	10
	PVBEK	Bekal	12^{0}23^{l}27.70^{ll}N; 75^{0}01^{l}52.62^{ll}E	10
	PVMAN	Mangalore	12^{0}47^{l}41.13^{ll}N; 74^{0}50^{l}48.62^{ll}E	95
	PVBAI	Baindur	13^{0}52^{l}23.50^{ll}N; 74^{0}36^{l}06.68^{ll}E	30
	PVKAR	Karwar	14^{0}48^{l}18.07^{ll}N; 74^{0}06^{l}44.24^{ll}E	35
	PVGOA	Goa	15^{0}08^{l}31.50^{ll}N; 73^{0}56^{l}46.58^{ll}E	15
	PVCHN	Chennai	13^{0}13^{l}53.11^{ll}N; 80^{0}19^{l}53.57^{ll}E	70
	PVVSK	Vishakpatnam	17^{0}41^l 05.39^{ll}N;83^{0}17^{l}44.25^{ll}E	10
	PVORI	Orissa	20^{0}15^{l}32.11^{ll}N; 86^{0}40^{l}46.55^{ll}E	20
Perna indica	PIKCHL	Kulachal	08^{0}10^{l}23.17^{ll}N; 77^{0}14^{l}46.34^{ll}E	30
	PIVZN	Vizhinjam	08^{0}22^{l}34.37^{ll}N; 76^{0}59^{l}10.23^{ll}E	45
	PIKOL	Kollam	08^{0}52^{l}34.53^{ll}N; 76^{0}34^{l}21.60^{ll}E	30
Crassostrea madrasensis	CMKOL	Kollam	08^{0}52^{l}34.53^{ll}N; 76^{0}34^{l}21.60^{ll}E	35
	CMERP	Ernakulam	09^{0}59^{l}47.46^{ll}N; 76^{0}13^{l}06.77^{ll}E	20
	CMCAL	Calicut	11^{0}29^{l}38.85^{ll}N; 75^{0}36^{l}53.15^{ll}E	15
	CMDAR	Kannur	11^{0}46^{l}43.88^{ll}N; 75^{0}27^{l}39.47^{ll}E	10
	CMPAD	Kasargod	12^{0}10^{l}08.77^{ll}N; 75^{0}27^{l}39.47^{ll}E	10
	CMTUT	Tuticorin	08^{0}48^{l}37.48^{ll}N; 78^{0}09^{l}56.12^{ll}E	35
Saccostrea cucullata	SCDAR	Kannur	11^{0}46^{l}43.88^{ll}N; 75^{0}27^{l}39.47^{ll}E	10
Pinctada fucata	PFTUT	Tuticorin	08^{0}48^{l}37.48^{ll}N; 78^{0}09^{l}56.12^{ll}E	10
Paphia malabarica	PMTUT	Tuticorin	08^{0}48^{l}37.48^{ll}N; 78^{0}09^{l}56.12^{ll}E	10
Paphia malabarica	PMKOL	Kollam	08^{0}52^{l}34.53^{ll}N; 76^{0}34^{l}21.60^{ll}E	10
Meritrix casta	MCKOL	Kollam	08^{0}52^{l}34.53^{ll}N; 76^{0}34^{l}21.60^{ll}E	10
Donax donax	DDTUT	Tuticorin	08^{0}48^{l}37.48^{ll}N; 78^{0}09^{l}56.12^{ll}E	05
Mercia opima	MOTUT	Tuticorin	08^{0}48^{l}37.48^{ll}N; 78^{0}09^{l}56.12^{ll}E	05
Sunetta scripta	SSVYP	Ernakulam	09^{0}59^{l}47.46^{ll}N; 76^{0}13^{l}06.77^{ll}E	15
Villorita cyprinoides	VCVAI	Vaikom	09^{0}43^{l}18.02^{ll}N; 76^{0}23^{l}19.52^{ll}E	10

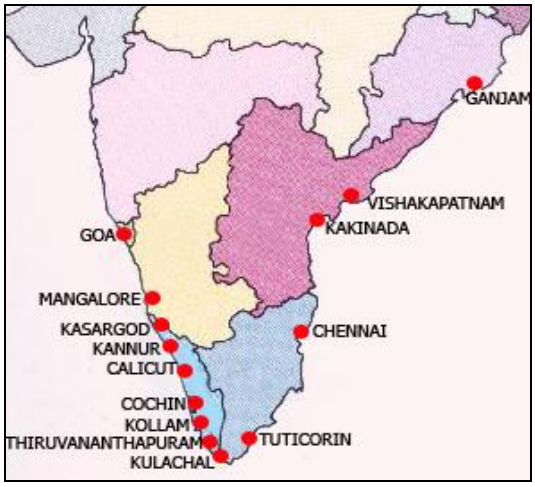


Fig 2: Map of South India showing the Specimen collection locations

Fig 3: Dissected specimen of *Perna indica* showing the adductor muscle tissues

3.2.1.1. Thankassery Bay

Thankassery Bay is well known for its extensive natural settlements of *P. viridis* and *P. indica*. The bay is located within the coordinates between $8^0 52^l 38^{ll}$ N, $76^0 34^l 24^{ll}$ E and $8^0 52^l 49^{ll}$ N, $76^0 34^{ll} 41^{ll}$ E which is a part of the Arabian Sea in Kollam district of Kerala state. The break water constructed out of the granite rocks and concrete tripods protect the bay from the heavy waves of the Sea. These hard structures form the major substratum for the attachment of the bivalves of the ecosystem. The bay harbors unique blend of flora and fauna similar to that of a coral ecosystem. The water is clear and transparent which often promotes the growth of sea weeds as well. Beside the extensive beds of *P. viridis* and *P. indica*, patchy beds of the edible oyster, *C. madrasensis* can also be seen. The area from Neendakara to Thankassery bay forms an important and unique ecological zone of green mussel and brown mussel which demarcate the populations of the two species. Both of these species co-occupy at this zone but, it is difficult to find an established population of *P. indica* towards the North of this zone; similarly it is very hard to find an established population of *P. viridis* towards the South of this zone. Though, there are some research works trying pull up the cause, the exact reason behind this phenomenon is not yet revealed. The productive water of the bay favors the growth of rich populations of *P. viridis* and *P. indica*, and hence, it forms one of the important fishery grounds of the same. Grown specimens of the mussels and the plankton samples were collected from this location for DNA isolation and larval screening respectively.

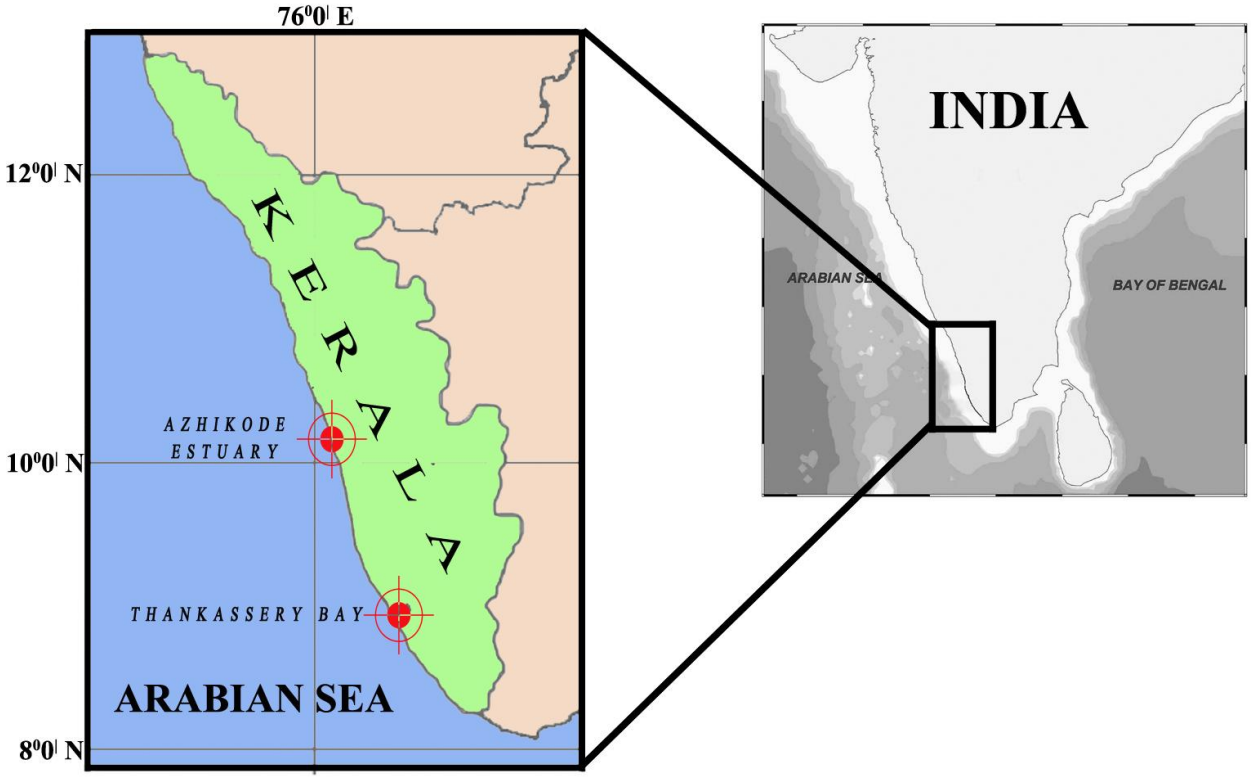


Fig 4: Map showing the study areas Thankassery Bay and Azhikode Estuary selected for the plankton screening and spat-fall prediction studies

3.2.1.2. Azhikode Estuary

Azhikode Estuary is located at the Northern end of the Ernakulam district of Kerala state within the coordinates between 10°11'11.00"N & 76°11'37.72"E and 10°11'09.65"N & 76°11'16.12"E. The upstream of the Estuary is connected to Periyar River, and it flows down and mixes into the Arabian Sea at Munambam bar-mouth, the border that demarcates the two districts, Ernakulam and Thrissur. The estuary is famous for the extensive natural beds of the edible oyster, *Crassostrea madrasensis*. It is one of the important fishery resources for the local fishermen, and there are a number of women self help groups (SHGs) that are actively engaged in the mariculture of this species using Raft method of oyster culture. The Estuary has an island known as 'Sathar Island' that harbors extensive beds of edible oysters. The island is made of laterite rocks which form the substratum of attachment for the oysters. Proximity of the Estuary to the Arabian Sea makes it susceptible to the tidal variations in water level at considerable extends. Besides the edible oyster beds, the Estuary also harbors very small patches of green mussel (*Perna viridis*) and rock oyster (*Saccostrea cucullata*) beds. The water is very productive and turbid with considerable amount of silt and mud input from the Periyar River. Adult specimens of the edible oysters for DNA isolation were collected from this area. Periodic collection of plankton biomass was also carried out from this location to screen for the presence of larvae of the target species.

3.2.2. Plankton Collection and Preservation

Plankton samples were collected from the coastal areas where prominent mussel and oyster beds occur. Plankton collection was made from Thankassery Bay to screen for the presence of *P.viridis* and *P.indica* larvae. Samples were collected from Azhikode Estuary to screen for the presence of *C.madrasensis* larvae. Collections were made periodically, starting from the month prior to the normal breeding season, and continued until the end of the season. A methodology was standardized for collecting the plankton samples. A towing plankton collection net made of bolting silk having 80µm mesh size and a mouth opening of 20cm diameter was used for collecting plankton. Horizontal hauling of the net was carried out approximately 1m below the water surface upto 100 m with the help of a motorized fiber craft at Thankassery Bay and a canoe at the Azhikode Estuary. The collected plankton sample was filtered through a 500µm meshed sieve in order to remove the unwanted planktonic forms that may contribute to the total plankton DNA. The plankton sample was then concentrated by sieving through a 50µm mesh followed by rinsing with fresh water to remove salt content and other contaminants such as salt and silt. Then a small portion of the sample was preserved in 4% formalin for microscopic analysis and the remaining sample was preserved in 100ml of 80 % Ethanol in a screw capped glass container for DNA analysis. The net volume of water filtered for collecting the plankton was calculated using the following formula (Goswami, 2004). Every m^3 volume of water filtered represents approximately 1000 liters of water. Therefore, approximately 12.5 tons of water could be filtered while towing the plankton net for a distance of 100 m.

Volume of water filtered (m^3) = Area of Net Opening × Distance of towing (d)

$$= \pi r^2 d$$

($1m^3$ = 1000 liters)

Fig 5: The plankton collection net being towed in the water at Azhikode Estuary

3.2.3. Microscopic Analysis of Plankton Samples

The plankton samples collected and preserved in 4% Formalin were observed under a compound microscope in order to identify the presence of any bivalve larvae. The preserved plankton samples in 100 ml borosilicate bottles were mixed thoroughly so that a subsample of homogeneous plankton representation shall be collected for microscopic analysis. About one ml of this homogeneous plankton mix was collected using a micro pipette attached with a wide-bore micro tip, and transferred to a watch glass. It was then diluted with a little water, and a few drops from the same were collected on a glass slide and observed under a compound microscope. The plankton samples confirmed for the presence of bivalve larvae through microscopic analysis were then subjected to molecular analysis using their counter parts preserved in ethanol.

3.3. Collection of Bivalve Larvae

The veliger larvae of the target bivalve species were collected from various sources, and preserved properly for further molecular analysis and standardization of detection methods being developed in the present work.

3.3.1. Voucher Collection of Bivalve Larvae

The veliger larvae of the bivalves namely *Perna viridis* and *Crassostrea madrasensis* which were transformed 48 hours post fertilization (hpf) were obtained from the Molluscs breeding facility of the Marine Hatchery in the Tuticorin Research Centre of Central Marine Fisheries Research Institute (CMFRI). Similarly, the veliger larvae of *Perna indica* (48 hpf) were obtained from the hatchery facility in Vizhinjam Research Centre, CMFRI. The larvae obtained were preserved in 80 % ethanol in a leak proof, airtight 30ml polypropylene storage container and kept in refrigerator.

3.3.2. Mussel Spawning in Wet Lab and Larval Collection

Ripe *P. viridis* samples in live condition were collected from Thankassery Bay during the last week of May 2011 and brought to the wet lab in CMFRI head quarters where the research work was carried out. The ripe mussels were induced to spawn following the strategy suggested by Sreenivasan *et al.* (1988). A thermal stimulation was applied in the mussel rearing fiber tank by raising the water temperature up to 4 ^{0}C (from 26 ^{0}C to 30 ^{0}C) using a water heater attached to a thermostat. The mussels started spawning after about 20 minutes of thermal stimulation. The male animals started ejecting spermatozoa as a white smoke followed by the females ejecting a jet of orange coloured ova (Figure 6). After 30 minutes of spawning the adult animals were removed from the spawning tank and the water containing both gametes were incubated in an air conditioned room. Samples of the incubated water were collected at an interval of 08 hours, 16 hours and 24 hours, and observed under compound microscope for

the presence of any bivalve larval stages. The transformed larvae at 48 hpf were collected from the water by filtering through a 50µm sieve, and preserved in 80 % ethanol for further research work to be carried out.

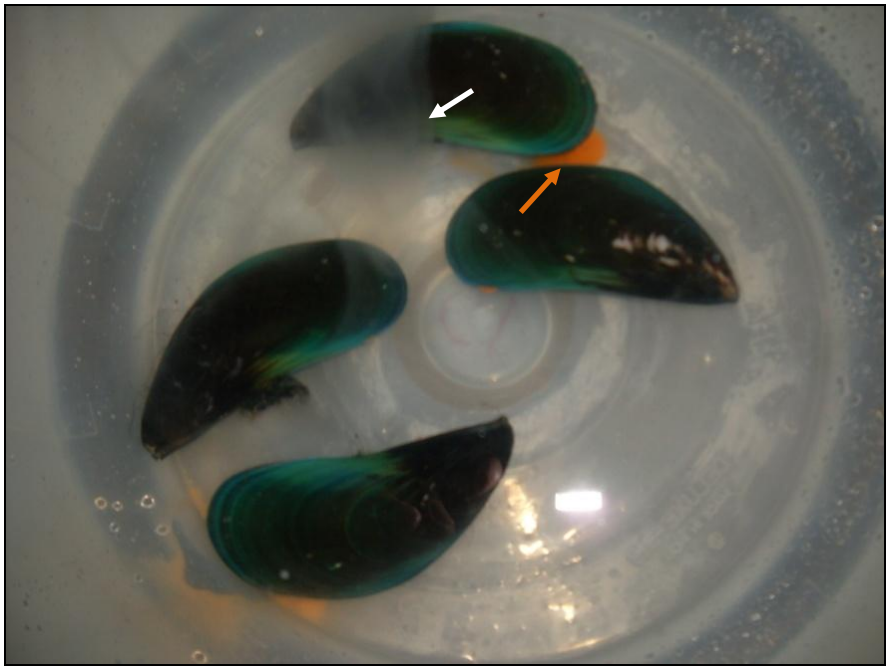


Figure 6: Spawning of green mussels. A white smoke of spermatozoa is marked with a white coloured arrow and the ova released are shown with an orange coloured arrow.

3.4. Molecular Studies in Bivalves

The objective of the research work is to design molecular markers that can specifically identify larvae of the bivalves of mariculture importance in the coastal waters so that their spat-fall may be predicted on time. In this regard, attempts were made to develop species specific DNA markers, based on Polymerase Chain Reaction (PCR), using which larvae of the bivalve species of interest could be distinguished and identified. The strategy was to amplify selected mitochondrial and nuclear genes through PCR, using universal primers, followed by sequence characterization. Then the characterized gene sequences were compared to identify a suitable gene region for developing species identification DNA markers. Species specific primers were designed from theese identified gene sequences for the development of Species Specific PCRs (SSPCRs). To increase the sensitivity, a two step Species Specific *nested* PCR (SS*n*PCR) was also designed. For this, the product of the first step PCR carried out using the universal primer is used as template for the second step amplification using the species specific primers.

3.4.1. Chemical Reagents Employed for Molecular Analysis and their Composition

3.4.1.1. DNA isolation reagents

3.4.1.1.1. TEN Lysis buffer

Chemical	Concentration / mass / volume in the final solution
Tris HCl	10mM
EDTA	1mM
NaCl	400mM
Double distilled water	100 ml

3.4.1.1.2. 10% SDS

Chemical	Concentration / mass / volume in the final solution
SDS detergent	10 g
Double distilled water	100 ml

3.4.1.1.3. 10% Proteinase-K

Chemical	Concentration / Volume in the final solution
ProteinaseK	100 mg
MilliQ water	1 Ml

3.4.1.1.4. Extraction Solution

Chemical	Concentration / mass / volume in the final solution
Phenol with pH 8	25 ml
Chloroform	24 ml
Isoamyl alcohol	1 ml

3.4.1.1.5. 3M Sodium Acetate

Chemical	Concentration / mass / volume in the final solution
Sodium Acetate Trihydrate	40.8 g
MilliQ water	100 ml

Note: pH should be adjusted to 5.2 with glacial acetic acid

3.4.1.1.6. 5M Sodium Chloride

Chemical	Concentration / mass / volume in the final solution
Sodium Chloride	29.2 g
MilliQ water	100 ml

3.4.1.1.7. 5% Chelex 100

Chemical	Concentration / mass / volume in the final solution
Chelex 100 (Bio-Rad)	5 g
MilliQ water	100 ml

3.4.1.1.8. 6M Sodium Iodide

Chemical	Concentration / mass / volume in the final solution
Sodium Iodide	89.9 g
MilliQ water	100 ml

3.4.1.1.9. 0.5M EDTA

Chemical	Concentration / mass / volume in the final solution
EDTA	18.6 g
Sodium hydroxide	2 g
MilliQ water	100 Ml

3.4.1.1.10. Silica Powder Suspension

Chemical	Concentration / mass / volume in the final solution
Silica Powder	10 g
6M Sodium Iodide	10 ml

Note: 10g Silica powder suspended in 100ml distilled water and shaken vigorously overnight then allowed settling for 12 hours. The silica powder without supernatant shall be used for preparing suspension with 6M Sodium Iodide

3.4.1.1.11. TEN wash buffer

Chemical	Concentration / mass / volume in the final solution
Tris	10 mM
EDTA	1 mM
Sodium Chloride	100 mM
Ethanol	50 %

3.4.1.1.12. 70% Ethanol

Chemical	Concentration / mass / volume in the final solution
Absolute alcohol	70 ml
MilliQ water	30 ml

3.4.1.1.13. TE buffer

Chemical	Concentration / mass / volume in the final solution
Tris Hcl	10 mM
EDTA	1 mM
Double distilled water	100 ml

3.4.1.2. Electrophoresis reagents

3.4.1.2.1. TBE buffer (10X)

Chemical	Concentration / mass / volume in the final solution
Tris base	890 mM
Boric Acid	890 mM
EDTA	20 mM
Double distilled water	1000 ml

3.4.1.2.2. Gel Loading Buffer

Chemical	Concentration / mass / volume in the final solution
1 % Bromophenol blue	2.5 ml
1 % Xylene cyanole	2.5 ml
30 % Glycerol	3 ml
Double distilled water	2 ml

3.4.1.2.3. Ethidium Bromide Dye

Chemical	Concentration / mass / volume in the final solution
Ethidium Bromide	100 mg
Double distilled water	10 ml

3.4.1.3. PCR reagents

3.4.1.3.1. PCR buffer (10X) (Supplied with Taq Polymerase enzyme)

Chemical	Concentration / mass / volume in the final solution
Tris Hcl – pH 9	100 mM
Potassium Chloride	500 mM
Magnesium Chloride	15 mM
Triton X-100	1 %

3.4.1.3.2. dNTP solution - 40 mM (10mM of each dNTP)

3.4.1.3.3. Oligo Nucleotides (10 µM)

3.4.1.3.4. Taq Polymerase (5 units / µl)

3.4.2. DNA isolation

Three different methodologies were tried to isolate genomic DNA from the preserved tissue samples, which includes phenol-chloroform method (Sambrook *et al.,* 1989), salting-out protocol (Miller *et al.,* 1988) and silica based isolation technique (Yue *et al*., 2001). All the three methods were compared for the quality and yield.

3.4.2.1. Phenol-Chloroform Isolation Protocol

In this method, approximately 20mg of adductor muscle tissue was chopped and kept for complete digestion in 500µl of TEN lysis buffer, 2% SDS and 100µg proteinase-K at 55^0C for about 1.5 hours. The denatured proteins from the digested tissues were removed by phenol-chloroform extraction followed by DNA precipitation using 3M Sodium Acetate (NaOAc) and absolute Ethanol. Phenol-chloroform extraction procedure was carried out twice as the bivalve tissues possess higher percentage of proteins. The precipitated nucleic acid was pelletized by centrifugation at 10000 rotations per minute (rpm), purified from salts using 70% Ethanol and then dissolved in 50µl of TE buffer ($Tris_{10}$ and $EDTA_1$) for further downstream applications.

3.4.2.2. Salting-Out Protocol

The tissue samples from which DNA to be extracted were digested by the method explained above. The denatured proteins from the digested tissues were precipitated down in the presence of 100 µl super saturated 6M Sodium Chloride (NaCl) solution. The precipitate was pelletized by centrifugation at 10000 rpm. The clear aqueous supernatant was transferred into a fresh microtube and then the DNA present in the same was precipitated by adding $1/10^{th}$ volume of 3M Sodium Acetate and an equal volume of absolute Ethanol. The precipitated nucleic acid was purified from salts using 70% Ethanol and dissolved in 50µl of TE buffer for further downstream applications.

3.4.2.3. Silica Based DNA Isolation Protocol

In silica based DNA isolation method about 5 mg of muscle tissue was chopped and kept in 1.5 ml microtube containing 200 µl of 5% Chelex 100 (Bio-Rad) and boiled for 10 min in a water bath. The solution was cool down to room temperature, added with 10 µl of 10% proteinase K, and incubated at 55^0C for one hour for the complete digestion of the tissue. This mix was centrifuged at 10000 rpm for 5 minutes and the supernatant alone was transferred into a new microtube. To this, 540 µl of 6M Sodium iodide and 8 µl Silica suspension were added and slightly vortexed and then briefly centrifuged. The pellet after removing the supernatant was suspended in1 ml of TEN wash buffer and centrifuged at 10000 rpm for 30 seconds. The pellet after removing the supernatant was air dried and suspended in 40 µl TE buffer, then centrifuged at 10000 rpm for one minute. The supernatant containing the DNA was pipetted out and used for further downstream applications.

3.4.2.4. DNA Isolation from the Plankton Samples

The method of cell lysis by the application of heat-shock was employed to release DNA from the plankton samples containing bivalve larvae for conducting the PCR diagnostics. In this procedure, about 20-40 mg of the ethanol preserved plankton sample was taken in a 1.5ml micro centrifuge tube and centrifuged at 5000 rpm for 5 minutes to make it into a pellet. The supernatant ethanol was removed and

the pellet was air dried to remove the residual alcohol content. The dried sample was re-suspended in 100µl Milli-Q water and it was incubated at 95^0C in a water bath for about 10 minutes. Then the sample was immediately kept in -20^0C freezer for about 10 minutes followed by thawing at room temperature. The resulting sample was centrifuged at 10000 rpm for 5 minutes and the supernatant alone was pipetted out without any debris, and immediately used as template for PCR.

3.4.2.5. Quantification, Quality Checking and Preservation

Quantification of the isolated DNA was carried out with a BioPhotometer plus™ (Eppendorf), and the quality of DNA was checked by electrophoresis using 0.8% agarose gel matrix in Tris-Borate EDTA (TBE) buffer. The isolated DNA samples were transferred into 1.2 ml screw capped poly propylene tubes, labeled properly with codes given to the original tissues and then stored at -20^0C for future use.

3.4.3. Selection of a Suitable Gene for Developing Species Identification DNA Markers

To select the suitable gene for developing species identification markers, the prospective genes such as Cytochrome Oxidase subunit 1 (CO1), 16S ribosomal RNA (16SrRNA), 18S ribosomal RNA (18SrRNA) and Internal Transcribed Spacer (ITS) were amplified using universal PCR primers and sequence characterized. Preliminary nucleotide sequence comparison of the amplified genes were carried out to study the level of sequence divergence, so that a suitable gene region, ideal for designing species specific PCR primers, could be identified.

The species *P. viridis* has a global distribution with widely distributed populations all over world than the other two candidate species namely, *P. indica* and *C. madrasensis* which are confined to Indian peninsula. Therefore, the DNA samples of *P. viridis* from different populations were used for the initial amplification of the prospective genes to gauge the level of sequence divergence in those genes. The gene selected after the sequence divergence analysis was then used for designing species specific PCR primers in the target species.

3.4.3.1. Polymerase Chain Reaction (PCR)

All PCRs were carried out in a 25µl reaction mix containing 1X standard Taq buffer, 200 µM dNTPs, 0.2 µM of each primer, one unit Taq polymerase (New England Biolabs-NEB, Ipswich, England) and 20 to 50ng of template DNA. The PCR consisted of an initial denaturation at 94^0C for 3 minutes followed by 30 - 40 thermal cycles consisting of denaturation at 94^0C for 30 seconds, annealing at temperature corresponding to the experimental requirement maintained for 30 seconds and extension at 72^0C for 30 - 45 seconds, and a final extension at 72^0C for 5 minutes. All the PCR reactions were carried out in an S1000 Thermal Cycler manufactured by Bio-Rad, USA.

3.4.3.2. PCR Product Purification and Sequencing

The PCR products were sequence characterized using the original universal primers used for amplification. The amplified PCR products were purified and eluted into 50 µl of nuclease free MilliQ water using QIAQUICK PCR Purification Kit (Qiagen, Germany) following the manufacturer's instruction manual. The purified PCR products were sequenced using dideoxy chain termination chemistry in 3730xl automated DNA analyzer (Applied Biosystems, California), at Scigenome Sequencing Service, Kochi.

3.4.3.3. PCR Amplification of Cytochrome Oxidase Subunit 1 (CO1)

The partial coding region of the mitochondrial gene Cytochrome Oxidase c subunit 1 (CO1) was amplified through a standard PCR technique using the custom synthesized universal primers LCO1490: GGTCAACAAATCATAAAGATATTGG and HCO2198: TAAACTTCAGGGTGACCAAAAAATCA (Folmer *et al*. 1994). DNA samples of the three target species, *P. viridis*, *P. indica* and *C. madrasensis* were used for PCR amplification employing this universal primer pair. The primers employed are robust in nature that can amplify the partial coding regions of CO1 gene across different molluscan families. Details of reaction mix composition and the thermal cycling conditions are given in Table 2.

Table 2: Details of PCR Conditions for Amplifying Mitochondrial CO1 Gene

Total Reaction Volume	Reaction Mix Composition	Thermal conditions of PCR
25µl	1x NEB Standard Taq buffer 200µM dNTPs. 0.2 µM of each primer 1U NEB Taq polymerase. 50ng of Template DNA.	94^0 for 03 min 94^0 for 30 sec 50^0 for 30 sec } 35 cycles 72^0 for 45 sec 72^0 for 05 min

3.4.3.4. PCR Amplification of 16S rRNA Gene

The mitochondrial gene of *P. viridis* coding for 16S ribosomal RNA (16S rRNA) was amplified partially through a standard PCR technique with the custom synthesized universal primers 16sarL: CGCCTGTTTAACAAAAACAT and 16sbrH: CCGGTCTGAACTCAGATCATGT (Palumbi *et al*. 1996). Details of reaction mix composition and the thermal cycling conditions are given in Table 3.

Table 3: Details of PCR Conditions for Amplifying Mitochondrial 16SrRNA Gene

Total Reaction Volume	Reaction Mix Composition	Thermal conditions of PCR
25µl	1x NEB Standard Taq buffer 200µM dNTPs. 0.2 µM of each primer 1U NEB Taq polymerase. 50ng of Template DNA.	94^0 for 03 min 94^0 for 30 sec 52^0 for 30 sec } 35 cycles 72^0 for 30 sec 72^0 for 05 min

3.4.3.5. PCR Amplification of Internal Transcribed Spacer I&II (ITS-I&II)

Approximately 900 bp of the ribosomal RNA coding region of *P. viridis* spanning 18SrRNA, ITS (Internal Transcribed Spacer) 1, 5.8SrRNA, ITS-2 and 28SrRNA of the nuclear genome was amplified using the universal primers **ITS5:** GGAAGTAAAAGTCGTAACAAGG and **ITS28:** CGCCGTTAC TAGGGGAATCCTTGTAAG (Wood *et al*. 2007). Details of reaction mix composition and the thermal cycling conditions are given in Table 4.

Table 4: Details of PCR Conditions for Amplifying ITS I & II

Total Reaction Volume	Reaction Mix Composition	Thermal conditions of PCR
25µl	1x NEB Standard Taq buffer 200µM dNTPs. 0.2 µM of each primer 1U NEB Taq polymerase. 50ng of Template DNA.	94^0 for 03 min 94^0 for 30 sec 50^0 for 30 sec } 35 cycles 72^0 for 1 min 72^0 for 10 min

3.4.3.6. PCR Amplification of 18SrRNA Gene

The nuclear gene of *P. viridis* coding for 18S ribosomal RNA (18SrRNA) was amplified partially through a standard PCR technique with the custom synthesized universal primers Myt18SF: CAACCTGGTTGATCCTGCCAGT and Myt18SR: CACCTCTAACACCGTAATACGA (Santaclara *et al*. 2006). The details of reaction mix compositions and the thermal cycling conditions are given in the Table 5.

Table 5: Details of PCR Conditions for Amplifying 18SrRNA Gene

Total Reaction Volume	Reaction Mix Composition	Thermal conditions of PCR
25µl	1x NEB Standard Taq buffer 200µM dNTPs 0.2 µM of each primer 1U NEB Taq polymerase 50ng of Template DNA.	94^0 for 03 min 94^0 for 30 sec 55^0 for 30 sec } 35 cycles 72^0 for 45 sec 72^0 for 05 min

3.4.3.7. Sequence Analysis

The amplified PCR products of all the four gene regions were purified and sequenced with original amplification primers as mentioned in the sub heading 3.4.3.2. The nucleotide sequences thus obtained were analyzed for the quality and integrity using the bioinformatics tool, Sequence Scanner v1.0, Applied Biosystems. Multiple overlapping base pair sequences due to the erroneous machine read were verified in five different DNA templates used for PCR amplification. Sequence editing such as addition or deletion of nucleotides and removal poor read sequences at the $5^{|}$ & $3^{|}$ flanking regions of the amplified segments were done with BioEdit 7.0 (Hall, 1999). The amplified gene sequences after all editing works

were deposited in the nucleotide database, GenBank using the software Sequin, NCBI. The nucleotide sequences of the genes generated for the five different templates after all editing works were compared with each other for the presence of INDELs, inter-specific sequence divergence and intra-specific sequence conservation. Those gene sequences having minimum INDELs, higher percentages of inter-specific sequence divergence and intra-specific sequence conservation (low intra-specific divergence) were identified since they are ideal for designing species identification DNA markers.

3.4.3.7.1. Inter-Species Sequence Divergence Analysis of *Perna sp.*

The percentage of sequence divergence between different species of *Perna* namely, *P. viridis, P. perna* and *P. canaliculus* (sequence data of the last two species were collected from GenBank, NCBI) were calculated separately for the sequences of CO1, 16SrRNA, 18SrRNA and ITS using pairwise distance calculation method in MEGA 5.2.2. The nucleotide data of 450bp length for 16SrRNA gene and 600bp length for the rest of the genes were used in the analysis. The calculations were conducted under 1000 bootstrap replications employing Kimura 2-parameter model (Kimura, 1980) of nucleotide substitutions. This provides an idea of inter-specific sequence variation between the closely related species by each DNA marker. The gene sequence that exhibits highest percentage of sequence divergence between the closely related species shall be selected for designing species identification DNA markers.

3.4.3.7.2. Intra-Species Sequence Divergence of *P. viridis* Populations

Based on the inter-specific sequence similarity analysis of the four different DNA markers, the CO1 was found to be having highest percentage of inter-species sequence variation. Hence the CO1 was only used for the intra-specific sequence divergence analyzis . The intra-specific sequence divergence of CO1 gene between the populations of *P. viridis* collected from different regions of India (Karwar, Mangalore, Kollam, Chennai and Orissa) and other countries (sequence data retrieved from GenBank, NCBI) were calculated through pairwise distance calculation method in MEGA 5.2.2. The CO1 nucleotide sequences spanning 608 bp length were used in the analysis under 1000 bootstrap replications employing Kimura 2-parameter model of nucleotide substitutions. This can provide an idea of sequence divergence percentage of the CO1 gene within different populations of the species so that the suitability of the gene for designing the species identification DNA markers can be elucidated.

3.4.4. Species Specific PCR (SSPCR)

Species Specific PCR (SSPCR) was designed for each target bivalve species that can accurately identify the target bivalve larvae in the plankton samples collected from the coastal waters. Sequence analysis of the prospective genes amplified using universal primers had indicated that the mitochondrial

CO1 gene was an ideal gene for SSPCR. Hence, the CO1 gene sequences characterized for the target species were used for designing SSPCR primers for the respective species.

3.4.4.1. 1. Species Specific PCR (SSPCR) primers for *P. viridis*

The CO1 gene sequences characterized for *P. viridis* in the present study and the CO1 sequence data retrieved from GenBank for other related bivalve species namely *P. indica, P. canaliculus, P. perna, P. picta, Meretrix casta, Villorita cyprinoides, Limnoperna sp., Balanus sp.,* and *Patella sp.*, were aligned and compared by ClustalW multiple alignment method (Thompson *et al.*1994) in BioEdit 7. The regions of inter-species multiple nucleotide variations and intra-species conserved regions were located by using the software, and four pairs of SSPCR primers were designed using the primer designing application, Primer-BLAST available in the NCBI web portal. Possibilities of nucleotide polymorphism at the primer annealing site was cross checked with the COI sequences of *P. viridis* from different continents (accession numbers JN179066, JN179053, GQ497835, DQ917599, DQ917590, DQ343590, DQ343587, DQ343581, DQ343573) available in GenBank.

Specificity PCR Assay

Specificity assessment of the primers designed for SSPCR was carried out with the organisms whose larval stages usually found in the habitat where *P. viridis* occurs. This includes *P. indica* (brown mussel), *Sunnetta scripta* (marine clam), *Paphia malabarica* (short-neck clam**)**, *Meritrix casta* (yellow clam), *Villorita cyprinoides* (black clam), *Crassostrea madrasensis* (Indian backwater oyster), *Saccostrea cucculata* (Indian rock oyster), *Pinctada fucata* (Pearl oyster), *Balanus sp*.(barnacles), *Patella sp.* (limpets) and *Parapenaeopsis stylifera* (Kiddi shrimp). A positive control PCR using the universal 18SrRNA primers, NSF1179: AATTTGACTCAACACGGG and NSR1642: GCGACGGGCGG TGTGTAC (Wuyts *et al*. 2001) was also set along with the specificity assay. The positive control PCR was conducted in a 25µl reaction mix having the same composition detailed under the subtitle 3.4.3.1. The thermal cycling conditions standardized for the SSPCR primers of *P. viridis* includes an initial denaturation at 94^0C for 3 minutes, followed by 35 - 40 cycles of denaturation at 94^0C for 30 seconds, annealing at 58^0C for 30 seconds, extension at 72^0C for 30 seconds and a final extension at 72^0C for 5 minutes. The possibilities for the false negative PCR amplification by the selected SSPCR primer due to the sequence variations at the primer binding region was verified and validated empirically by conducting PCR with 96 DNA samples of *P. viridis* collected from different locations (Table 3.1.).

3.4.4.1.2. Species Specific PCR (SSPCR) primers for *P. indica*

The sequences of mitochondrial CO1 gene of *P. indica* characterized in the present study and that of other related bivalve species namely *P. perna, P. picta, P. viridis, P. canaliculus, Meretrix casta,*

Villorita cyprinoides, Limnoperna sp., Balanus sp., and *Patella sp.,* collected from GenBank were aligned and compared by ClustalW multiple alignment method. The regions of inter-species multiple nucleotide variations and intra-species conserved regions were manually located and three pairs of SSPCR primers were designed by using the online tool OligoCalc (Kibbe, 2007) taking care to avoid the chances of formation of secondary structures.

Specificity PCR Assay

Specificity assay was conducted to select the most specific PCR primers of *P. indica*. DNA samples from the bivalves and other organisms whose larval stages usually found in the habitat of *P. indica* was collected and used in the specificity assay. This includes *P. viridis, Sunnetta scripta, Paphia malabarica, Meritrix casta, Villorita cyprinoides, Crassostrea madrasensis, Saccostrea cucculata, Pinctada fucata, Balanus sp*., *Patella sp.* and *Parapenaeopsis stylifera.* A positive control PCR using the universal 18S rRNA primers, NSF1179 and NSR1642 was also set along with the specificity PCR assay. The thermal cycling conditions standardized for the SSPCR primers of *P. indica* consists of an initial denaturation at 94^0C for 3 minutes, followed by 35 - 40 cycles of denaturation at 94^0C for 30 seconds, annealing at 50^0C for 30 seconds, extension at 72^0C for 30 seconds and a final extension at 72^0C for 5 minutes. The selected SSPCR primers were used to amplify 96 DNA templates belonging to the individuals of *P. indica* collected from different populations along the Indian peninsula (Table 3.1.) in order to confirm that there is no false negative amplification.

3.4.4.1.3. Species Specific PCR (SSPCR) primers for *C. madrasensis*

The CO1 sequences characterized for *C. madrasensis* along with that retrieved from the GenBank for the related bivalve species such as *C. gryphoides, Saccostrea cucculata, Isognomon sp.* (mangrove oyster), *Pinctada fucata, P. margaritifera, Perna indica, P. viridis, Meretrix casta, Villorita cyprinoides, Balanus sp.* and *Patella sp.* were compared using ClustalW multiple alignment method. The distinct sequence regions with inter-species multiple nucleotide variations and intra-species conserved regions were located and such regions were used for designing four pairs of species specific primers using the primer designing application Primer-BLAST available in the NCBI web portal.

Specificity PCR

Specificity of these primers was verified by a specificity PCR assay in which the DNA isolated from different bivalve species occurring in the habitat of *C. madrasensis* were subjected for PCR amplification. The bivalves used in specificity PCR includes *Crassostrea gryphoides, Saccostrea cucculata, Pinctada fucata, P. margaritifera* (black lip pearl oyster), *Isognomon sp., Perna viridis, Perna indica, Meretrix meretrix, M. casta, Paphia malabarica, Villorita cyprinoides, Patella sp.,* and *Balanus*

sp.. A positive control PCR was also set in this specificity PCR assay using the universal 18S rRNA primers NSF1179 & NSR1642. The thermal cycling conditions standardized for the SSPCR primers of *C. madrasensis* includes an initial denaturation at 94^0C for 3 minutes, followed by 35 - 40 cycles of denaturation at 94^0C for 30 seconds, annealing at 58^0C for 30 seconds, extension at 72^0C for 30 seconds and a final extension at 72^0C for 5 minutes. The possibilities for the false negative PCR amplification by the selected SSPCR primer due to the sequence variations at the primer binding region was verified and validated empirically by conducting PCR with 96 DNA samples of *C. madrasensis* collected from different locations (Table 3.1.).

3.4.4.2. Species Specific *nested* PCR (SS*n*PCR)

There is the possibility of low sensitivity when identification of the target bivalve larvae in the field collected plankton samples are carried out using the SSPCR system. The plankton samples having very low number of target larvae will yield the total plankton DNA with low ratios of target DNA. The SSPCR with relatively low concentrations of the target DNA may generate varying PCR results due to the random fluctuations in the PCR priming efficiency (Chandler *et al*. 2003). Therefore, a Species Specific *nested* PCR (SS*n*PCR) was designed to increase the sensitivity of identification of target bivalve larvae from plankton samples. It is performed in two steps; first one is the primary enrichment of target DNA with universal CO1 primers, LCO1490 and HCO2198 using the reaction mix composition and thermal cycling conditions as detailed under the subtitle 3.4.3.3. The second step is a nested PCR with the SSPCR primers of the respective target bivalve species and the diluted PCR product of the first step PCR as template DNA. The nested PCR reactions were performed as per the standardized reaction conditions given under the subtitles 3.4.4.1.1 to 3.4.4.1.3.

3.4.4.2.1. Standardization of SS*n*PCR with Simulated Plankton Sample

The reaction conditions for SS*n*PCR were standardized using a simulated plankton sample for obtaining an optimum result. A plankton sample devoid of bivalve larvae (collected prior to the normal spawning season) was simulated to a real-time plankton sample with known number of bivalve larvae. This was done by adding larvae (48 hpf) of all three species to a 40 mg aliquot of the plankton, and then carrying out SS*n*PCR for the standardization of the same. The PCR product obtained in the first step PCR is diluted serially to prepare DNA template material for the second step PCR. Dilutions of the first PCR products ranging from 1 to 1/25 were prepared and then used as template for the second step nested PCR. The template dilutions at which optimum PCR yield obtained is selected for further screening process of plankton samples using SS*n*PCR.

3.4.4.3. Sensitivity of SSPCR and SS*n*PCR

The minimum concentration of target DNA required in a pool of plankton DNA to produce an observable PCR amplicon by the SSPCR as well as the SS*n*PCR is arrived at through the sensitivity PCR assay. The experimental plankton DNA samples which contains varying concentrations of target DNA ranging from 1 pico gram (0.001 nano gram) to 250 nano gram were subjected to sensitivity PCR assay. The plankton DNA (500 ng/µl) and the target species DNA (500 ng/µl) were serially diluted with MilliQ water in order to get a range of diluted templates. Then these diluted DNA samples of plankton and target species were mixed at pre determined proportions in order to get a final plankton DNA mix containing the desirable concentration of target DNA (Table 6). The plankton DNA samples thus prepared containing a range of varying concentrations of target DNA were subjected to SSPCR and SS*n*PCR using species specific primers corresponding to the target species under the standardized reaction conditions. The positive PCR result produced at the lowest concentration of target DNA present in the plankton DNA mix was identified separately for the SSPCR and SS*n*PCR.

Minimum number of larvae of the target species required in a plankton sample to produce an observable PCR amplicon by the SSPCR and SS*n*PCR system was worked out through the sensitivity PCR assay. Plankton samples devoid of bivalve larvae were used for sensitivity measurements. Ten aliquots of the plankton samples each containing approximately 40 mg of plankton biomass was prepared. Veliger larvae of target the bivalve species (48 hpf) were added into these aliquots in a progression of 1, up to 5 numbers in the first 5 aliquots and in a progression of 10, up to 50 numbers in the remaining 5 aliquots. DNA was extracted from these mixtures by salting out protocol (Miller *et al*., 1988), diluted to form a concentration of approximately 4-5ng/µl, and then subjected to SSPCR and SS*n*PCR tests. The minimum number of bivalve larvae required to produce PCR amplicons of observable concentration in the ordinary agarose gel electrophoresis (AGE) were identified in this way.

3.5. Plankton Screening Using the SSPCR and SSnPCR

The ethanol preserved plankton samples were subjected to screening for the presence of larvae of the target species through SSPCR and SS*n*PCR.

3.5.4. Identification of Individual Veliger Larvae Using SSPCR

The bivalve veliger larvae present in the ethanol preserved plankton samples were sorted out using a glass micro-capillary tube under a compound microscope. DNA was isolated from the individual veliger larvae using the heat treatment method explained in the subhead 3.4.2.4. Then these DNA samples were subjected to SSPCR specific for *P. viridis*, *P. indica* and *C. madrasensis* in order to identify the species of the veliger larvae.

3.5.5. Identification of Veliger Larvae in the Whole Plankton Sample

Screening of the unsorted whole plankton samples collected from the study areas was carried out using SSPCR and SS*n*PCR systems developed in the current study. The plankton samples collected from Thankassery Bay and Azhikode Estuary during 2011 and 2012 were used for DNA isolation as explained in the subhead 3.4.2.4., and then subjected to SSPCR and SS*n*PCR for *P. viridis*, *P. indica* and *C. madrasensis.* For this, around 1.5 ml of homogeneously mixed subsample was collected from the100 ml preserved plankton sample for obtaining 20-40 mg of plankton biomass. This aliquot of plankton biomass was then subjected to plankton screening using SSPCR and SS*n*PCR. Five different aliquots were collected from each preserved plankton sample for the analysis so that the percentage of representation of the larvae can be predicted from the number of positive results obtained from all the five samples.

Table 6: Dilutions of DNA Templates Made for Conducting Sensitivity PCR

Conc. of Plankton DNA (ng/µl) (A)	Volume (µl) of Plankton DNA taken for dilution (B)	Conc. Of mussel / oyster DNA (ng/µl) (C)	Volume (µl) of mussel / oyster DNA taken for dilution (D)	Final volume of DNA mix (B+D)	Conc. of the final DNA mix (ng/µl) (A+C/2)	Conc. of Plankton DNA in the final DNA mix (ng/µl) (A/2)	Conc. of mussel / oyster DNA in the final DNA mix (ng/µl) (C/2)
1	2.5	500	2.5	5	250.5	0.5	250
100	2.5	400	2.5	5	250	50	200
200	2.5	300	2.5	5	250	100	150
300	2.5	200	2.5	5	250	150	100
350	2.5	150	2.5	5	250	175	75
400	2.5	100	2.5	5	250	200	50
450	2.5	50	2.5	5	250	225	25
480	2.5	20	2.5	5	250	240	10
485	2.5	15	2.5	5	250	242.5	7.5
490	2.5	10	2.5	5	250	245	5
495	2.5	5	2.5	5	250	247.5	2.5
500	2.5	4	2.5	5	252	250	2
500	2.5	2	2.5	5	251	250	1
500	2.5	1	2.5	5	250.5	250	0.5
500	2.5	0.5	2.5	5	250.25	250	0.25
500	2.5	0.2	2.5	5	250.1	250	**0.10**
500	2.5	0.1	2.5	5	250.05	250	0.05
500	2.5	0.02	2.5	5	250.01	250	0.01
500	2.5	0.01	2.5	5	250.005	250	0.005
500	2.5	0.002	2.5	5	250.001	250	0.001

3.6. Prediction of Spat-fall and Spat Collection

The minimum number of veliger larvae required in the whole plankton samples that can be detected through the SSPCR and SS*n*PCR systems are different as evident from the sensitivity measurements explained under the sub head 3.4.4.3. A positive result in the SS*n*PCR with the whole plankton sample represents the presence of very low numbers of target species larvae at the sampling site which can be considered as an indication of the beginning of spawning season or the availability of the ripe individuals. Similarly, a positive result in the SSPCR with the whole plankton sample represents the presence of more numbers of veliger larvae per unit volume of water filtered for collecting the plankton samples. Therefore, the period in which continuous positive results obtained in both SS*n*PCR and SSPCR tests indicates the continuous availability of target species veliger larvae at significant numerical density. The probability of spat-fall will be high in this period.

Correlations were made between the results obtained from larval screening tests and the field observation of the spat settlement on the rocky substratum at the sampling sites. Oyster Rens were installed in Azhikode Estuary for obtaining the spat settlement. Rens were placed by the end of October 2011 based on the results obtained from the larval screening tests conducted with the plankton samples. This field study was conducted in cooperation with the Molluscan Fisheries Division of CMFRI under which the oyster Rens were installed and monitored through a World Bank assisted project. A spat-fall prediction method was thus formulated correlating the laboratory results and the field observations.

CHAPTER 4

RESUSLTS

4.1. Mussel Spawning

In addition to the voucher collection of the veliger larvae of *Perna viridis* (green mussel), *Perna indica* (brown mussel) and *Crassostrea madrasensis* (edible oyster), the larvae of *P. viridis* were also produced in the wet laboratory through induced mussel spawning by thermal stimulation (raising the water temperature from 26 ^{0}C to 30 ^{0}C). The veliger larvae transformed 48 hours post fertilization (hpf) were collected and preserved in 95% Ethanol. This was useful as a ready stock in-hand to conduct the sensitivity PCR assays of the bivalve larval detection. The microscopic view of the preserved veliger larvae are given below.

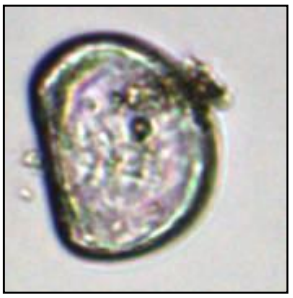


Fig.7: Veliger larva of *Perna viridis*

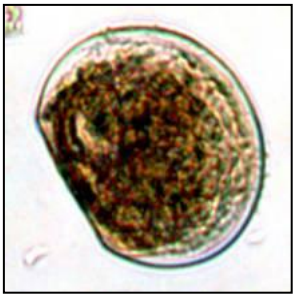


Fig.8: Veliger larva of *Perna indica*

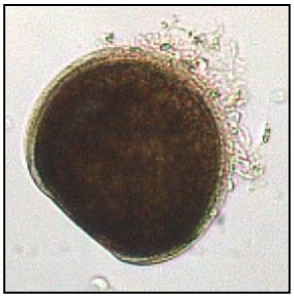
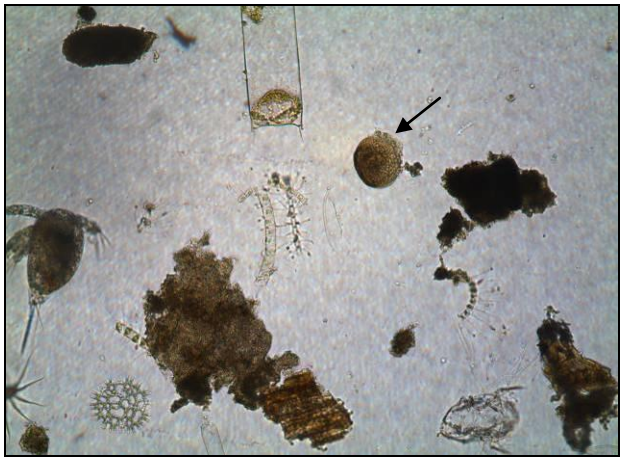


Fig.9: Veliger larva of *Crassostrea madrasensis*

4.2. Microscopic analysis of plankton samples

The plankton samples collected from Thankassery bay and Azhikode estuary were preserved in ethanol and formalin for genetic studies and microscopic analysis respectively. The preserved plankton samples were observed under the compound microscope for the presence of bivalve larvae. Microscopic view of the plankton samples showing the presence of bivalve veliger larvae are provided in the Figures 10 & 11.



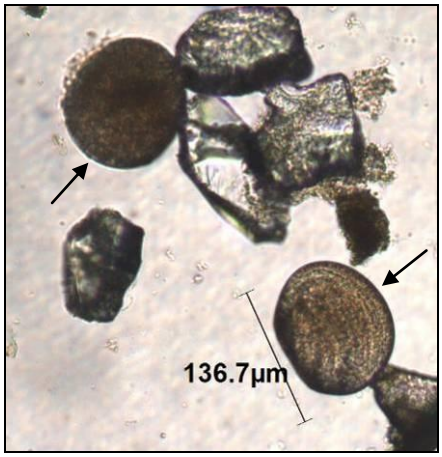


Figure 10 & 11: Microscopic view of the plankton sample collected using an 80µ plankton mesh showing different planktonic organisms and debris beyond the mesh size. Bivalve veliger larvae are shown with arrow marks.

4.3. DNA Isolation

Three different DNA extraction methods were tried in this study for getting quality DNA from different tissues. In the 'phenol-chloroform isolation protocol', the rate of protein removal was increased by doubling the phenol-chloroform extraction step, but, the yield of DNA got reduced due to the loss of aqueous phase during the extraction process. In the 'salting-out protocol' the DNA yield was high, but there was the presence of considerable quantity of protein-bound salt in the final DNA solution even after repeated treatment with washing buffer. In 'Silica based DNA isolation' though, the DNA yield was commendable, the final DNA extract was often found to be having traces of silica. PCR inhibition could be noticed when the DNA from silica based extraction method is used. Therefore, the Phenol-chloroform method was used for DNA isolation from tissue samples to get better quality DNA and Salting out protocol was used for DNA isolation from plankton samples.

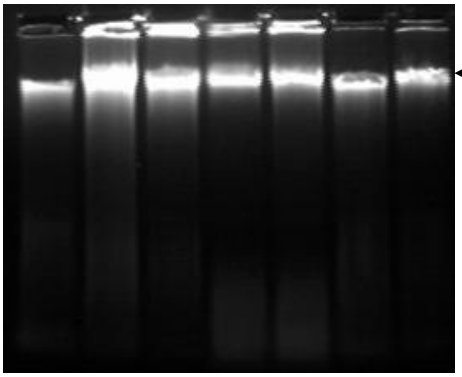


Figure 12: Agarose gel image showing the high molecular weight genomic DNA isolated from *Perna viridis* using Phenol - Chloroform DNA isolation method

4.4. Selection of a suitable gene for developing species identification DNA markers

A preliminary nucleotide sequence comparison was carried out with the mitochondrial and nuclear genes of *P. viridis* in order to identify a suitable gene region for developing species identification DNA markers. The mitochondrial genes coding for the protein Cytochrome Oxidase subunit 1 (CO1) and 16S ribosomal RNA (16SrRNA), a nuclear gene coding for 18S ribosomal RNA (18SrRNA) and the Internal Transcribed Spacers (ITS1&2) located in between the 18SrRNA, 5.8SrRNA and 28SrRNA were sequence characterized in *P. viridis* (Table 7). All of these nucleotide sequences generated in the present study were deposited in GenBank, NCBI using the online tool, Sequin, after making necessary editing at the 3' and 5' flanking regions. The details of sequence depositions are provided in the Table 8.

4.4.1. Inter-Species Sequence Comparison

The nucleotide sequences of CO1, 16SrRNA, 18SrRNA and ITS1&2 generated in this study for *P. viridis* and the same retrieved from GenBank for *P. perna* and *P. canaliculus* were used to calculate the percentage of sequence divergence using pair-wise distance calculation method in MEGA 5.2.2 (Table 9,10,11,12). A comparison of the sequence divergence percentage between different gene sequences and between different species revealed that the CO1 gene sequence has the highest divergence percentage (24.6% between *P. viridis* and *P. canaliculus*; 23.4% between *P. viridis* and *P. perna*) followed by 16SrRNA gene (23.2% between *P. viridis* and *P. canaliculus*; 22.5% between *P. viridis* and *P. perna*), ITS1&2 region (14.2% between *P. viridis* and *P. perna*; 12% between *P. viridis* and *P. canaliculus*) and the least by 18SrRNA gene (only 0.2% between *P. viridis, P. perna* and *P. canaliculus*).This indicate that the nucleotide sequence of the mitochondrial CO1 gene region is more polymorphic among closely related bivalve species. The gene sequence coding for 16SrRNA and the nuclear DNA of ITS region are characterized with a number of inter-specific INDELs that too contributed to the total percentage of divergence between the closely related species. Intra-specific INDELs are found to be common in the ITS region whereas, it is absent in the case of the 480 bp sequence of 16SrRNA used in the comparison.

Table 7: Properties of the gene sequences generated for *P. viridis, P. indica* and *C. madrasensis.*

Name of the Species		*Crassostrea madrasensis*	*Perna indica*	*Perna viridis*	*Perna viridis*	*Perna viridis*	*Perna viridis*
Gene / DNA symbol		CO1	CO1	CO1	16SrRNA	18SrRNA	ITS
Length of PCR product (bp)		≈ 700	≈ 700	≈ 700	≈ 480	≈ 900	≈ 900
Characterized gene size (bp)		654	659	651	480	662	793
Molecular Weight (kda)		396	399	394	290	401	482
Number of each nucleoside	A	151	170	160	149	162	192
	C	119	102	82	65	161	210
	G	133	135	141	114	175	205
	T	251	252	268	152	163	186
Percentage composition of Purines and Pyramidines	A+T %	61.47	64.04	65.75	62.71	49.17	47.67
	G+C %	38.53	35.96	34.25	37.29	50.83	52.33
Percentile similarity of the sequences in pair wise sequence alignment (BLAST search) with the earlier submissions in GenBank for the same species		99%	99%	99%	99%	99%	99%
Sequence query coverage		97%	100%	95%	98%	98%	97%

Table 8: Nucleotide sequence submissions made with GenBank, NCBI

Gene / DNA sequence definition	Species	Location	Accession Number
Mitochondrial cytochrome c oxidase subunit1, partial coding sequence	*Perna viridis*	Calicut	JF520789
	”	”	JF520812
	”	Chennai	JF520790
	”	”	JF520791
	”	”	JF520792
	”	”	JF520793
	”	Goa	JF520794
	”	”	JF520795
	”	”	JF520796
	”	”	JF520797
	”	Karwar	JF520798
	”	”	JF520799
	”	”	JF520800
	”	”	JF520801
	”	Kollam	JF520802
	”	”	JF520803
	”	”	FJ428756
	”	”	FJ428757
	”	”	FJ428758
	”	Mangalore	JF520804
	”	”	JF520805
	”	”	JF520806
	”	”	JF520807
	”	Orissa	JF520808
	”	”	JF520809
	”	”	JF520810
	”	”	JF520811
	Perna indica	Kulachal	FJ428753
	”	”	FJ428754
	”	”	FJ428755
	Crassostrea madrasensis	Ernakulam	FJ428750
	”	”	FJ428751
	”	”	FJ428752
	Meretrix casta	Kollam	JQ773441
	Villorita cyprinoides	Vaikom	JQ773442
Mitochondrial 16S ribosomal RNA, partial coding sequence	*Perna viridis*	Chennai	KT862016
	”	Karwar	KT862017
	”	Kollam	KT862018
	”	Mangalore	KT862019
	”	Orissa	KT862020
Nuclear 18S ribosomal RNA, partial coding sequence	”	Chennai	KT862021
	”	Karwar	KT862022
	”	Kollam	KT862023
	”	Mangalore	KT862024
	”	Orissa	KT862025
Partial coding sequence of 18SrRNA, ITS1, 5.8SrRNA, ITS2 and partial coding sequence of 28SrRNA	”	Chennai	KT862026
	”	Karwar	KT862027
	”	Kollam	KT862028
	”	Mangalore	KT862029
	”	Orissa	KT862030

Table 9: Pairwise distance calculated between the gene coding sequences of CO1 belonging to *P. viridis, P.perna and P. canaliculus*

Sl. No.	Species	Accession No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*Perna viridis*	JF520793		0.002	0.002	0.000	0.002	0.000	0.004	0.004	0.021	0.022	0.021	0.022	0.022	0.021
2		JF520803	0.002		0.000	0.002	0.000	0.002	0.004	0.003	0.022	0.023	0.022	0.022	0.022	0.021
3		JF520807	0.002	0.000		0.002	0.000	0.002	0.004	0.003	0.022	0.023	0.022	0.022	0.022	0.021
4		JF520811	0.000	0.002	0.002		0.002	0.000	0.004	0.004	0.021	0.022	0.021	0.022	0.022	0.021
5		JF520812	0.002	0.000	0.000	0.002		0.002	0.004	0.003	0.022	0.023	0.022	0.022	0.022	0.021
6		DQ917612	0.000	0.002	0.002	0.000	0.002		0.004	0.004	0.021	0.022	0.021	0.022	0.022	0.021
7		DQ343587	0.010	0.008	0.008	0.010	0.008	0.010		0.004	0.022	0.023	0.022	0.022	0.022	0.022
8		JF520801	0.008	0.007	0.007	0.008	0.007	0.008	0.012		0.022	0.023	0.022	0.022	0.022	0.022
9	*Perna perna*	KC692007	0.222	0.224	0.224	0.222	0.224	0.222	0.224	0.227		0.004	0.007	0.019	0.020	0.019
10		HG005374	0.229	0.231	0.231	0.229	0.231	0.229	0.231	0.234	0.008		0.007	0.020	0.020	0.020
11		DQ917617	0.217	0.219	0.219	0.217	0.219	0.217	0.219	0.222	0.028	0.033		0.020	0.021	0.020
12	*Perna canaliculus*	HG005373	0.239	0.239	0.239	0.239	0.239	0.239	0.243	0.241	0.189	0.191	0.196		0.003	0.002
13		DQ917613	0.241	0.241	0.241	0.241	0.241	0.241	0.246	0.244	0.196	0.198	0.203	0.005		0.004
14		DQ917609	0.236	0.236	0.236	0.236	0.236	0.236	0.241	0.239	0.191	0.193	0.198	0.003	0.008	

The estimates of evolutionary divergence between the CO1 sequences of different species of *Perna* calculated using Kimura 2-parameter in MEGA5. The values should be multiplied with 100 to get the percentage values of variation. Standard error estimates are shown above the diagonal. Highest value of variation between *P. viridis* and *P. perna* is highlighted in yellow; highest value of variation between *P. viridis* and *P. canaliculus* is highlighted in orange.

Table 10: Pairwise distance calculated between the gene coding sequences of 16S rRNA belonging to *P. viridis, P.perna and P. canaliculus*

Sl. No.	Species	Accession No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*Perna viridis*	KT862016		0.000	0.002	0.003	0.002	0.013	0.004	0.003	0.020	0.020	0.020	0.021	0.020	0.020
2		KT862017	0.000		0.002	0.003	0.002	0.013	0.004	0.003	0.020	0.020	0.020	0.021	0.020	0.020
3		KT862018	0.002	0.002		0.002	0.003	0.013	0.003	0.002	0.020	0.020	0.020	0.020	0.020	0.020
4		KT862019	0.005	0.005	0.002		0.002	0.013	0.003	0.002	0.019	0.019	0.019	0.020	0.020	0.020
5		KT862020	0.002	0.002	0.005	0.002		0.013	0.004	0.003	0.020	0.020	0.020	0.020	0.020	0.020
6		AY262338	0.075	0.075	0.073	0.073	0.075		0.013	0.013	0.026	0.026	0.026	0.027	0.027	0.027
7		GQ472157	0.007	0.007	0.005	0.005	0.007	0.073		0.002	0.019	0.019	0.019	0.020	0.020	0.020
8		GQ472154	0.005	0.005	0.002	0.002	0.005	0.070	0.002		0.020	0.020	0.020	0.020	0.020	0.020
9	*Perna perna*	DQ923878	0.150	0.150	0.148	0.145	0.148	0.225	0.145	0.148		0.000	0.000	0.013	0.012	0.012
10		DQ923879	0.150	0.150	0.148	0.145	0.148	0.225	0.145	0.148	0.000		0.000	0.013	0.012	0.012
11		DQ923880	0.150	0.150	0.148	0.145	0.148	0.225	0.145	0.148	0.000	0.000		0.013	0.012	0.012
12	*Perna canaliculus*	AB265681	0.156	0.156	0.153	0.151	0.154	0.232	0.151	0.153	0.065	0.065	0.065		0.002	0.002
13		GU324135	0.153	0.153	0.151	0.148	0.151	0.229	0.148	0.151	0.063	0.063	0.063	0.002		0.000
14		U22886	0.153	0.153	0.151	0.148	0.151	0.229	0.148	0.151	0.063	0.063	0.063	0.002	0.000	

The estimates of evolutionary divergence between nucleotide sequences of the gene coding for 16S ribosomal RNA of different species of *Perna* calculated using Kimura 2-parameter in MEGA5. The values should be multiplied with 100 to get the percentage values of variation. Standard error estimates are shown above the diagonal. Highest value of variation between *P. viridis* and *P. perna* is highlighted in yellow; highest value of variation between *P. viridis* and *P. canaliculus* is highlighted in orange.

Table 11: Pairwise distance calculated between the gene coding sequences of 18S rRNA belonging to *P. viridis, P.perna and P. canaliculus*

Sl. No.	Species	Accession No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*Perna viridis*	KT862021		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.002	0.002	0.002	0.002
2		KT862022	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.002	0.002	0.002	0.002
3		KT862023	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.002	0.002	0.002	0.002
4		KT862024	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.002	0.002	0.002	0.002	0.002	0.002
5		KT862025	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.002	0.002	0.002	0.002	0.002	0.002
6		DQ640525	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.002	0.002	0.002	0.002	0.002	0.002
7		EF613234	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.002	0.002	0.002	0.002	0.002	0.002
8		DQ640528	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.002	0.002	0.002	0.002	0.002	0.002
9	*Perna perna*	DQ640520	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002		0.000	0.000	0.000	0.000	0.000
10		DQ640519	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000		0.000	0.000	0.000	0.000
11		DQ640517	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.000		0.000	0.000	0.000
12	*Perna canaliculus*	DQ640521	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.000		0.000	0.000
13		DQ640522	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000		0.000
14		DQ640523	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000	0.000	

The estimates of evolutionary divergence between nucleotide sequences of the gene coding for 18S ribosomal RNA of different species of *Perna* calculated using Kimura 2-parameter in MEGA5. The values should be multiplied with 100 to get the percentage values of variation. Standard error estimates are shown above the diagonal.

Table 12: Pairwise distance calculated between the gene coding sequences of ITS belonging to *P. viridis*, *P.perna and P. canaliculus*

Sl. No.	Species	Accession No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*Perna viridis*	KT862026		0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.017	0.017	0.017	0.016	0.016	0.016
2		KT862027	0.002		0.000	0.000	0.000	0.002	0.002	0.002	0.018	0.018	0.018	0.016	0.016	0.016
3		KT862028	0.002	0.000		0.000	0.000	0.002	0.002	0.002	0.018	0.018	0.018	0.016	0.016	0.016
4		KT862029	0.002	0.000	0.000		0.000	0.002	0.002	0.002	0.018	0.018	0.018	0.016	0.016	0.016
5		KT862030	0.002	0.000	0.000	0.000		0.002	0.002	0.002	0.018	0.018	0.018	0.016	0.016	0.016
6		KP892896	0.000	0.002	0.002	0.002	0.002		0.000	0.000	0.017	0.017	0.017	0.016	0.016	0.016
7		DQ924550	0.000	0.002	0.002	0.002	0.002	0.000		0.000	0.017	0.017	0.017	0.016	0.016	0.016
8		DQ924539	0.000	0.002	0.002	0.002	0.002	0.000	0.000		0.017	0.017	0.017	0.016	0.016	0.016
9	*Perna perna*	DQ924559	0.139	0.142	0.142	0.142	0.142	0.139	0.139	0.139		0.000	0.000	0.011	0.011	0.011
10		DQ924547	0.139	0.142	0.142	0.142	0.142	0.139	0.139	0.139	0.000		0.000	0.011	0.011	0.011
11		DQ924543	0.139	0.142	0.142	0.142	0.142	0.139	0.139	0.139	0.000	0.000		0.011	0.011	0.011
12	*Perna canaliculus*	DQ924553	0.118	0.120	0.120	0.120	0.120	0.118	0.118	0.118	0.062	0.062	0.062		0.000	0.000
13		DQ924552	0.118	0.120	0.120	0.120	0.120	0.118	0.118	0.118	0.062	0.062	0.062	0.000		0.000
14		DQ924551	0.118	0.120	0.120	0.120	0.120	0.118	0.118	0.118	0.062	0.062	0.062	0.000	0.000	

The estimates of evolutionary divergence between nucleotide sequences of the gene coding for 16S ribosomal RNA of different species of *Perna* calculated using Kimura 2-parameter in MEGA5. The values should be multiplied with 100 to get the percentage values of variation. Standard error estimates are shown above the diagonal. Highest value of variation between *P. viridis* and *P. perna* is highlighted in yellow; highest value of variation between *P. viridis* and *P. canaliculus is* highlighted in orange.

Table 13: Pairwise distance calculated for CO1 gene sequences between different populations of *Perna viridis*

Sl. No.	Location	Accession Number	1	2	3	4	5	6	7	8	9	10	11	12
1	Chennai	JF520793		0.003	0.003	0.002	0.002	0.002	0.002	0.004	0.002	0.002	0.002	0.002
2	Chennai	JF520790	0.005		0.003	0.002	0.002	0.003	0.003	0.004	0.003	0.003	0.003	0.002
3	Chennai	JF520792	0.007	0.005		0.003	0.003	0.003	0.003	0.004	0.003	0.003	0.003	0.003
4	Chennai	JF520791	0.002	0.003	0.005		0.000	0.002	0.002	0.003	0.002	0.002	0.002	0.000
5	Goa	JF520796	0.002	0.003	0.005	0.000		0.002	0.002	0.003	0.002	0.002	0.002	0.000
6	Goa	JF520797	0.003	0.005	0.007	0.002	0.002		0.002	0.004	0.002	0.000	0.002	0.002
7	Karwar	JF520799	0.003	0.005	0.007	0.002	0.002	0.003		0.004	0.002	0.002	0.002	0.002
8	Karwar	JF520801	0.008	0.010	0.012	0.007	0.007	0.008	0.008		0.003	0.004	0.004	0.003
9	Kollam	JF520802	0.003	0.005	0.007	0.002	0.002	0.003	0.003	0.005		0.002	0.002	0.002
10	Kollam	JF520803	0.003	0.005	0.007	0.002	0.002	0.000	0.003	0.008	0.003		0.002	0.002
11	Mangalore	JF520807	0.003	0.005	0.007	0.002	0.002	0.003	0.003	0.008	0.003	0.003		0.002
12	Mangalore	JF520805	0.002	0.003	0.005	0.000	0.000	0.002	0.002	0.007	0.002	0.002	0.002	
13	Calicut	JF520812	0.002	0.003	0.005	0.000	0.000	0.002	0.002	0.007	0.002	0.002	0.002	0.000
14	Calicut	JF520789	0.003	0.005	0.007	0.002	0.002	0.003	0.003	0.008	0.003	0.003	0.003	0.002
15	Onissa	JF520810	0.005	0.003	0.002	0.003	0.003	0.005	0.005	0.010	0.005	0.005	0.005	0.003
16	Onissa	JF520808	0.008	0.010	0.012	0.007	0.007	0.008	0.008	0.010	0.008	0.008	0.008	0.007
17	Onissa	JF520811	0.003	0.002	0.003	0.002	0.002	0.003	0.003	0.008	0.003	0.003	0.003	0.002
18	Chennai	DQ917612	0.003	0.002	0.003	0.002	0.002	0.003	0.003	0.008	0.003	0.003	0.003	0.002
19	Philippines	DQ917599	0.007	0.008	0.010	0.005	0.005	0.007	0.007	0.008	0.007	0.007	0.007	0.005
20	Thailand	DQ917590	0.010	0.012	0.013	0.008	0.008	0.010	0.010	0.012	0.010	0.010	0.010	0.008
21	Venezuela	DQ343590	0.012	0.013	0.015	0.010	0.010	0.012	0.012	0.013	0.012	0.012	0.012	0.010
22	USA	DQ343587	0.010	0.012	0.013	0.008	0.008	0.010	0.010	0.012	0.010	0.010	0.010	0.008
23	Australia	DQ343581	0.007	0.008	0.010	0.005	0.005	0.007	0.007	0.008	0.007	0.007	0.007	0.005

The estimates of evolutionary divergence between the CO1 sequences of different populations of *Perna viridis* calculated using Kimura 2-parameter in MEGA5. The values should be multiplied with 100 to get the percentage values of variation. Standard error estimates are shown above the diagonal. Highest value of intra-specific variation of *P. viridis* is highlighted in yellow.

Sl. No.	Location	Accession Number	13	14	15	16	17	18	19	20	21	22	23
1	Chennai	JF520793	0.002	0.002	0.003	0.004	0.002	0.002	0.003	0.004	0.004	0.004	0.003
2	Chennai	JF520790	0.002	0.003	0.002	0.004	0.002	0.002	0.004	0.004	0.005	0.005	0.004
3	Chennai	JF520792	0.003	0.003	0.002	0.004	0.002	0.002	0.004	0.005	0.005	0.005	0.004
4	Chennai	JF520791	0.000	0.002	0.002	0.003	0.002	0.002	0.003	0.004	0.004	0.004	0.003
5	Goa	JF520796	0.000	0.002	0.002	0.003	0.002	0.002	0.003	0.004	0.004	0.004	0.003
6	Goa	JF520797	0.002	0.002	0.003	0.004	0.002	0.002	0.003	0.004	0.004	0.004	0.003
7	Karwar	JF520799	0.002	0.002	0.003	0.004	0.002	0.002	0.003	0.004	0.004	0.004	0.003
8	Karwar	JF520801	0.003	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
9	Kollam	JF520802	0.002	0.002	0.003	0.003	0.002	0.002	0.003	0.004	0.004	0.004	0.003
10	Kollam	JF520803	0.002	0.002	0.003	0.004	0.002	0.002	0.003	0.004	0.004	0.004	0.003
11	Mangalore	JF520807	0.002	0.002	0.003	0.004	0.002	0.002	0.003	0.004	0.004	0.004	0.003
12	Mangalore	JF520805	0.000	0.002	0.002	0.003	0.002	0.002	0.003	0.004	0.004	0.004	0.003
13	Calicut	JF520812		0.002	0.002	0.003	0.002	0.002	0.003	0.004	0.004	0.004	0.003
14	Calicut	JF520789	0.002		0.003	0.004	0.002	0.002	0.003	0.004	0.004	0.004	0.003
15	Orissa	JF520810	0.003	0.005		0.004	0.001	0.001	0.004	0.004	0.005	0.004	0.004
16	Orissa	JF520808	0.007	0.008	0.010		0.004	0.004	0.002	0.003	0.003	0.003	0.002
17	Orissa	JF520811	0.002	0.003	0.002	0.008		0.000	0.003	0.004	0.004	0.004	0.003
18	Chennai	DQ917612	0.002	0.003	0.002	0.008	0.000		0.003	0.004	0.004	0.004	0.003
19	Philippines	DQ917599	0.005	0.007	0.008	0.002	0.007	0.007		0.002	0.003	0.002	0.000
20	Thailand	DQ917590	0.008	0.010	0.012	0.005	0.010	0.010	0.003		0.002	0.003	0.002
21	Venezuela	DQ343590	0.010	0.012	0.013	0.007	0.012	0.012	0.005	0.002		0.004	0.003
22	USA	DQ343587	0.008	0.010	0.012	0.005	0.010	0.010	0.003	0.007	0.008		0.002
23	Australia	DQ343581	0.005	0.007	0.008	0.002	0.007	0.007	0.000	0.003	0.005	0.003	

The estimates of evolutionary divergence between the CO1 sequences of different populations of *Perna viridis* calculated using Kimura 2-parameter in MEGA5. The values should be multiplied with 100 to get the percentage values of variation. Standard error estimates are shown above the diagonal. Highest value of intra-specific variation of *P. viridis* is highlighted in yellow.

4.4.2. Intra-Species Sequence Comparison

The CO1 gene sequence that showed higher percentage of inter-specific sequence divergence was selected for screening the intra-specific sequence divergence. The CO1 sequences of different populations of *P. viridis* from twelve different regions were used for calculating the intra-specific sequence divergence percentage using the pairwise distance calculation method in MEGA 5.2.2 (Table 13). The populations of *P. viridis* collected from seven different locations of Indian peninsula viz., Karwar, Goa, Mangalore, Calicut, Kollam, Chennai and Orissa, and five other international populations viz., Philippines, Venezuela, Thailand, USA and Australia were used in the analysis. This analysis revealed that the sequence divergence percentage between the twelve different populations of *P. viridis* is actually very low in value. The maximum sequence divergence in CO1 was only1.5% (between Chennai and Venezuela populations).This is indicative of the high degree of sequence conservation in *P. viridis* at the intra-species level. Therefore, the CO1 gene which showed higher percentage of inter-specific sequence divergence and higher degree of intra-specific sequence conservation was used for designing species specific DNA markers.

4.5. Species Specific PCR (SSPCR) Primers

Mitochondrial CO1 gene sequence characterized for each of the target species were used to design species specific PCR (SSPCR) primers for the respective species. Four pairs of SSPCR primers each were designed for *P. viridis* and *C. madrasensis* and three pairs for *Perna indica*. Details of these SSPCR primers and their respective position in the mitochondrial CO1 gene are given below in the Table 14 and in the nucleotide sequence illustrations 1, 2 and 3. As can be seen in the illustrations, all of the primer binding region are conserved within the species but are polymorphic between species.

Table 14: List of species specific PCR (SSPCR) primers designed for *P. viridis, P. indica* and *C. madraensis*

Primer Name	Primer Sequence (5$^{|}$ - 3$^{|}$)	T_a (^{0}C)	Product Size
Perna viridis			
PVCO1F265 PVCO1R539	GGCACCTAATGCTTTGTACT TTAAAAGAACACCGGTTACG	58	274 bp
PVCO1F140 PVCO1R345	GTTGTAGTAACAACTCATGCAT GTATGGTACAACCCAGAAGATA	52	205 bp
PVCO1F227 PVCO1R433	CTTCCATTATGTATTGGTGGTG ACCTAATAAAGAACTCAATCCA	48	206 bp
PVCO1F31 PVCO1R290	GTTAATGGGGAGAAGGCTTA TAAGTAAGTACAAAGCATTAGGTG	58	259 bp
Perna indica			
PICO1F284 PICO1R575	CTCCTAATGCTCTCTATTTAT AGAACAGGTACAGAAATAATC	50	291 bp
PICO1F168 PICO1R334	CCGTTGCTTATTGGGGCCTTTGGA GTTCAACCTGTACCAGCCCCC	58	166 bp
PICO1F270 PICO1R526	AGGGGGCTGGTACAGGTTGAACT TCCTCCAGCAAGAACAGGTACAGAA	56	257 bp

Crassostrea madrasensis			
CMCO1F66 CMCO1R315	GCTGAAGGCTGTATAACCCG GAATAAGTTGATAGCGGCGG	56	294 bp
CMCO1F77 CMCO1R315	TATAACCCGGGGGCTAAGTT GAATAAGTTGATAGCGGCGG	58	283 bp
CMCO1F76 CMCO1R409	GTATAACCCGGGGGCTAAGT AGCCAAATGCAGCCTTAAAA	56	334 bp
CMCO1F76 CMCO1R278	GTATAACCCGGGGGCTAAGT GTGAGCCTGGTAAAACCCAA	56	203 bp

4.5.1. Specificity PCR assay

The specificity PCR assay was conducted to identify the suitable SSPCR primer pair for each target species from among the multiple primer pairs designed (Table 9). The specificity of the SSPCR primers were compared using the Agarose Gel Electrophoresis (AGE) images of the PCR amplicons produced by each SSPCR primer pair against other species included in specificity PCR assay. The SSPCR primer pair which specifically amplified the target species DNA alone was considered as the most specific pair and was selected for further screening tests.

Out of the four pairs of SSPCR primers designed for *Perna viridis,* PVCO1F265 & PVCO1R539 were found to be the most specific PCR primer combination for the species. This primer pair produced 274 bp PCR amplicon only with *P. viridis* DNA at an annealing temperature of 58^0C. The positive control PCR set with the PCR primer for 18SrRNA resulted in 450 bp PCR amplicon, which indicated that there was no PCR inhibition occurring in the assay conducted. Result of this specificity PCR assay is shown in the figure 13.

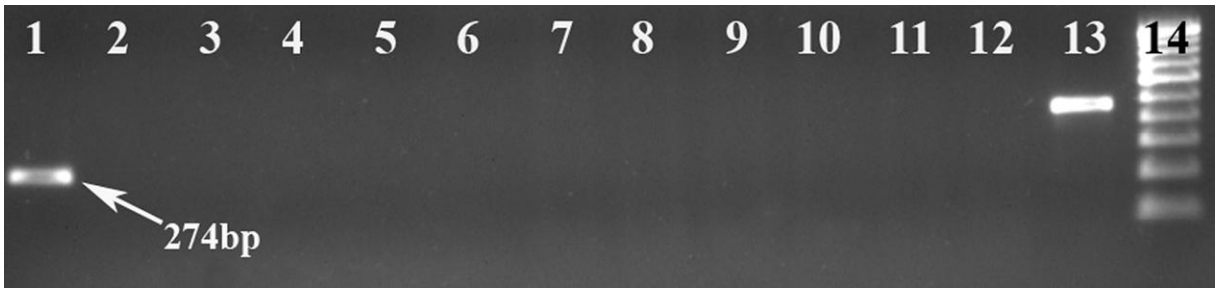
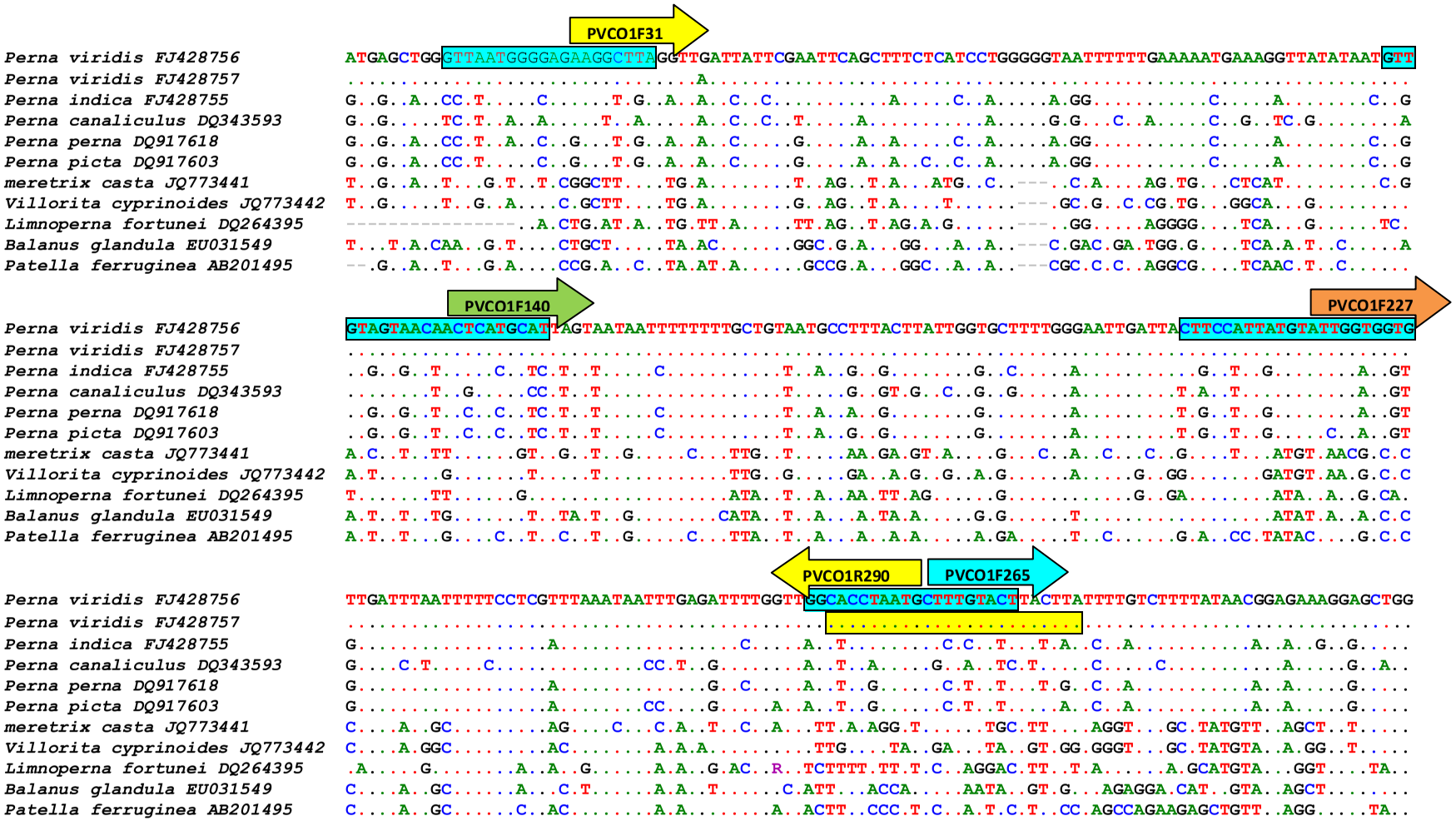


Figure 13: Agarose gel image showing the PCR products of specificity PCR assay conducted with *Perna viridis* specific PCR primers PVCO1F265 & PVCO1R539. Lane 1: *Perna viridis,* Lane 2: *Perna indica,* Lane 3: *Paphia malabarica,* Lane 4: *Meritrix casta,* Lane 5: *Sunnetta scripta,* Lane 6: *Villorita cyprinoides,* Lane 7: *Saccostrea cucculata,* Lane 8: *Crassostrea madrasensis,* Lane 9: *Pinctada fucata,* Lane 10: *Patella sp*., Lane 11: *Balanus sp.,* Lane 12: *Parapenaeopsis stylifera,* Lane 13: 18SrRNA positive control, Lane 14: 100 bp DNA ladder

Illustrations of the aligned CO1 nucleotide sequences of *Perna viridis*, *Perna indica*, *Crassostrea madrasensis* and other related species showing the respective positions of SSPCR primers

Illustration 1: A comparison of the partial coding region of Cytochrome Oxidase c Subunit 1 (CO1) of different bivalve species showing the corresponding positions of each SSPCR primers designed for *Perna viridis*. A dot in the illustration represents the conserved nucleotide position of the above sequence. The position of each primer pair is marked with block arrows in a particular colour code

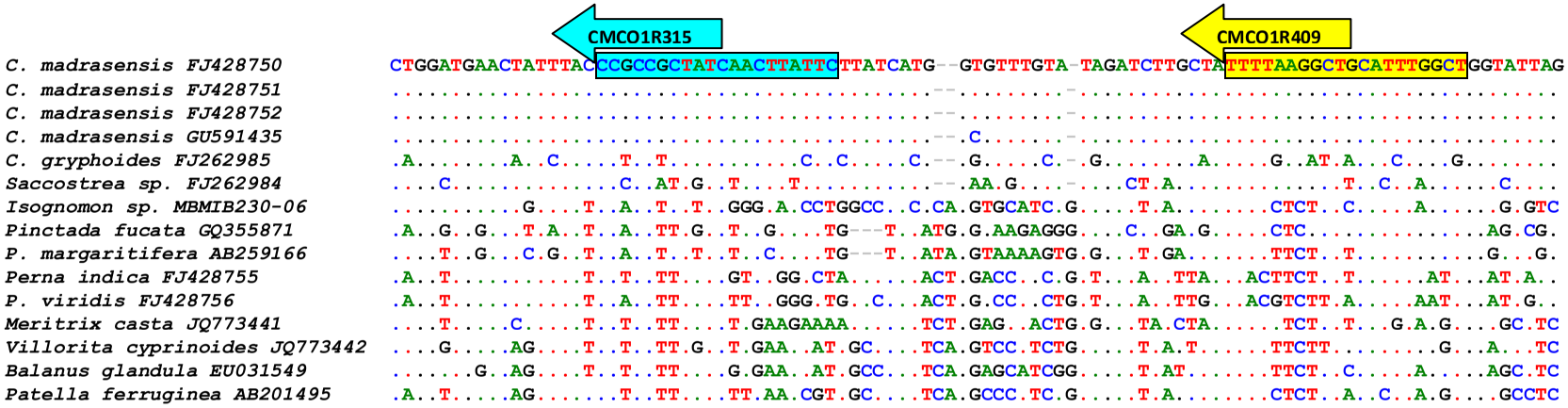


PVCO1R345
PVCO1R433
PVCO1R433
PVCO1R539
Perna viridis FJ428756
Perna viridis FJ428757
Perna indica FJ428755
Perna canaliculus DQ343593
Perna perna DQ917618
Perna picta DQ917603
meretrix casta JQ773441
Villorita cyprinoides JQ773442
Limnoperna fortunei DQ264395
Balanus glandula EU031549
Patella ferruginea AB201495

Illustration 2: A comparison of the partial coding region of Cytochrome Oxidase c Subunit 1 (CO1) of different bivalve species showing the corresponding positions of each SSPCR primers designed for *Perna indica*. A dot in the illustration represents the conserved nucleotide position of the above sequence. The position of each primer pair is marked with block arrows in a particular colour code.

Perna indica FJ428754
Perna indica FJ428755
Perna perna DQ917618
Perna picta DQ917603
Perna viridis FJ428756
Perna canaliculus DQ343593
meretrix casta JQ773441
Villorita cyprinoides JQ773442
Limnoperna fortunei DQ264395
Balanus glandula EU031549
Patella ferruginea AB201495
PICO1R575
PICO1R526

Illustration 3: A comparison of the partial coding region of Cytochrome Oxidase c Subunit 1 (CO1) of different bivalve species showing the corresponding positions of each SSPCR primers designed for *Crassostrea madrasensis*. A dot in the illustration represents the conserved nucleotide position of the above sequence. The position of each primer pair is marked with block arrows in a particular colour code.



Among the three pairs of SSPCR primers designed for *Perna indica*, PICO1F284 and PICO1R575 were found to be the most specific primer combination for the species. This primer pair produced 291bp PCR amplicon with *P. indica* DNA only at an annealing temperature of 50^0C. There was no PCR inhibition occurring which was evident from the 18SrRNA positive control PCR set in the assay. Result of this specificity PCR assay is shown in the figure 14.

Figure 14: Agarose gel image showing the PCR products of specificity PCR assay conducted using the *Perna indica* specific PCR primers PICO1F284 and PICO1R575. Lane 1: *Perna indica*, Lane 2: *Perna viridis*, Lane 3: *Sunnetta scripta*, Lane 4: *Paphia malabarica*, Lane 5: *Meritrix casta*, Lane 6: *Villorita cyprinoides*, Lane 7: *Crassostrea madrasensis*, Lane 8: *Saccostrea cucculata*, Lane 9: *Pinctada fucata*, Lane 10: *Balanus sp.*, Lane 11: *Patella sp.*, Lane 12: *Parapenaeopsis stylifera*, Lane 13: positive control, Lane 14: 100 bp DNA ladder

Out of the four pairs of SSPCR primers designed for *Crassostrea madrasensis,* CMCO1F66 and CMCO1R315 were found to be the most specific PCR primer combination for the species. This primer pair produced 294 bp PCR amplicon only with *C. madrasensis* DNA at an annealing temperature of 58^0C. No PCR inhibition occurred in assay which is evident through 18SrRNA positive control PCR set in the assay. Result of this specificity PCR assay is shown in the figure 15.

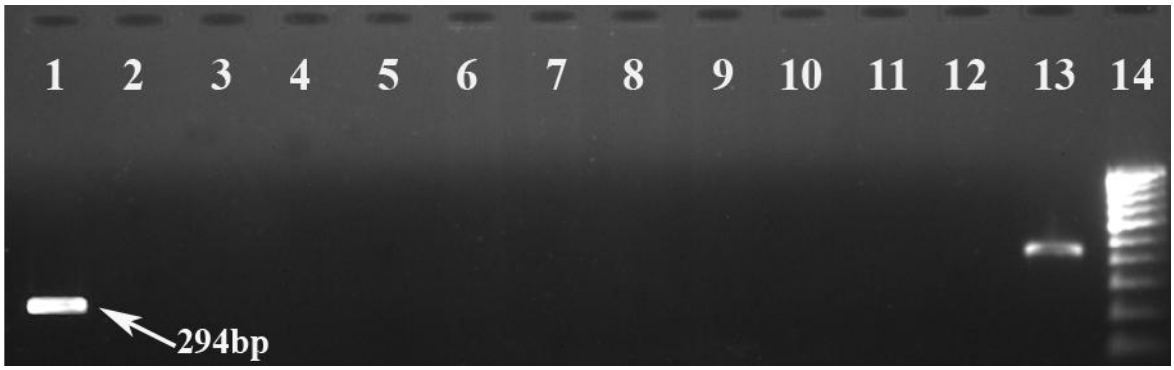


Figure 15: Agarose Gel image showing the PCR products of specificity PCR assay conducted for *Crassostrea madrasensis* specific PCR primers, CMCO1 F66 & CMCO1 R315. Lane 1*: Crassostrea madrasensis,* Lane 2: *Saccostrea cucculata, Lane* 3: *Pinctada fucata,* Lane 4: *Pinctada margaritifera,* Lane 5: *Isognomon ephippium*, Lane 6: *Paphia malabarica,* Lane 7: *Meritrix casta,* Lane 8: *Villorita cyprinoides,* Lane 9: *Perna viridis,* Lane 10: *Perna indica*., Lane 11: *Patella sp.,* Lane 12: *Balanus sp.,* Lane 13: positive control, Lane 14: 100 bp DNA ladder

Comparison of the sequence of the selected SSPCR primers of *P. viridis* with that of the *P. viridis* populations from different continents that are available in GenBank has proved that the primer binding region is highly conserved within in the species with negligible number of nucleotide polymorphisms (Table 15). Distant populations are not yet reported in the case of *P. indica* and *C. madrasensis.* Consistency of the amplification by the selected SSPCR primers in each target species were empirically validated by carrying out PCR amplification of 96 DNA samples of each species, collected from different maritime states of India (Table 1). All of these tests conducted have produced positive results indicating that the primer binding region is highly conserved within the species, and ruled-out the possibility of false negative amplification. These SSPCR primers were used for further screening of the respective target species in the field collected plankton samples.

4.6. Species specific *nested* PCR (SS*n*PCR)

Nested PCR system can increase the sensitivity level of the larval detection when the field collected plankton samples have very lower number of target species larvae. In the present study, a few plankton samples which had low number of target larvae (Exa: THA1102 in the Table 16 & 17), showed false negative results when SSPCR was used for larval detection from the total plankton DNA.

Concentration of the SSPCR products generated with these DNA samples were too low to be detected in ordinary Agarose Gel Electrophoresis (AGE). In order to overcome this issue, nested PCR systems were designed for each target species that can detect the presence of very low number of target larvae in the whole plankton sample. The species specific *nested* PCR (SS*n*PCR) consisted of two steps, the first step is carried out with the universal PCR primers to amplify the partial coding region of mitochondrial CO1 gene, and the second step is done with the SSPCR primers which are internal to the product of first reaction. In addition, sequencing of the first step PCR product with original amplification primers was done to ascertain the false positive result.

Table 15: A comparison of SSPCR primer sequence of *P. viridis* populations from various locations

GenBank Accession No.& Place of origin.	PVCO1F265 5′GGCACCTAATGCTTTGTACT 3′	PVCO1R539 5′TTAAAAGAACACCGGTTACG 3′
JN179078 India	GGCACCTAATGCTTTGTACT	TTAAAAGAACACCGGTTACG
JF520801 Karwar-India	....	....
JF520796 Goa-India	....	....
JF520807 Mangalore-India	....	....
JF520812 Calicut-India	....	....
JF520802 Kollam-India	....	....
JF520793 Chennai-India	....	...................A
JF520811 Orissa-India	....	...................A
JN179066 Singapore	....	....
JN179062 Hongkong	................T.	....
JN179053 Florida	....	....
GQ497838 Jamaica	....	.............A......
GQ497837 Tampa Bay	....	.............A......
GQ497835 Trinidad	....	....
DQ917599 Philippines	....	....
DQ917590 Thailand	....	....
DQ343590 Venezuela	....	....
DQ343587 USA	....	....
DQ343581 Australia	....	....
DQ343573 Hongkong	....	....

A dot in the parenthesis indicates the conserved nucleotide status of the above sequence

4.6.1. SS*n*PCR with simulated plankton sample

The reaction conditions for SS*n*PCR were standardized using a simulated plankton sample for obtaining optimum result. A plankton sample collected prior to the normal spawning season was simulated to a real-time plankton sample with known number of bivalve larvae. This was done by adding larvae (48 hpf) of all the three species to a 40 mg aliquot of the plankton and then carrying out SS*n*PCR for the standardization of the same. As the primers used in first step SS*n*PCR are universal in nature there is a possibility for the augmentation of mitochondrial CO1 gene of many planktonic invertebrates along with *P. viridis*. Higher concentrations of this PCR amplicons may cause template inhibition if they are used directly for the second step PCR. Hence, the PCR product obtained in the first step PCR was diluted appropriately to prepare DNA template for the second step PCR. Result of the above amplification trials revealed that dilutions between $1/8^{th}$ and $1/12^{th}$ do have reasonable DNA concentration that will in turn

produce a detectable level of amplification in the second step nested PCR. First step PCR product with the dilution rate below and beyond this range causes reduced PCR amplification at the second step nested PCR. The $1/10^{th}$ dilution of the first PCR product worked fairly in all the tests conducted, and produced optimum results. Hence, in all the experiments and tests conducted with SS*n*PCR the first step PCR product was diluted to $1/10^{th}$ dilution, and template for the second step nested PCR was taken from it.

4.6.2. Sensitivity PCR assay

The minimum concentration of target DNA required in a pool of plankton DNA to produce an observable PCR amplicon by the SSPCR and also in SS*n*PCR system was arrived using sensitivity PCR assay. The experimental plankton DNA samples which contains varying concentrations of target DNA ranging from 1 pico gram to 250 nano gram (Table 6) were subjected to sensitivity PCR assay. Results of the assay with the DNA of the same plankton samples showed that a minimum concentration of 2 ng/µl and 0.1 ng/µl target DNA are required for the SSPCR and SS*n*PCR test respectively in order to bring out an observable PCR amplicon.

Minimum number of larvae of the target species required in a plankton sample to produce an observable PCR amplicon by the SSPCR and SS*n*PCR system was also worked out through the sensitivity PCR assay. The assay set with 10 numbers of plankton aliquots each containing different numbers of veliger larvae of the target species were subjected to SSPCR and SS*n*PCR. The results showed that the SSPCR test can detect a minimum of 20 numbers of veliger larvae (48 hpf) in a plankton mix of 40mg (Figure 16), and in the same way the SS*n*PCR can detect even a single veliger larvae from a plankton mix of 40mg (Figure 17).

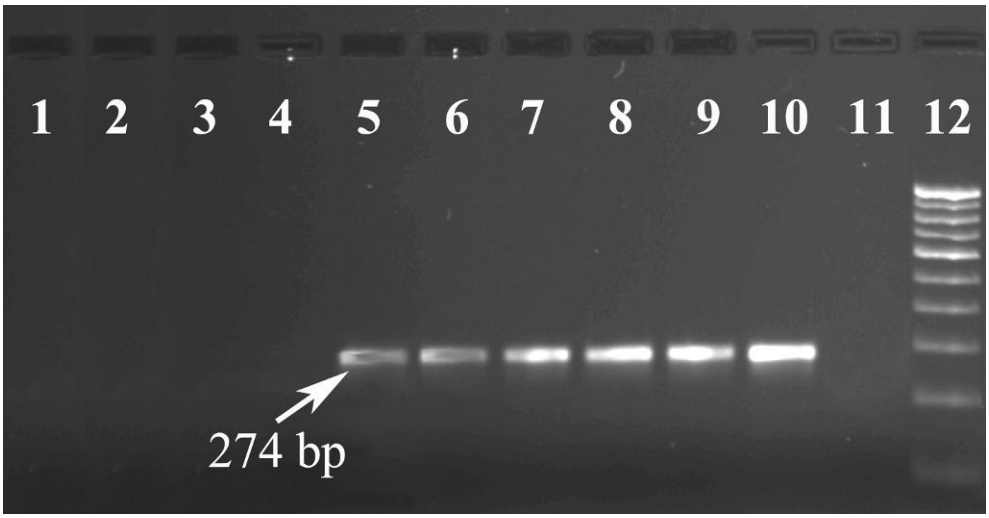


Figure 16: Agarose gel image showing the SSPCR products of sensitivity PCR assay in which different numbers of *P. viridis* larvae were added to ~40mg of plankton tissue. Lane1: 1 larva, Lane2: 5 larvae, Lane 3: 10 larvae, Lane 4:15 larvae, Lane 5: 20 larvae, Lane 6: 25 larvae, Lane 7: 30 larvae, Lane8: 35 larvae, Lane 9: 40 larvae, Lane 10: positive control set with *P. viridis* DNA, Lane 11: negative control with no DNA and Lane12: 100bp DNA ladder.

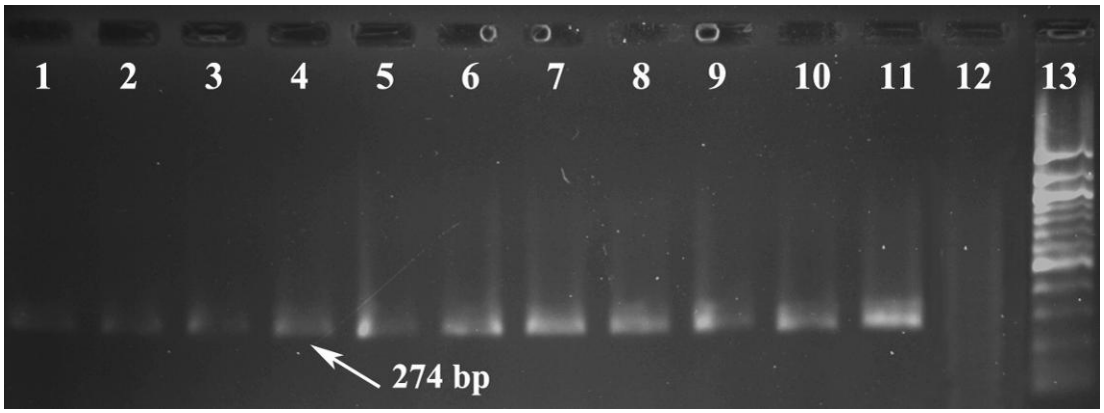


Figure 17: Agarose gel image showing the II[nd] step SS*n*PCR products of sensitivity PCR assay in which different numbers of *P.viridis* larvae were added to ~40mg of plankton tissue. Lane1: 1 larva, Lane2: 2 larvae, Lane3:3 larvae, Lane4: 4 larvae, Lane5: 5 larvae, Lane 6: 10 larvae, Lane7: 15 larvae, Lane8: 20 larvae, Lane 9: 25 larvae, Lane10: 30 larvae, Lane11: positive control set with *P. viridis* DNA, Lane12: negative control with no DNA and Lane13: 100bp DNA ladder.

4.7. Plankton screening

The Results of the SSPCR tests conducted to identify the individual larva of *P. viridis, P .indica* and *C. madrasensis* sorted from the plankton samples are shown in the figures 18, 19 and 20. The ethanol preserved whole plankton samples were subjected to screening for the presence of larvae of the target species through SSPCR and SS*n*PCR. In order to get results with less error, SSPCR and SS*n*PCR tests were conducted with five different aliquots from every plankton sample. The number of positive results obtained from five different aliquots shows the percentage of representation of larvae in a unit volume of preserved plankton sample. Graphical interpretation of the results are made (Fig. 21 - 26) based on the season of collection and percentage representation of the bivalve larvae in each preserved sample.

4.7.1. SSPCR with individual larva sorted from planktons

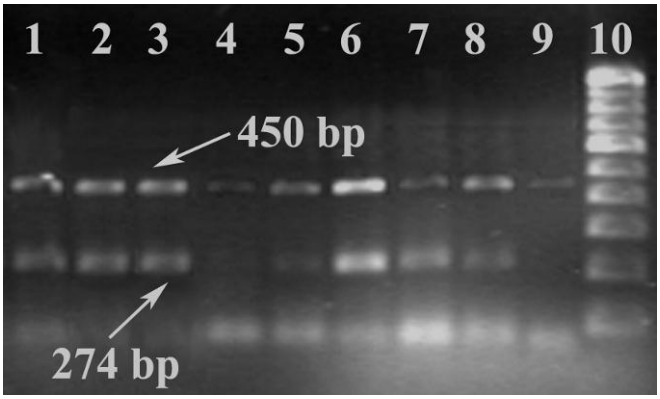


Figure 18: Image showing the PCR products of SSPCR conducted with the veliger larvae isolated from the plankton sample THA1205 collected from Thankassery Bay using the *P. viridis* specific primers. Lane 1 to 9: PCR amplicons of 18S rRNA positive control PCR (450 bp) and the PCR amplicons of SSPCR (274 bp) loaded in the same lane, Lane 10: 100 bp DNA ladder.

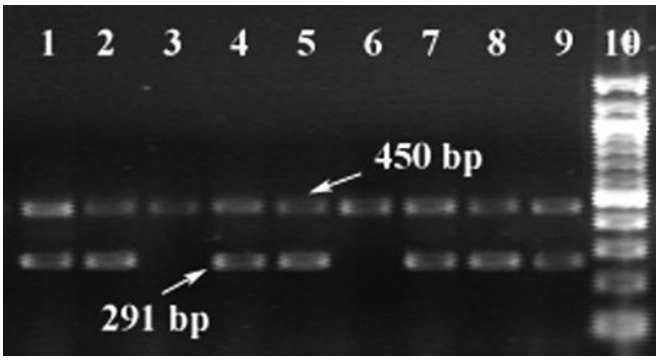


Figure 19: Image showing the PCR products of SSPCR conducted with the veliger larvae isolated from the plankton sample THA1205 collected from Thankassery Bay using the *P. indica* specific primers. Lane 1 to 9: PCR amplicons of 18S rRNA positive control PCR (450 bp) and the PCR amplicons of SSPCR (291 bp) loaded in the same lane, Lane 10: 100 bp DNA ladder.

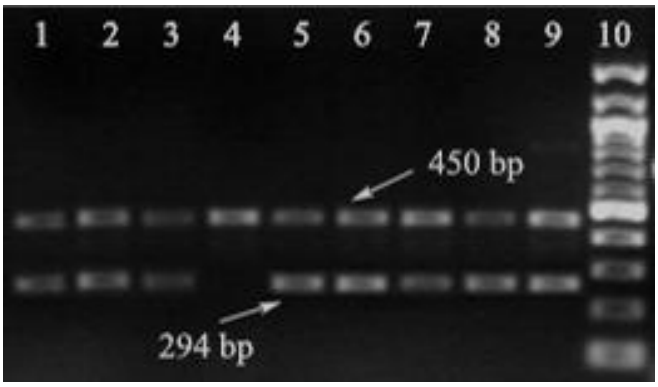


Figure 20: Image showing the PCR products of SSPCR conducted with the veliger larvae isolated from the plankton sample SAT1205 collected from Moothakunnam estuary using the *C. madrasensis* specific primers. Lane 1 to 9: PCR amplicons of 18S rRNA positive control PCR (450 bp) and the PCR amplicons of SSPCR (294 bp) loaded in the same lane, Lane 10: 100 bp DNA ladder.

Result of SSPCR tests for each target species with the veliger larva sorted from the plankton samples THA1205 and SAT1205 clearly identifies the species of the larva. It is evident from the size of the PCR products which agree with the expected size of corresponding species. Irrespective of the multiple species representation of the sorted veliger larvae from the plankton samples, only the DNA of target species larvae produced specified size PCR product and the DNA of the larvae of non target species did not produce any specific PCR product. SSPCR using the *P. viridis* specific primers with the DNA of the veliger larvae isolated from the plankton sample produced 274 bp PCR products except for the samples 4 & 9, as shown in the Figure 18. SSPCR using the *P. indica* specific primers with the DNA of the veliger larvae isolated from the plankton sample produced 291 bp PCR products except for the samples 3 & 6, as shown in the Figure 19. Similarly, SSPCR using the *C. madrasensis* specific primers with the DNA of the veliger larvae isolated from the plankton sample produced 294 bp PCR products except for the sample 4, as shown in the Figure 20. The positive control set with all the tests produced PCR product of the expected size (450 bp), indicating that there has been no PCR inhibition or lower concentration of target DNA even in the cases of SSPCR tests with negative results.

4.7.2. SS*n*PCR with plankton samples

The results of SS*n*PCR conducted with the wild collected plankton samples containing random numbers of target species larvae are provided in the Tables 16 and 18. In most cases the first step PCR is

positive as the primer used in first step PCR is universal and it amplifies the DNA of multiple bivalve species. In the case of second step nested PCR, only the sample having the target DNA produce a positive result. The SS*n*PCR could detect even a single veliger larvae in a plankton biomass of 40mg as evident from the sensitivity PCR assay conducted before. In order to get results with less error, SS*n*PCR tests were conducted with five different aliquots from every plankton sample. Analysis of the results of SS*n*PCR with plankton samples collected from Thankassery Bay shows the presence of *P. viridis* larvae from May second week to July first week in 2011 and 2012 (Fig. 21&22). Similarly, as evident in the SS*n*PCR results given in the Table 16 the presence of *Perna indica* could be noticed in the water from the first week of May until the first week of July in 2011 (Fig. 23) and from the last week of May to the second week of June in 2012 (Fig. 24). Analysis of the results of SS*n*PCR with plankton samples collected from Azhikode Estuary shows the occurrence of *C. madrasensis* larvae in the third week of September until the second week of December 2011 (Table 18 & Fig. 25). But, the continuous larval detection in all of the plankton samples is evident only in the plankton samples collected during the first three weeks of October 2011. After this period the larval detection in the plankton samples becomes irregular, indicating the decreased or the lower numerical density of *C. madrsensis* larvae in the water body. SS*n*PCR with the plankton samples collected during 2012 shows the presence of *C. madrsensis* larvae from the second week of September until the second week of December (Fig. 26) with a similar trend of occurrence as in the previous year.

4.7.3. SSPCR with plankton samples

SSPCR was conducted with the wild collected plankton samples which contain random numbers of target species larvae. As evident from the sensitivity PCR assay conducted before, there is the requirement of a threshold number of target species larvae in the field collected plankton sample in order to produce an observable PCR amplicon. In order to get results with lesser error, SSPCR tests were conducted with five different aliquots from every plankton sample and the results are provided in the Tables 17 and 19.

Analysis of the results of SSPCR with plankton samples collected from Thankassery Bay shows the presence of *P. viridis* larvae during the initial three weeks of June in 2011 and 2012 (Fig. 21&22). Similarly, as evident in the SSPCR results given in the Table 17, the presence of *Perna indica* could be noticed in the water from the last week of May until the third week of June in 2011(Fig. 23) and from the last week of May to the second week of June in 2012 (Fig. 24). Analysis of the results of SSPCR with whole plankton samples collected from Azhikode Estuary shows the presence of *C. madrasensis* larvae during the initial three weeks of October in 2011 and 2012 (Table 19; Fig. 25&26).

Table 16: SS*n*PCR of plankton samples collected from Thankassery Bay for the detection of the larvae of *Perna viridis* and *Perna indica* during May to August of 2011 & 2012 respectively

Sl. No	Plankton sample code	Season of Collection	Wet weight of Plankton (in mg)	Dry weight of Plankton (in mg)	Sub – Sample Number	Positive control PCR (18SrRNA)	*Perna viridis*			*Perna indica*		
							Ist step PCR (CO1)	**Nested PCR** (species specific)	**Result** (presence/ absence of larvae)	**Ist step** PCR (CO1)	**Nested PCR** (species specific)	**Result** (presence/ absence of larvae)
1	THA1101	May 2011	32.11	08.56	I	+	+	-	Absent	+	-	Absent
2	"	"	40.55	11.00	II	+	+	-	Absent	+	-	Absent
3	"	"	36.55	09.95	III	+	+	-	Absent	+	+	Present
4	"	"	40.11	10.26	IV	+	+	-	Absent	+	+	Present
5	"	"	36.54	09.24	V	+	+	-	Absent	+	-	Absent
6	THA1102	May 2011	40.20	10.10	I	+	+	-	Absent	+	-	Absent
7	"	"	40.11	10.54	II	+	+	+	Present	+	+	Present
8	"	"	36.44	09.74	III	+	+	-	Absent	+	-	Absent
9	"	"	32.54	08.45	IV	+	+	-	Absent	+	+	Present
10	"	"	36.22	09.25	V	+	+	-	Absent	+	-	Absent
11	THA1103	May 2011	36.79	09.78	I	+	+	-	Absent	+	+	Present
12	"	"	41.01	10.56	II	+	+	+	Present	+	+	Present
13	"	"	37.21	09.97	III	+	+	+	Present	+	+	Present
14	"	"	40.14	10.30	IV	+	+	-	Absent	+	+	Present
15	"	"	40.22	10.01	V	+	+	+	Present	+	+	Present
16	THA1104	June 2011	28.45	07.21	I	+	+	+	Present	+	+	Present
17	"	"	20.47	05.21	II	+	-	+	Present	-	+	Present
18	"	"	32.44	06.67	III	+	+	+	Present	+	+	Present
19	"	"	33.41	06.81	IV	+	+	+	Present	+	+	Present
20	"	"	20.45	05.61	V	+	+	+	Present	+	+	Present
21	THA1105	June 2011	40.17	10.21	I	+	+	+	Present	+	+	Present
22	"	"	44.65	11.54	II	+	+	+	Present	+	+	Present
23	"	"	40.55	10.10	III	+	+	+	Present	+	+	Present
24	"	"	44.32	11.61	IV	+	+	+	Present	+	+	Present
25	"	"	40.14	10.47	V	+	+	+	Present	+	+	Present
26	THA1106	June 2011	36.71	09.56	I	+	+	+	Present	+	+	Present
27	"	"	37.22	09.01	II	+	+	+	Present	+	+	Present
28	"	"	40.81	10.20	III	+	+	+	Present	+	+	Present
29	"	"	37.55	09.75	IV	+	+	+	Present	+	+	Present
30	"	"	37.51	09.11	V	+	-	+	Present	-	+	Present
31	THA1107	June 2011	40.64	10.98	I	+	+	+	Present	+	+	Present
32	"	"	48.24	13.41	II	+	+	+	Present	+	-	Absent
33	"	"	49.52	12.87	III	+	+	-	Absent	+	+	Present
34	"	"	49.71	13.01	IV	+	+	+	Present	+	+	Present
35	"	"	48.61	12.99	V	+	+	-	Absent	+	-	Absent
36	THA1108	July 2011	37.11	09.95	I	+	+	-	Absent	+	+	Present
37	"	"	40.24	10.14	II	+	+	+	Present	+	+	Present
38	"	"	40.81	10.30	III	+	+	+	Present	+	-	Absent
39	"	"	36.22	09.99	IV	+	+	-	Absent	+	-	Absent
40	"	"	48.44	12.89	V	+	+	-	Absent	+	+	Present
41	THA1109	July 2011	24.88	08.10	I	+	+	-	Absent	+	-	Absent

42	"	"	28.41	07.32	II	+	+	-	Absent	+	-	Absent
43	"	"	29.17	07.55	III	+	+	-	Absent	+	-	Absent
44	"	"	34.00	08.54	IV	+	+	-	Absent	+	-	Absent
45	"	"	25.12	06.71	V	+	+	-	Absent	+	-	Absent
46	THA1110	July 2011	39.21	09.98	I	+	+	-	Absent	+	-	Absent
47	"	"	40.11	10.11	II	+	+	-	Absent	+	-	Absent
48	"	"	39.21	09.57	III	+	+	-	Absent	+	-	Absent
49	"	"	40.01	10.20	IV	+	+	-	Absent	+	-	Absent
50	"	"	39.88	10.38	V	+	+	-	Absent	+	-	Absent
51	THA1111	July 2011	41.27	10.96	I	+	+	-	Absent	+	-	Absent
52	"	"	44.27	11.41	II	+	+	-	Absent	+	-	Absent
53	"	"	43.81	11.05	III	+	+	-	Absent	+	-	Absent
54	"	"	40.72	10.47	IV	+	+	-	Absent	+	-	Absent
55	"	"	40.61	10.24	V	+	+	-	Absent	+	-	Absent
56	THA1112	Aug 2011	24.11	06.01	I	+	-	-	Absent	-	-	Absent
57	"	"	20.41	05.14	II	+	+	-	Absent	+	-	Absent
58	"	"	20.44	05.30	III	+	+	-	Absent	+	-	Absent
59	"	"	21.31	05.51	IV	+	+	-	Absent	+	-	Absent
60	"	"	22.01	05.91	V	+	+	-	Absent	+	-	Absent
61	THA1113	Aug 2011	20.84	05.01	I	+	+	-	Absent	+	-	Absent
62	"	"	21.09	05.41	II	+	+	-	Absent	+	-	Absent
63	"	"	17.52	04.21	III	+	+	-	Absent	+	-	Absent
64	"	"	20.47	05.22	IV	+	+	-	Absent	+	-	Absent
65	"	"	17.67	04.54	V	+	-	-	Absent	-	-	Absent
66	THA1114	Aug 2011	12.73	03.11	I	+	+	-	Absent	+	-	Absent
67	"	"	12.55	03.32	II	+	+	-	Absent	+	-	Absent
68	"	"	21.04	05.21	III	+	+	-	Absent	+	-	Absent
69	"	"	21.17	05.55	IV	+	+	-	Absent	+	-	Absent
70	"	"	20.49	04.41	V	+	-	-	Absent	-	-	Absent
71	THA1201	May 2012	40.51	10.41	I	+	+	-	Absent	+	-	Absent
72	"	"	40.92	10.10	II	+	+	-	Absent	+	-	Absent
73	"	"	36.44	09.65	III	+	+	-	Absent	+	+	Present
74	"	"	36.14	09.98	IV	+	+	-	Absent	+	-	Absent
75	"	"	40.77	10.10	V	+	+	-	Absent	+	-	Absent
76	THA1202	May 2012	43.94	11.00	I	+	+	-	Absent	+	+	Present
77	"	"	45.04	11.35	II	+	+	+	Present	+	-	Absent
78	"	"	40.85	10.47	III	+	+	+	Present	+	+	Present
79	"	"	40.64	10.44	IV	+	+	-	Absent	+	+	Present
80	"	"	40.17	10.98	V	+	+	-	Absent	+	-	Absent
81	THA1203	May 2012	40.66	10.78	I	+	-	+	Present	-	+	Present
82	"	"	44.27	11.20	II	+	+	+	Present	+	+	Present
83	"	"	44.62	11.54	III	+	+	-	Absent	+	+	Present
84	"	"	42.92	10.65	IV	+	+	+	Present	+	+	Present
85	"	"	43.98	11.28	V	+	+	-	Absent	+	+	Present
86	THA1204	June 2012	41.82	10.41	I	+	-	+	Present	-	+	Present
87	"	"	42.00	10.54	II	+	+	+	Present	+	+	Present
88	"	"	40.55	10.21	III	+	-	+	Present	-	+	Present

89	"	"	41.21	10.68	IV	+	+	+	Present	+	+	Present
90	"	"	44.10	11.47	V	+	-	+	Present	-	+	Present
91	THA1205	June 2012	21.33	05.36	I	+	+	+	Present	+	+	Present
92	"	"	21.74	05.88	II	+	+	+	Present	+	+	Absent
93	"	"	25.17	06.94	III	+	+	+	Present	+	+	Present
94	"	"	17.21	04.88	IV	+	+	+	Present	+	+	Present
95	"	"	20.41	05.21	V	+	+	+	Present	+	+	Present
96	THA1206	June 2012	32.44	08.65	I	+	+	+	Absent	+	-	Absent
97	"	"	32.66	08.14	II	+	-	+	Present	-	+	Present
98	"	"	30.85	07.88	III	+	+	+	Present	+	-	Absent
99	"	"	36.41	09.00	IV	+	+	+	Present	+	-	Absent
100	"	"	36.55	09.11	V	+	+	+	Present	+	-	Absent
101	THA1207	June 2012	40.77	10.21	I	+	+	+	Present	+	+	Present
102	"	"	40.61	10.54	II	+	-	+	Present	-	-	Absent
103	"	"	39.57	09.88	III	+	+	-	Absent	+	+	Present
104	"	"	39.89	09.30	IV	+	+	-	Absent	+	-	Absent
105	"	"	33.41	08.30	V	+	+	+	Present	+	-	Absent
106	THA1208	July 2012	12.11	03.41	I	+	-	-	Absent	-	-	Absent
107	"	"	12.41	03.01	II	+	-	+	Present	-	-	Absent
108	"	"	16.77	04.21	III	+	+	-	Absent	+	-	Absent
109	"	"	13.51	03.30	IV	+	+	+	Present	+	-	Absent
110	"	"	12.82	03.41	V	+	-	-	Absent	-	-	Absent
111	THA1209	July 2012	20.44	05.01	I	+	+	-	Absent	+	-	Absent
112	"	"	16.83	04.21	II	+	-	-	Absent	-	-	Absent
113	"	"	16.41	04.10	III	+	+	-	Absent	+	-	Absent
114	"	"	15.89	04.32	IV	+	+	-	Absent	+	-	Absent
115	"	"	16.47	04.50	V	+	+	-	Absent	+	-	Absent
116	THA1210	July 2012	20.41	05.00	I	+	+	-	Absent	+	-	Absent
117	"	"	16.22	04.10	II	+	+	-	Absent	+	-	Absent
118	"	"	16.22	04.11	III	+	+	-	Absent	+	-	Absent
119	"	"	20.72	05.47	IV	+	+	-	Absent	+	-	Absent
120	"	"	13.51	03.42	V	+	+	-	Absent	+	-	Absent
121	THA1211	July 2012	12.57	03.74	I	+	+	-	Absent	+	-	Absent
122	"	"	13.04	03.16	II	+	-	-	Absent	-	-	Absent
123	"	"	16.41	04.10	III	+	+	-	Absent	+	-	Absent
124	"	"	16.72	04.21	IV	+	+	-	Absent	+	-	Absent
125	"	"	13.21	03.22	V	+	-	-	Absent	-	-	Absent
126	THA1212	Aug 2012	16.42	04.14	I	+	-	-	Absent	-	-	Absent
127	"	"	13.20	03.98	II	+	+	-	Absent	+	-	Absent
128	"	"	16.12	04.14	III	+	+	-	Absent	+	-	Absent
129	"	"	17.52	04.89	IV	+	+	-	Absent	+	-	Absent
130	"	"	12.05	03.99	V	+	-	-	Absent	-	-	Absent
131	THA1213	Aug 2012	12.84	03.21	I	+	-	-	Absent	-	-	Absent
132	"	"	12.62	03.47	II	+	+	-	Absent	+	-	Absent
133	"	"	12.14	03.28	III	+	+	-	Absent	+	-	Absent
134	"	"	16.42	04.14	IV	+	+	-	Absent	+	-	Absent
135	"	"	12.82	03.24	V	+	-	-	Absent	-	-	Absent

136	THA1214	Aug 2012	08.45	02.01	I	+	+	-	Absent	+	-	Absent
137	"	"	08.22	02.77	II	+	-	-	Absent	-	-	Absent
138	"	"	12.41	03.24	III	+	+	-	Absent	+	-	Absent
139	"	"	12.92	03.38	IV	+	+	-	Absent	+	-	Absent
140	"	"	09.66	02.74	V	+	+	-	Absent	+	-	Absent

Table 17: SSPCR of plankton samples collected from Thankassery Bay for the detection of the larvae of *Perna viridis* and *Perna indica* during May to August of 2011 & 2012 respectively

Sl. No	Plankton sample code	Season of Collection	Wet weight of Plankton (in mg)	Dry weight of Plankton (in mg)	Sub - sample number	Positive control PCR (18SrRNA)	*Perna viridis*		*Perna indica*	
							SSPCR	**Result** (presence/ absence of larvae)	**SSPCR**	**Result** (presence/ absence of larvae)
1	THA1101	May 2011	40.42	10.20	I	+	-	Absent	-	Absent
2	"	"	41.31	10.00	II	+	-	Absent	-	Absent
3	"	"	44.21	11.01	III	+	-	Absent	-	Absent
4	"	"	34.55	09.94	IV	+	-	Absent	-	Absent
5	"	"	40.14	10.26	V	+	-	Absent	-	Absent
6	THA1102	May 2011	37.21	09.32	I	+	-	Absent	-	Absent
7	"	"	36.54	09.96	II	+	-	Absent	-	Absent
8	"	"	40.54	10.20	III	+	-	Absent	-	Absent
9	"	"	37.22	09.86	IV	+	-	Absent	-	Absent
10	"	"	38.11	09.80	V	+	-	Absent	-	Absent
11	THA1103	May 2011	40.12	10.01	I	+	-	Absent	+	Present
12	"	"	40.65	10.00	II	+	-	Absent	+	Present
13	"	"	40.45	10.10	III	+	-	Absent	-	Absent
14	"	"	31.54	09.65	IV	+	-	Absent	+	Present
15	"	"	40.22	10.45	V	+	-	Absent	+	Present
16	THA1104	June 2011	21.04	05.14	I	+	-	Absent	+	Present
17	"	"	24.32	06.60	II	+	+	Present	+	Present
18	"	"	24.11	06.45	III	+	+	Present	+	Present
19	"	"	22.00	05.95	IV	+	+	Present	+	Present
20	"	"	23.90	06.10	V	+	+	Present	+	Present
21	THA1105	June 2011	43.51	11.63	I	+	+	Present	+	Present
22	"	"	43.01	11.23	II	+	+	Present	+	Present
23	"	"	40.11	10.45	III	+	+	Present	+	Present
24	"	"	41.20	10.56	IV	+	+	Present	+	Present
25	"	"	44.30	11.23	V	+	+	Present	+	Present
26	THA1106	June 2011	35.60	09.12	I	+	+	Present	+	Present
27	"	"	36.01	09.20	II	+	+	Present	+	Present
28	"	"	33.22	09.12	III	+	+	Present	+	Present
29	"	"	40.92	10.01	IV	+	+	Present	+	Present
30	"	"	41.05	10.00	V	+	+	Present	-	Absent
31	THA1107	June 2011	49.63	13.05	I	+	-	Absent	-	Absent
32	"	"	46.33	12.41	II	+	-	Absent	-	Absent
33	"	"	43.11	11.99	III	+	-	Absent	-	Absent
34	"	"	47.00	13.04	IV	+	-	Absent	-	Absent
35	"	"	48.46	13.01	V	+	-	Absent	-	Absent

36	THA1108	July 2011	39.55	10.80	I	+	-	Absent	-	Absent
37	"	"	35.66	09.30	II	+	-	Absent	-	Absent
38	"	"	40.01	10.50	III	+	-	Absent	-	Absent
39	"	"	41.09	10.25	IV	+	-	Absent	-	Absent
40	"	"	38.10	09.95	V	+	-	Absent	-	Absent
41	THA1109	July 2011	28.70	07.10	I	+	-	Absent	-	Absent
42	"	"	25.45	08.40	II	+	-	Absent	-	Absent
43	"	"	26.85	07.11	III	+	-	Absent	-	Absent
44	"	"	27.00	06.99	IV	+	-	Absent	-	Absent
45	"	"	28.00	08.01	V	+	-	Absent	-	Absent
46	THA1110	July 2011	40.65	10.44	I	+	-	Absent	-	Absent
47	"	"	41.22	10.22	II	+	-	Absent	-	Absent
48	"	"	42.30	10.50	III	+	-	Absent	-	Absent
49	"	"	40.98	10.00	IV	+	-	Absent	-	Absent
50	"	"	39.85	10.21	V	+	-	Absent	-	Absent
51	THA1111	July 2011	42.00	11.36	I	+	-	Absent	-	Absent
52	"	"	41.92	11.00	II	+	-	Absent	-	Absent
53	"	"	40.31	10.96	III	+	-	Absent	-	Absent
54	"	"	43.11	11.21	IV	+	-	Absent	-	Absent
55	"	"	40.13	10.85	V	+	-	Absent	-	Absent
56	THA1112	Aug 2011	21.98	05.91	I	+	-	Absent	-	Absent
57	"	"	24.10	05.95	II	+	-	Absent	-	Absent
58	"	"	20.91	05.00	III	+	-	Absent	-	Absent
59	"	"	23.55	05.89	IV	+	-	Absent	-	Absent
60	"	"	25.22	06.10	V	+	-	Absent	-	Absent
61	THA1113	Aug 2011	17.50	04.53	I	+	-	Absent	-	Absent
62	"	"	20.68	04.90	II	+	-	Absent	-	Absent
63	"	"	19.41	04.78	III	+	-	Absent	-	Absent
64	"	"	21.11	05.01	IV	+	-	Absent	-	Absent
65	"	"	19.23	04.68	V	+	-	Absent	-	Absent
66	THA1114	Aug 2011	19.11	03.84	I	+	-	Absent	-	Absent
67	"	"	19.45	03.88	II	+	-	Absent	-	Absent
68	"	"	23.92	05.50	III	+	-	Absent	-	Absent
69	"	"	23.41	05.62	IV	+	-	Absent	-	Absent
70	"	"	20.55	03.47	V	+	-	Absent	-	Absent
71	THA1201	May 2012	37.11	09.81	I	+	-	Absent	-	Absent
72	"	"	41.92	10.23	II	+	-	Absent	-	Absent
73	"	"	44.01	10.10	III	+	-	Absent	-	Absent
74	"	"	41.03	09.93	IV	+	-	Absent	-	Absent
75	"	"	39.86	09.59	V	+	-	Absent	-	Absent
76	THA1202	May 2012	43.21	11.74	I	+	-	Absent	-	Absent
77	"	"	42.96	11.00	II	+	-	Absent	-	Absent
78	"	"	40.41	10.69	III	+	-	Absent	-	Absent
79	"	"	42.03	10.94	IV	+	-	Absent	-	Absent
80	"	"	44.61	11.12	V	+	-	Absent	-	Absent
81	THA1203	May 2012	43.94	11.02	I	+	-	Absent	+	Present
82	"	"	42.64	11.35	II	+	-	Absent	+	Present

83	"	"	40.56	10.96	III	+	-	Absent	+	Present
84	"	"	44.30	11.65	IV	+	-	Absent	+	Present
85	"	"	43.95	11.00	V	+	-	Absent	+	Present
86	THA1204	June 2012	41.45	10.43	I	+	+	Present	+	Present
87	"	"	41.25	10.10	II	+	+	Present	+	Present
88	"	"	42.17	10.50	III	+	+	Present	+	Present
89	"	"	41.65	10.36	IV	+	+	Present	+	Present
90	"	"	44.41	11.01	V	+	+	Present	+	Present
91	THA1205	June 2012	21.09	05.09	I	+	+	Present	+	Present
92	"	"	28.22	06.78	II	+	+	Present	+	Present
93	"	"	28.66	07.02	III	+	+	Present	+	Present
94	"	"	25.63	05.62	IV	+	+	Present	+	Present
95	"	"	20.41	05.21	V	+	+	Present	+	Present
96	THA1206	June 2012	24.60	08.04	I	+	+	Present	-	Absent
97	"	"	24.10	08.04	II	+	+	Present	-	Absent
98	"	"	23.11	08.19	III	+	+	Present	-	Absent
99	"	"	31.11	07.95	IV	+	+	Present	-	Absent
100	"	"	36.45	09.10	V	+	+	Present	-	Absent
101	THA1207	June 2012	37.10	09.62	I	+	-	Absent	-	Absent
102	"	"	38.21	09.19	II	+	-	Absent	-	Absent
103	"	"	40.20	10.00	III	+	-	Absent	-	Absent
104	"	"	37.11	09.20	IV	+	-	Absent	-	Absent
105	"	"	34.00	08.93	V	+	-	Absent	-	Absent
106	THA1208	July 2012	09.23	02.32	I	+	-	Absent	-	Absent
107	"	"	13.11	02.91	II	+	-	Absent	-	Absent
108	"	"	20.65	05.10	III	+	-	Absent	-	Absent
109	"	"	09.21	02.20	IV	+	-	Absent	-	Absent
110	"	"	13.44	03.01	V	+	-	Absent	-	Absent
111	THA1209	July 2012	21.30	04.69	I	+	-	Absent	-	Absent
112	"	"	12.57	04.00	II	+	-	Absent	-	Absent
113	"	"	20.13	04.69	III	+	-	Absent	-	Absent
114	"	"	16.41	03.97	IV	+	-	Absent	-	Absent
115	"	"	20.32	05.00	V	+	-	Absent	-	Absent
116	THA1210	July 2012	18.11	04.90	I	+	-	Absent	-	Absent
117	"	"	17.51	04.41	II	+	-	Absent	-	Absent
118	"	"	17.86	04.63	III	+	-	Absent	-	Absent
119	"	"	25.10	06.00	IV	+	-	Absent	-	Absent
120	"	"	20.31	03.99	V	+	-	Absent	-	Absent
121	THA1211	July 2012	15.10	03.60	I	+	-	Absent	-	Absent
122	"	"	16.22	03.90	II	+	-	Absent	-	Absent
123	"	"	17.54	04.33	III	+	-	Absent	-	Absent
124	"	"	16.21	04.00	IV	+	-	Absent	-	Absent
125	"	"	15.42	03.82	V	+	-	Absent	-	Absent
126	THA1212	Aug 2012	16.32	04.23	I	+	-	Absent	-	Absent
127	"	"	17.11	04.44	II	+	-	Absent	-	Absent
128	"	"	16.14	04.10	III	+	-	Absent	-	Absent
129	"	"	20.41	05.10	IV	+	-	Absent	-	Absent

130	"	"	17.51	04.25	V	+	-	Absent	-	Absent
131	THA1213	Aug 2012	12.11	02.90	I	+	-	Absent	-	Absent
132	"	"	12.54	03.51	II	+	-	Absent	-	Absent
133	"	"	16.10	03.66	III	+	-	Absent	-	Absent
134	"	"	17.21	04.51	IV	+	-	Absent	-	Absent
135	"	"	12.84	02.99	V	+	-	Absent	-	Absent
136	THA1214	Aug 2012	09.10	02.00	I	+	-	Absent	-	Absent
137	"	"	08.11	02.51	II	+	-	Absent	-	Absent
138	"	"	12.04	03.01	III	+	-	Absent	-	Absent
139	"	"	13.47	03.40	IV	+	-	Absent	-	Absent
140	"	"	12.21	02.59	V	+	-	Absent	-	Absent

Table 18: SS*n*PCR of plankton samples collected from Azhikode Estuary for the detection of the larvae of *Crassostrea madrasensis* during the months from September to December of 2011 & 2012 respectively

Sl. No	Plankton sample code	Season of Collection	Wet weight of Plankton (in mg)	Dry weight of Plankton (in mg)	Sub – Sample number	Positive control PCR (18SrRNA)	***Crassostrea madrasensis***		
							Ist step PCR (CO1)	**Nested PCR** (species specific)	**Result** (presence/ absence of larvae)
1	SAT1101	Sep 2011	24.00	06.01	I	+	+	-	Absent
2	"	"	24.68	06.45	II	+	+	-	Absent
3	"	"	20.45	05.51	III	+	+	-	Absent
4	"	"	24.86	06.10	IV	+	+	-	Absent
5	"	"	21.45	05.32	V	+	+	-	Absent
6	SAT1102	Sep 2011	40.10	10.05	I	+	+	-	Absent
7	"	"	32.98	08.95	II	+	+	-	Absent
8	"	"	33.65	08.41	III	+	+	-	Absent
9	"	"	39.78	10.10	IV	+	+	-	Absent
10	"	"	40.11	09.88	V	+	+	-	Absent
11	SAT1103	Sep 2011	33.54	08.05	I	+	+	+	Present
12	"	"	24.11	06.11	II	+	+	-	Absent
13	"	"	25.01	06.92	III	+	+	-	Absent
14	"	"	28.44	07.11	IV	+	+	+	Present
15	"	"	28.18	07.81	V	+	+	+	Present
16	SAT1104	Oct 2011	39.78	09.55	I	+	+	+	Present
17	"	"	36.88	08.67	II	+	-	+	Present
18	"	"	36.79	08.47	III	+	+	+	Present
19	"	"	37.88	08.98	IV	+	+	+	Present
20	"	"	37.54	09.27	V	+	+	+	Present
21	SAT1105	Oct 2011	37.55	08.91	I	+	+	+	Present
22	"	"	32.41	08.16	II	+	+	+	Present
23	"	"	40.85	10.00	III	+	+	+	Present
24	"	"	36.75	09.75	IV	+	+	+	Present
25	"	"	40.54	10.08	V	+	+	+	Present
26	SAT1106	Oct 2011	37.65	09.10	I	+	+	+	Present
27	"	"	36.89	08.91	II	+	+	+	Present
28	"	"	40.24	10.11	III	+	+	+	Present
29	"	"	40.01	09.18	IV	+	+	+	Present

30	"	"	39.65	09.24	V	+	-	+	Present
31	SAT1107	Oct 2011	40.24	10.40	I	+	+	+	Present
32	"	"	40.12	10.98	II	+	+	+	Present
33	"	"	40.21	10.11	III	+	+	-	Absent
34	"	"	40.55	10.24	IV	+	+	+	Present
35	"	"	38.45	09.93	V	+	+	-	Absent
36	SAT1108	Nov 2011	28.91	07.29	I	+	+	-	Absent
37	"	"	31.45	08.15	II	+	+	+	Present
38	"	"	36.54	08.16	III	+	+	-	Absent
39	"	"	28.94	07.66	IV	+	+	+	Present
40	"	"	36.41	08.95	V	+	+	+	Present
41	SAT1109	Nov 2011	40.51	10.12	I	+	+	-	Absent
42	"	"	40.43	10.95	II	+	+	+	Present
43	"	"	40.23	10.09	III	+	+	-	Absent
44	"	"	40.55	10.41	IV	+	+	-	Absent
45	"	"	40.37	10.90	V	+	+	+	Present
46	SAT1110	Nov 2011	28.15	07.12	I	+	+	-	Absent
47	"	"	33.21	08.10	II	+	+	-	Absent
48	"	"	32.45	07.11	III	+	+	-	Absent
49	"	"	36.00	08.00	IV	+	+	-	Absent
50	"	"	28.45	07.97	V	+	+	-	Absent
51	SAT1111	Nov 2011	33.01	07.95	I	+	+	+	Present
52	"	"	36.49	09.10	II	+	+	-	Absent
53	"	"	33.15	08.90	III	+	+	+	Present
54	"	"	37.05	09.11	IV	+	+	-	Absent
55	"	"	37.51	09.41	V	+	+	-	Absent
56	SAT1112	Dec 2011	25.11	06.13	I	+	-	-	Absent
57	"	"	24.61	06.01	II	+	+	+	Present
58	"	"	20.88	05.75	III	+	+	+	Present
59	"	"	24.01	06.01	IV	+	+	-	Absent
60	"	"	23.92	05.99	V	+	+	-	Absent
61	SAT1113	Dec 2011	20.45	05.23	I	+	+	-	Absent
62	"	"	17.02	04.20	II	+	+	+	Present
63	"	"	20.61	05.14	III	+	+	-	Absent
64	"	"	17.11	04.92	IV	+	+	-	Absent
65	"	"	20.65	05.03	V	+	-	-	Absent
66	SAT1114	Dec 2011	17.21	04.91	I	+	+	-	Absent
67	"	"	16.31	04.00	II	+	+	-	Absent
68	"	"	20.76	05.70	III	+	+	-	Absent
69	"	"	20.42	05.14	IV	+	+	-	Absent
70	"	"	17.57	04.77	V	+	-	-	Absent
71	SAT1201	Sep 2012	40.11	10.18	I	+	+	-	Absent
72	"	"	39.54	09.06	II	+	+	-	Absent
73	"	"	36.10	09.89	III	+	+	-	Absent
74	"	"	28.41	06.45	IV	+	+	-	Absent
75	"	"	40.21	10.02	V	+	+	-	Absent
76	SAT1202	Sep 2012	39.21	09.21	I	+	+	+	Present
77	"	"	28.94	07.25	II	+	+	+	Present

78	"	"	35.11	08.50	III	+	+	-	Absent
79	"	"	36.45	09.01	IV	+	+	-	Absent
80	"	"	36.01	08.55	V	+	+	-	Absent
81	SAT1203	Sep 2012	39.45	09.30	I	+	-	-	Absent
82	"	"	39.51	09.10	II	+	+	-	Absent
83	"	"	40.12	10.00	III	+	+	-	Absent
84	"	"	40.21	10.45	IV	+	+	+	Present
85	"	"	40.39	10.12	V	+	+	+	Present
86	SAT1204	Oct 2012	40.87	10.01	I	+	-	+	Present
87	"	"	37.21	09.21	II	+	+	+	Present
88	"	"	40.41	10.11	III	+	-	+	Present
89	"	"	37.15	09.92	IV	+	+	+	Present
90	"	"	40.22	10.12	V	+	-	+	Present
91	SAT1205	Oct 2012	37.61	09.44	I	+	+	+	Present
92	"	"	40.78	10.05	II	+	+	+	Present
93	"	"	37.15	09.10	III	+	+	+	Present
94	"	"	32.55	08.41	IV	+	+	+	Present
95	"	"	40.81	10.02	V	+	+	+	Present
96	SAT1206	Oct 2012	39.54	09.52	I	+	+	+	Present
97	"	"	39.14	09.30	II	+	-	+	Present
98	"	"	33.41	08.69	III	+	+	+	Present
99	"	"	37.52	09.22	IV	+	+	+	Present
100	"	"	37.55	09.11	V	+	+	+	Present
101	SAT1207	Oct 2012	40.01	09.62	I	+	+	+	Present
102	"	"	40.24	09.78	II	+	-	-	Absent
103	"	"	39.64	09.42	III	+	+	+	Present
104	"	"	39.89	09.65	IV	+	+	-	Absent
105	"	"	40.44	09.12	V	+	+	+	Present
106	SAT1208	Nov 2012	33.41	08.25	I	+	-	-	Absent
107	"	"	29.61	07.15	II	+	-	+	Present
108	"	"	28.22	07.00	III	+	+	+	Present
109	"	"	29.10	06.85	IV	+	+	-	Absent
110	"	"	24.58	06.15	V	+	-	-	Absent
111	SAT1209	Nov 2012	20.45	04.99	I	+	+	+	Present
112	"	"	20.78	05.01	II	+	-	-	Absent
113	"	"	17.44	04.82	III	+	+	-	Absent
114	"	"	17.54	04.26	IV	+	+	-	Absent
115	"	"	20.45	05.45	V	+	+	-	Absent
116	SAT1210	Nov 2012	20.87	05.17	I	+	+	+	Present
117	"	"	20.61	04.65	II	+	+	-	Absent
118	"	"	21.45	05.03	III	+	+	-	Absent
119	"	"	20.81	04.10	IV	+	+	-	Absent
120	"	"	17.20	04.49	V	+	+	+	Present
121	SAT1211	Nov 2012	13.01	03.10	I	+	+	-	Absent
122	"	"	16.20	03.78	II	+	-	-	Absent
123	"	"	16.14	04.10	III	+	+	-	Absent
124	"	"	16.19	03.97	IV	+	+	-	Absent

125	"	"	16.52	04.05	V	+	-	-	Absent
126	SAT1212	Dec 2012	12.92	03.55	I	+	-	-	Absent
127	"	"	17.01	04.86	II	+	+	+	Present
128	"	"	16.51	04.30	III	+	+	-	Absent
129	"	"	16.21	04.95	IV	+	+	-	Absent
130	"	"	17.01	04.16	V	+	-	-	Absent
131	SAT1213	Dec 2012	13.05	03.40	I	+	-	-	Absent
132	"	"	16.98	03.89	II	+	+	+	Present
133	"	"	17.01	03.74	III	+	+	-	Absent
134	"	"	17.12	04.14	IV	+	+	-	Absent
135	"	"	16.41	04.10	V	+	-	-	Absent
136	SAT1214	Dec 2012	08.40	02.70	I	+	+	-	Absent
137	"	"	12.61	02.91	II	+	-	-	Absent
138	"	"	13.01	03.41	III	+	+	-	Absent
139	"	"	16.04	03.90	IV	+	+	-	Absent
140	"	"	05.78	03.49	V	+	+	-	Absent

Table 19: SSPCR of plankton samples collected from Azhikode Estuary for the detection of the larvae of *Crassostrea madrasensis* during September to December of 2011 & 2012 respectively

Sl. No	Plankton sample code	Season of Collection	Wet weight of Plankton (in mg)	Dry weight of Plankton (in mg)	Sub - Sample number	Positive control PCR (18SrRNA)	***Crassostrea madrasensis***	
							SSPCR	**Result** (presence/ absence of larvae)
1	SAT1101	Sep 2011	20.10	05.01	I	+	-	Absent
2	"	"	21.00	05.20	II	+	-	Absent
3	"	"	23.91	06.11	III	+	-	Absent
4	"	"	20.51	05.10	IV	+	-	Absent
5	"	"	20.96	05.70	V	+	-	Absent
6	SAT1102	Sep 2011	37.91	09.55	I	+	-	Absent
7	"	"	22.04	06.90	II	+	-	Absent
8	"	"	36.54	09.11	III	+	-	Absent
9	"	"	40.61	10.20	IV	+	-	Absent
10	"	"	41.00	10.88	V	+	-	Absent
11	SAT1103	Sep 2011	23.67	07.55	I	+	-	Absent
12	"	"	28.00	06.41	II	+	-	Absent
13	"	"	26.89	07.00	III	+	-	Absent
14	"	"	27.65	07.22	IV	+	-	Absent
15	"	"	29.10	07.34	V	+	-	Absent
16	SAT1104	Oct 2011	36.22	09.10	I	+	+	Present
17	"	"	35.96	08.41	II	+	+	Present
18	"	"	36.00	08.89	III	+	+	Present
19	"	"	36.22	09.10	IV	+	+	Present
20	"	"	35.21	08.91	V	+	+	Present
21	SAT1105	Oct 2011	35.11	09.11	I	+	+	Present
22	"	"	36.01	08.96	II	+	+	Present
23	"	"	40.27	09.90	III	+	+	Present

24	"	"	36.22	09.15	IV	+	+	Present
25	"	"	39.58	09.87	V	+	+	Present
26	SAT1106	Oct 2011	35.98	08.20	I	+	+	Present
27	"	"	36.01	08.11	II	+	+	Present
28	"	"	37.95	09.11	III	+	+	Present
29	"	"	38.11	08.78	IV	+	+	Present
30	"	"	40.08	09.74	V	+	+	Present
31	SAT1107	Oct 2011	40.11	10.80	I	+	-	Absent
32	"	"	44.21	11.00	II	+	-	Absent
33	"	"	43.51	10.45	III	+	-	Absent
34	"	"	43.92	10.97	IV	+	-	Absent
35	"	"	43.66	11.03	V	+	-	Absent
36	SAT1108	Nov 2011	28.45	06.99	I	+	-	Absent
37	"	"	35.41	08.55	II	+	-	Absent
38	"	"	31.10	07.66	III	+	-	Absent
39	"	"	35.68	08.66	IV	+	-	Absent
40	"	"	39.71	09.55	V	+	-	Absent
41	SAT1109	Nov 2011	41.08	10.22	I	+	-	Absent
42	"	"	44.21	11.75	II	+	-	Absent
43	"	"	40.20	09.99	III	+	-	Absent
44	"	"	40.61	10.21	IV	+	-	Absent
45	"	"	44.06	11.00	V	+	-	Absent
46	SAT1110	Nov 2011	24.17	06.22	I	+	-	Absent
47	"	"	32.12	07.88	II	+	-	Absent
48	"	"	32.00	07.96	III	+	-	Absent
49	"	"	35.00	08.21	IV	+	-	Absent
50	"	"	33.51	08.11	V	+	-	Absent
51	SAT1111	Nov 2011	36.10	08.55	I	+	-	Absent
52	"	"	39.91	09.55	II	+	-	Absent
53	"	"	37.04	09.10	III	+	-	Absent
54	"	"	36.10	08.71	IV	+	-	Absent
55	"	"	36.45	08.61	V	+	-	Absent
56	SAT1112	Dec 2011	24.01	05.91	I	+	-	Absent
57	"	"	23.95	05.95	II	+	-	Absent
58	"	"	20.00	05.00	III	+	-	Absent
59	"	"	23.96	05.89	IV	+	-	Absent
60	"	"	24.51	06.10	V	+	-	Absent
61	SAT1113	Dec 2011	19.86	04.53	I	+	-	Absent
62	"	"	20.11	04.90	II	+	-	Absent
63	"	"	21.30	04.78	III	+	-	Absent
64	"	"	20.91	05.01	IV	+	-	Absent
65	"	"	17.22	04.68	V	+	-	Absent
66	SAT1114	Dec 2011	13.01	03.84	I	+	-	Absent
67	"	"	12.94	03.88	II	+	-	Absent
68	"	"	23.44	05.50	III	+	-	Absent
69	"	"	21.33	05.62	IV	+	-	Absent
70	"	"	13.01	03.47	V	+	-	Absent

71	SAT1201	Sep 2012	37.11	09.81	I	+	-	Absent
72	"	"	36.22	08.66	II	+	-	Absent
73	"	"	40.09	10.10	III	+	-	Absent
74	"	"	23.65	06.45	IV	+	-	Absent
75	"	"	39.86	09.59	V	+	-	Absent
76	SAT1202	Sep 2012	35.21	08.54	I	+	-	Absent
77	"	"	29.00	07.44	II	+	-	Absent
78	"	"	35.87	09.10	III	+	-	Absent
79	"	"	35.66	08.65	IV	+	-	Absent
80	"	"	36.11	08.79	V	+	-	Absent
81	SAT1203	Sep 2012	35.55	09.10	I	+	-	Absent
82	"	"	39.65	09.55	II	+	-	Absent
83	"	"	38.85	09.75	III	+	-	Absent
84	"	"	40.61	10.21	IV	+	-	Absent
85	"	"	36.84	09.68	V	+	-	Absent
86	SAT1204	Oct 2012	36.21	09.24	I	+	+	Present
87	"	"	37.22	09.68	II	+	+	Present
88	"	"	40.22	10.50	III	+	+	Present
89	"	"	40.18	10.09	IV	+	+	Present
90	"	"	40.31	09.84	V	+	+	Present
91	SAT1205	Oct 2012	37.10	09.10	I	+	+	Present
92	"	"	37.11	09.65	II	+	+	Present
93	"	"	36.17	08.45	III	+	+	Present
94	"	"	35.19	08.92	IV	+	+	Present
95	"	"	40.01	09.55	V	+	+	Present
96	SAT1206	Oct 2012	35.77	08.04	I	+	+	Present
97	"	"	36.21	09.10	II	+	+	Present
98	"	"	36.22	09.19	III	+	+	Present
99	"	"	35.84	09.10	IV	+	+	Present
100	"	"	36.02	09.10	V	+	+	Present
101	SAT1207	Oct 2012	35.78	09.62	I	+	-	Absent
102	"	"	34.98	09.19	II	+	-	Absent
103	"	"	40.30	10.00	III	+	-	Absent
104	"	"	35.41	09.20	IV	+	-	Absent
105	"	"	36.10	08.93	V	+	-	Absent
106	SAT1208	Nov 2012	31.57	07.60	I	+	-	Absent
107	"	"	26.10	06.45	II	+	-	Absent
108	"	"	25.74	06.45	III	+	-	Absent
109	"	"	26.41	06.75	IV	+	-	Absent
110	"	"	28.11	06.59	V	+	-	Absent
111	SAT1209	Nov 2012	20.41	05.11	I	+	-	Absent
112	"	"	24.01	05.96	II	+	-	Absent
113	"	"	19.17	04.69	III	+	-	Absent
114	"	"	20.16	04.00	IV	+	-	Absent
115	"	"	19.78	05.00	V	+	-	Absent
116	SAT1210	Nov 2012	20.11	04.90	I	+	-	Absent
117	"	"	17.21	04.41	II	+	-	Absent
118	"	"	17.00	04.63	III	+	-	Absent

119	"	"	24.19	06.00	IV	+	-	Absent
120	"	"	15.68	03.99	V	+	-	Absent
121	SAT1211	Nov 2012	23.14	03.60	I	+	-	Absent
122	"	"	16.24	03.90	II	+	-	Absent
123	"	"	17.00	04.33	III	+	-	Absent
124	"	"	15.41	04.00	IV	+	-	Absent
125	"	"	16.21	03.82	V	+	-	Absent
126	SAT1212	Dec 2012	17.08	04.23	I	+	-	Absent
127	"	"	16.88	04.44	II	+	-	Absent
128	"	"	16.40	04.10	III	+	-	Absent
129	"	"	20.43	05.10	IV	+	-	Absent
130	"	"	16.83	04.25	V	+	-	Absent
131	SAT1213	Dec 2012	12.61	02.90	I	+	-	Absent
132	"	"	15.47	03.51	II	+	-	Absent
133	"	"	13.21	03.66	III	+	-	Absent
134	"	"	20.14	04.51	IV	+	-	Absent
135	"	"	12.11	03.99	V	+	-	Absent
136	SAT1214	Dec 2012	08.24	02.00	I	+	-	Absent
137	"	"	13.21	02.51	II	+	-	Absent
138	"	"	12.04	03.01	III	+	-	Absent
139	"	"	13.60	03.40	IV	+	-	Absent
140	"	"	16.09	03.59	V	+	-	Absent

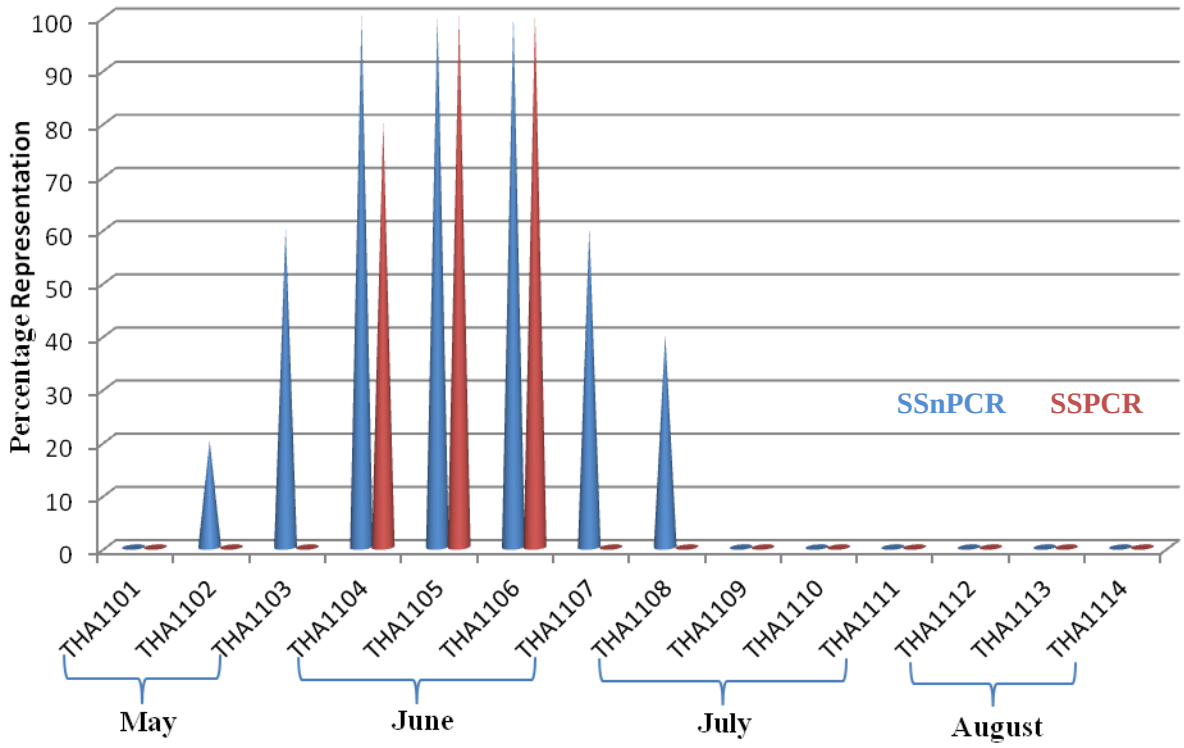


Fig 21: Detection of *P. viridis* larvae using SSnPCR and SSPCR in the plankton samples collected from Thankassery Bay during 2011

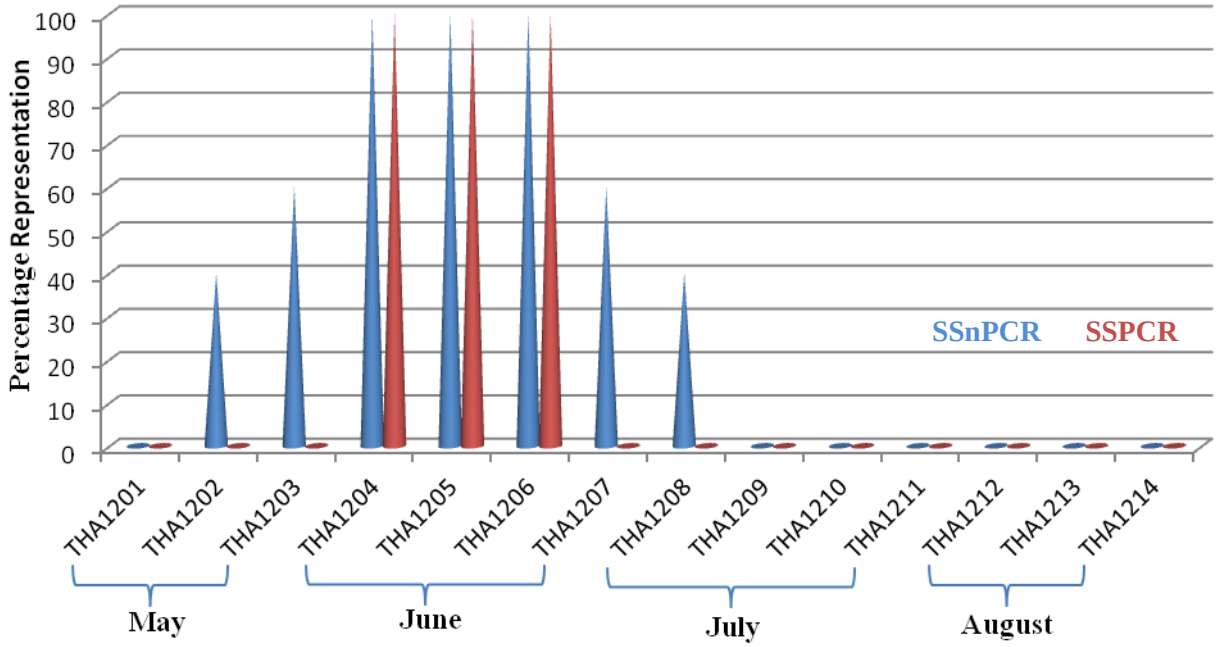


Fig 22: Detection of *P. viridis* larvae using SSnPCR and SSPCR in the plankton samples collected from Thankassery Bay during 2012

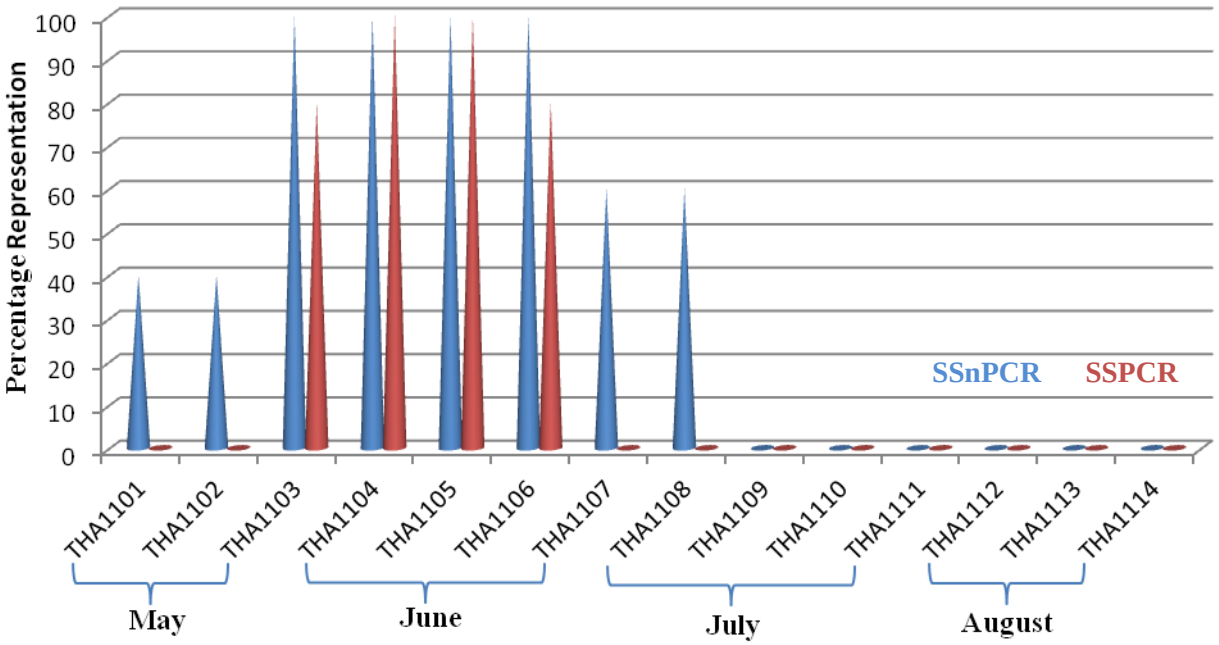


Fig 23: Detection of *P. indica* larvae using SSnPCR and SSPCR in the plankton samples collected from Thankassery Bay during 2011

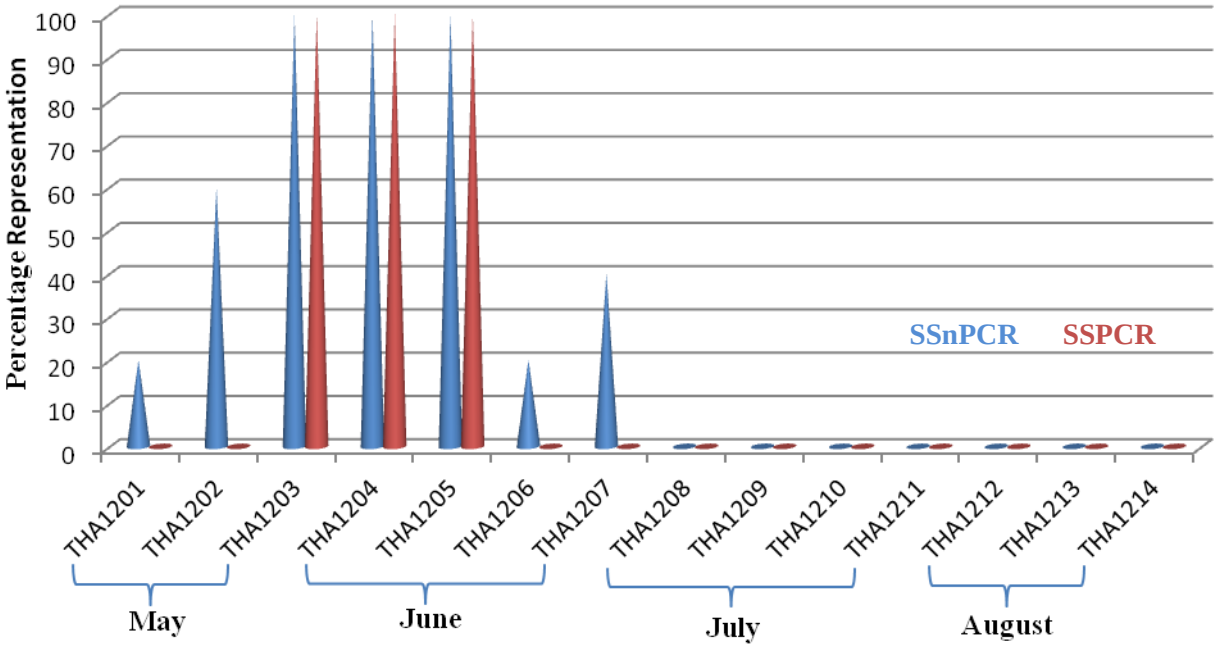


Fig 24: Detection of *P. indica* larvae using SSnPCR and SSPCR in the plankton samples collected from Thankassery Bay during 2012

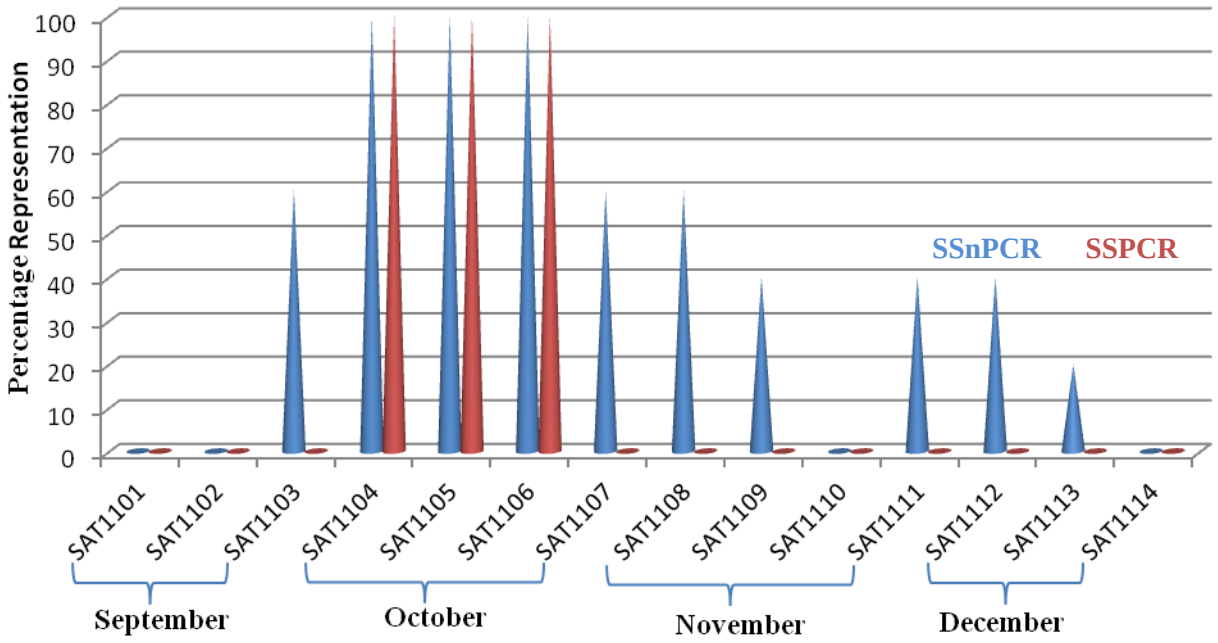


Fig 25: Detection of *C. madrasensis* larvae using SSnPCR and SSPCR in the plankton samples collected from Azhikode Estuary during 2011

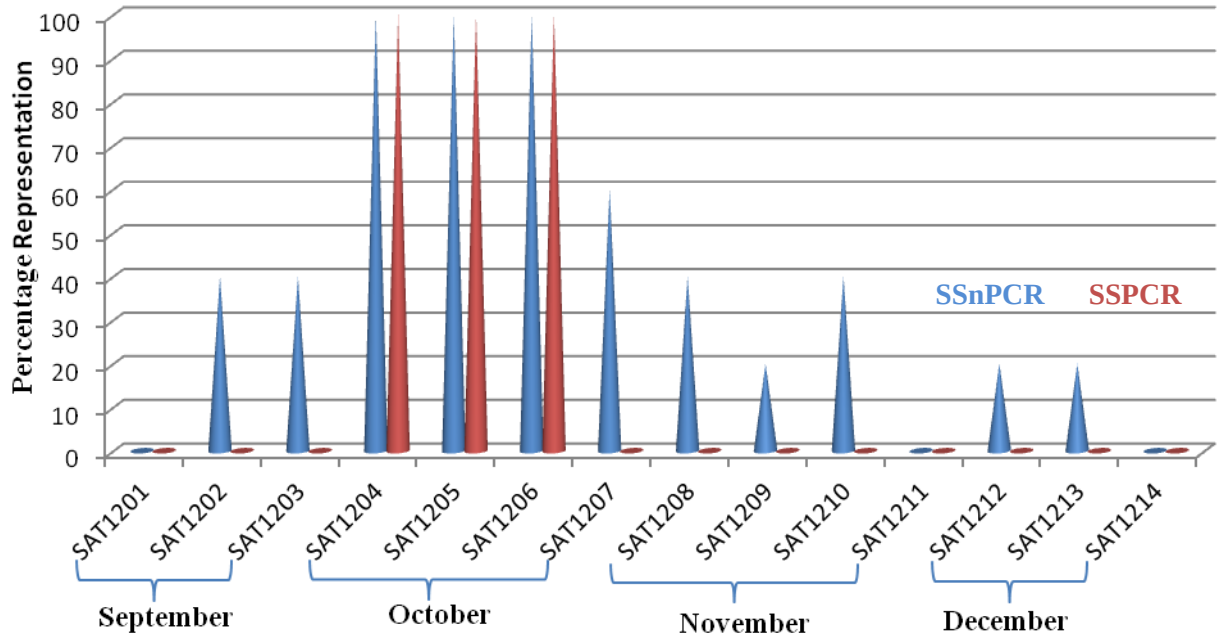


Fig 26: Detection of *C. madrasensis* larvae using SSnPCR and SSPCR in the plankton samples collected from Azhikode Estuary during 2012

4.8. Prediction of spat-fall and field observations

Continuous larval detection through SS*n*PCR and positive results in SSPCR with the successive plankton samples are the indicators for the impending massive spat-fall of the target bivalve species. In this study, the continuous occurrence of the larvae of *P. viridis* and *P. indica* at a considerable numerical density at Thankassery Bay was observed during the month of June until the last week. The mussel spats of around 5 mm size were found to appear on the rocky break water of Thankassery Bay at the end of July and in the beginning of August in the same year (Figures 27 & 28). This shows the positive correlation of larval detection using SSPCR and SS*n*PCR with the succeeding spat-fall and spat settlement on the rocky substratum of the study area.

Results of the SSPCR and SS*n*PCR for the detection of *C. madrasensis* larvae in the plankton samples collected from Azhikode Estuary showed the continuous occurrence of the larvae at considerable numerical density during the month of October. Hence, by the end of October 2011, spat collectors in the form of Rens (Figure 29 & 30) were installed in the Azhikode Estuary in order to collect the spats of *C. madrasensis*. The spat settlement on the Rens could be noticed by the end of the November and in the beginning of December (Figure 31). Oyster spats are also found to appear on the rocky substratum of Azhikode Estuary during the same period (Figure 32).


Figure 27 & 28: Images showing the *P. indica* and *P. viridis* spats settled on the rocky intertidal zone in Thankassery Bay, Kollam

Figure 29: Image showing the *C. madrasensis* rack culture farm set in the Azhikode Estuary.

Figure 30: Picture showing an Oyster farm set in Azhikode Estuary with exposed Rens during low tide. Rens with Oyster shells are hung down from the bamboo poles for the settlement of the oyster larvae.

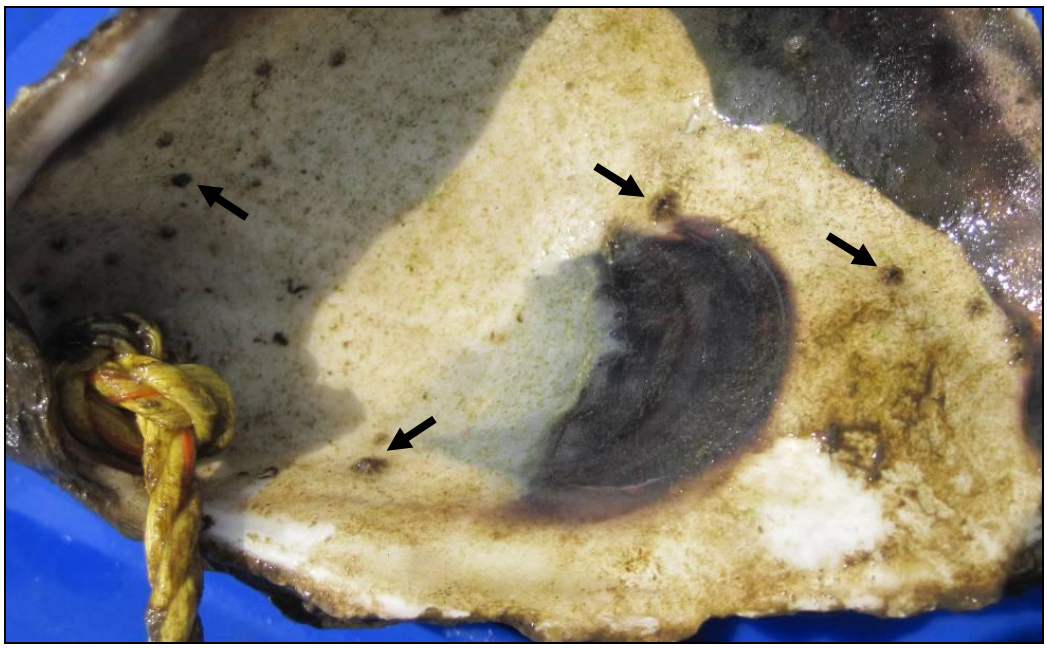


Figure 31: Picture showing an oyster Ren with the newly settled *C. madrasensis* larvae. The settled larvae are pointed with arrow marks.

Figure 32: Picture showing the growing *C. madrasensis* spat settled on a rocky substratum in Azhikode Estuary. The spat is pointed with an arrow mark.

CHAPTER 5

DISCUSSION

The present research work has come forth with a molecular technique that can be conveniently used to precisely detect the presence of the veliger larvae of three bivalve species of aquaculture importance in India such as *Perna viridis, Perna indica* and *Crassostrea madrasensis* from their natural habitats. This bivalve larval detection technology could be used as a management tool in the alternative method of spat collection i.e., spat collection using cultch materials during the breeding season of the bivalve species of interest.

5.1. Appropriateness of the Methodology Used

The main focus of the present research work was to develop an appropriate molecular marker to identify the target bivalve larval species from plankton samples so that the spat-fall of the particular species can be predicted with the highest probability. Various types of molecular methods have been developed over the last four decades (Garland and Zimmer, 2002) to identify or differentiate different species of mussels and oysters. This includes allozyme electrophoresis, immunologic recognition, DNA based techniques such as restriction fragment length polymorphism (RFLP), single-stranded conformational polymorphism (SSCP) and a wide range of PCR techniques (Baldwin *et al.*, 1996; Toro, 1998; Morgan and Rogers, 2001; Klinbunga *et al.*, 2003, Livi *et al.*, 2006). Many of these markers are handicapped since they need individually separated bivalve larvae to produce the results (Wang *et al.*, 2007, Zhan *et al.*, 2008). Physical separation of the bivalve larvae from such a large variety of larval forms, and its species identification are practically impossible.

Simply, a species identification marker may not accomplish the objective of the present work. The molecular marker being developed should not superimpose with the genetic profile of any other organism as the current work deals with the screening of plankton samples formed of a mix of organisms. It should have sensitivity, specificity, speed, and should be technology friendly for automation. Specificity of these markers can get compromised when the test is conducted with the samples like plankton. In the present work, PCR based DNA markers were developed as they can meet most of the above mentioned requirements. Species specific PCR (SSPCR) and species specific nested PCR (SS*n*PCR) were developed towards this end. Since the SSPCR primers developed are species specific, they can be used to amplify the DNA of the target bivalve species from a mixture of plankton DNA. Hence, the tedious process of bivalve larval sorting and morphology based species identification could be avoided.

Development of the PCR based DNA markers in this study required optimization of the DNA isolation protocols from the preserved bivalve tissue samples and plankton samples; identification of the appropriate gene for designing PCR based DNA markers; designing of SSPCR and SS*n*PCR primers;

assessment and optimization of the specificity and sensitivity of the SSPCR and SS*n*PCR; spat fall prediction in the field collected plankton samples using the SSPCR and SS*n*PCR; and finally, assessment of the reliability of spat fall prediction.

Quality of the DNA isolated is very important for obtaining consistent result in the molecular experiments, and the same is equally important for longer periods of storage. In the present study, three different genomic DNA isolation methodologies were tried and compared for quality and yield from the tissue samples. This includes the phenol-chloroform method (Sambrook *et al.,* 1989), salting-out protocol (Miller *et al.,* 1988) and silica based DNA isolation technique (Yue *et al.,* 2001). Among these three methods employed, the phenol-chloroform isolation method by Sambrook *et al.*(1989) was found to yield better quality DNA with less protein content. Though, the bivalve meat is high in protein content, the two time treatment of the digested tissue with the organic extraction component i.e., the phenol has enhanced the protein removal. But, this procedure reduced the volume of aqueous extract leading to a marginal lowering of the total DNA yield. Quality of the extracted DNA was found to be far better when compared with the procedure of single extraction using phenol. The salting out procedure of DNA isolation of Miller *et al.*(1988) is also a well-known method commonly used for DNA extraction from many of the aquatic organisms. This method is usually employed to isolate DNA from blood cells and the soft tissues like fin clips of fish (Turtinen and Juran, 1998, Usman *et al.,* 2013) where the protein content is comparatively less. The salt in the super saturated salt solution used in this method is likely to remain in the final DNA solution and its purity is often compromised. Removal of protein and other contaminants may be inefficient. It often demands RNase treatment and repeated alcohol precipitation before it can be used for further downstream applications (Noguera *et al.,* 2001, Mirmomeni *et al.,* 2010). The presence of salt in the final DNA solution in this method is less desirable even if the DNA yield is better. The silica based DNA isolation method developed by Yue *et al.,* in 2001 was mainly intended for isolating DNA from the formalin preserved old tissues. Even though this method provides far better quality and quantity of DNA, the trace quantities of silica or chaotropic salts in the final DNA solution shall hamper its further downstream applications like PCR. Similar problem was noticed in the present study also, where the trace of silica inhibited the PCR, when the DNA isolated by silica based method was used. Hence, phenol-chloroform method was found to be the best method of DNA isolation from bivalve tissues in this study and therefore the method was followed throughout the study.

In the case of plankton samples, cell disruption by heat shock was employed for faster release of DNA. It is a convenient and simplified procedure which can reduce the time required for plankton screening. The total DNA yield from the plankton samples is very critical for the tests conducted for

sensitivity analysis. Hence, the salting-out procedure was employed for DNA isolation from the plankton samples for conducting sensitivity analysis.

Blair *et al*. (2006) had reported the development of species specific PCR markers to discriminate the tissue samples of three *Perna* species namely *P. viridis*, *P. canaliculus* and *P. perna.* They were verified for their applicability in the context of the present study. It was found to produce nonspecific amplicons with the DNA of the bivalves and the other organisms used in the specificity assay conducted in the present work, under the reaction conditions prescribed by the author. The larval detection from the plankton samples using these primers will be less sensitive, and therefore, this primer pair cannot be used for the larval identification of *P. viridis* from the plankton samples. Though, Dias *et al*. (2013) have developed a genus specific PCR probe that can amplify the DNA of three species of *Perna* to produce PCR product of distinct size for each species, it was not supported with the trials using plankton samples collected from the field. Therefore, in the present study, PCR based markers were developed afresh for *P. viridis* and *P. indica.*

Studies on the specific detection of *C. madrasensis* larvae from the plankton samples are particularly lacking, even though, there are a number of reports about the molecular larval detection techniques for other *Crassostrea* species. There are reports of specific PCR primers for different species belonging to the genus *Crassostrea* except *C. madrasensis*. Detection of the larvae of *C. rhizophorae* and *C.brasiliana* for the purpose of spat collection by Ludwig *et al.*(2011); specific identification of *Crassostrea* and *Ostrea* species based on multiplex PCR technique by Cross *et al.*(2006); detection of veliger larvae of *C. gigas* from the ballast water samples for the screening of invasive species by Patil *et al.*(in 2005); detection and differentiation of *C. gigas* and *C. sikamea* species based on sequence specific PCR primers by Banks *et al.*(1993) are some examples. However, similar studies have not yet been reported in the case of *C. madrasensis*, which is an important mariculture species in India. In the present study, PCR based DNA markers for the specific identification of veliger larvae of *C. madrasensis* from the plankton samples have been developed for predicting the spat-fall.

5.2. Gene selection and Marker Development

The genes commonly used in molecular taxonomy are CO1, 16S rRNA, 18S rRNA and ITS 1&2. The appropriateness of these genes for designing species identification markers for *P. viridis*, *P. indica* and *C. madrasensis* were verified. These genes were PCR amplified and sequence characterized in *P. viridis* in order to assess the trend of variation shown by each one of them, and to select the gene for marker development. Most important characteristic of the gene required for designing DNA markers for species identification is the high rate of inter-species sequence divergence and high rate of intra-species

sequence conservation. The chances for the cross species DNA amplification by the species specific PCR primers (SSPCR primers) will be very low when these primers are designed from the regions where there is high rate of inter-species nucleotide sequence variation or divergence. Consistency of the PCR amplification by the SSPCR primers would be high when the primers are designed at the locations where intra-species sequence conservation is high.

The SSPCR primers developed in the present work were expected to show very high specificity towards the target species, as they were designed after comparing their gene sequences with the closely related species and the common planktonic organisms. Specificity of the SSPCR primers developed was validated through specificity PCR assay in which the DNA samples of the related bivalves and other planktonic organisms usually seen in the same habitat were included. Similarly, sensitivity of these primers was validated through sensitivity PCR assay, which involves the screening of experimental plankton samples containing different numbers of target species larvae. The SSPCR based markers developed in this study were found to be specific for the precise identification of the larvae of *P. viridis*, *P. indica* and *C. madrasensis* in the plankton samples collected from coastal waters. Species specific nested PCR (SS*n*PCR) was also developed to enhance the sensitivity. Usefulness of these markers were assessed through field level validation.

In the present work, level of the sequence divergence between different species of *Perna* were analyzed for each gene (CO1, 16S rRNA, 18S rRNA and ITS 1&2) using the pairwise distance calculation method applying the Kimura-2 parameter (K2P) in MEGA 5. Among the gene sequences used for the analysis, the CO1 sequences showed highest degree of nucleotide divergence between the species. It was found that the CO1 gene had a nucleotide divergence of 23.4% between *P. viridis* & *P. perna* and 24.6% between *P. viridis* & *P. canaliculus* (Table 9). The nucleotide sequences coding for 16SrRNA stood second in the percentage of nucleotide divergence. It showed a divergence of 22.5% between *P. viridis* & *P. perna* and 23.2% between *P. viridis* & *P. canaliculus* (Table 10). The sequences of intergenic spacers came third in the percentage of nucleotide divergence, and it showed 14.2% between *P. viridis* & *P. perna* and 12% between *P. viridis* & *P. canaliculus* (Table 12). The gene coding for 18S rRNA showed the least degree of sequence divergence (2% between *P. viridis*, *P. perna* and *P. canaliculus*). Since the CO1 gene showed high rate of sequence divergence between different *Perna* species, it was then selected for the sequence variation analysis at intra-specific level. The highest percentage of CO1 gene sequence divergence calculated between different populations of *P. viridis* was only 1.5%, between the Chennai and Venezuela populations; indicating higher degree of sequence conservation between populations.

The most commonly used genes for species identification in the case of bivalves are CO1 and 16SrRNA. It has been opined by Hebert *et al.*(2003) and Trivedi *et al.*(2012) that the higher degree of sequence variation shown by these genes makes them a better choice for identification of the closely related species. The sequence analysis conducted in the present study showed that there is only a small difference in the divergence percentage between the CO1 and 16S rRNA gene sequences of different *Perna* species. Even though, the 16SrRNA gene sequences have also been used for species identification in many bivalves (Thomas *et al.,* 2011, Jen *et al.,* 2008, Bendezu *et al.,* 2005) the CO1 gene was selected in current work for designing the SSCR primers. The CO1 gene have been reported to have higher percentage of inter-specific sequence divergence and phylogenetic signals better than the other mitochondrial genes with an evolutionary rate three times greater than that of 16SrRNA gene (Feng *et al.,* 2011). Though, the intergenic spacers and the18SrRNA gene are also used for species identification in many bivalve species (Rasmussen and Morrissey, 2008, Wang and Guo, 2008, Espineira *et al.,* 2009, Zieritz *et al.,* 2012), their nucleotide divergence was found to be low in the present study. Moreover, the intra-species sequence variation of the CO1 gene (divergence between various populations) of *P. viridis* estimated using the pair wise distance method in this study showed only negligible value (Table 13) indicative of the higher degree of intra-specific sequence conservation of the CO1 gene. The properties like higher degree of inter-specific sequence variation and intra-specific sequence conservation shown by the CO1 gene revealed in the sequence analysis of *P. viridis* was utilized to design the species specific DNA markers (SSPCR) for the species. Due to these properties of the CO1 gene it was used to design the SSPCR primers for *P. indica* and *C. madrasensis* also.

5.3. Specificity of the SSPCR primers

SSPCR primers for the amplification of CO1 of *P. viridis* and *C. madrasensis* were designed using Primer-BLAST in NCBI assigning stringent parameters which make them highly specific. The chances for non-specific annealing of the primers were reduced considerably by designing them to have comparatively high melting temperature of 58^0C and GC content of 50%. The polymorphic nucleotide positions (the nucleotide positions which are variable) impart uniqueness and specificity to the designed primers of the respective species. The inter-species polymorphic or variable nucleotide positions at the 3| end of the primer are critical in determining its species specificity. The chance for non-specific PCR amplification is more if the inter-species sequence variation towards the 3| end of the primer is less. All of the SSPCR primers were selected in such a way that they do possess more inter-species variation at the 3| end (Illustrations 1, 2&3). The primer sequence outputs obtained from Primer-BLAST for CO1 sequences of *P. indica* were found to be having less number of inter-species polymorphic sites especially at the 3| end of the primer. Therefore, the CO1 sequence regions of *P. indica* where there is higher level of inter-species nucleotide variations were manually located and such regions were utilized for designing the

SSPCR primers using OligoCalc (Kibbe, 2007). The primers designed in this way were analyzed for the chances of formation of secondary structures and self-annealing dimers.

Specificity of the primers was finally checked using the specificity PCR assay. The SSPCR primers developed for the target species were tested against the DNA of the organisms that are usually seen in the same and similar habitat of the target species so that the chance of cross species PCR amplification could be verified. A positive control PCR using the universal primers (Wuyts *et al.,* 2001) amplifying the 18SrRNA gene was also conducted for every DNA sample included in the specificity assay. This is to confirm that the non-amplification of the test DNA by the SSPCR primer was only because of the species specificity of the primer pair and not because of the failure in PCR due to poor DNA quality. A negative control PCR without the addition of any particular DNA was also included in the specificity assay in order to check the presence of DNA contaminations in the reagents used for the assay. Out of the four pairs of specific primers designed using Primer-BLAST and specificity PCR assay carried out, PVCO1F265 & PVCO1R539 were found to be the most specific primer pair for *P. viridis* and CMCO1F66 & CMCO1R315 for *C. madrasensis*. Out of the three pairs of SSPCR primers designed and compared for *P.indica* using OligoCalc, the primers PICO1F284 & PICO1R575 were found to be most specific to the species. On comparing the primer binding region of the target species and the other closely related species, it could be observed that the sequence of the primer pair PVCO1F265 & PVCO1R539 spanning 40 bp nucleotides contain 17 numbers of polymorphic sites; the sequence of primer pair PICO1F284 & PICO1R575 spanning 42 bp nucleotides contain 14 polymorphic sites; and the sequence of primer pair CMCO1F66 & CMCO1R315 spanning 40 bp nucleotides contain 13 numbers of polymorphic sites. This avows the high rate of inter-species sequence divergence of the primers.

The intra-species sequence variation or divergence at the primer annealing location is another important criterion which influences the species specificity of the SSPCR primers. There is a possibility for non-amplification in PCR, if the primer designed region has intra-species nucleotide sequence variation, leading to false negative PCR result. Since this can affect the utility of the SSPCR primers developed, a comparison was done for the sequences of selected SSPCR primer binding region of *P. viridis* from different locations (Table 15). This has revealed a high level of sequence conservation within the populations collected from Indian peninsula with the exception of a 3$^{|}$ terminal nucleotide substitution (**A** instead of **G**) in the case of the individuals from Chennai and Orissa for the SSPCR primer PVCO1R539. The complementary nucleotide to the 3$^{|}$ terminal of PVCO1R539 primer in Chennai and Orissa populations is **'T'**, instead of **'C'** in other populations. Hence, the possibility of false-negative PCR in Chennai and Orissa populations due to this mismatch at the 3$^{|}$ end of the primer binding region had to be ruled out. Simsek (2000) and Kwok (1990) have reported that the PCR amplification efficiency is not

affected by G/T nucleotide mismatch at the 3^{l} end. In the present study, this was tested and proved through an empirical analysis in which 96 numbers of DNA belonging to different populations of *P. viridis* were PCR amplified using this SSPCR primer pair. All the tests, including that conducted for the DNA templates from the populations of Chennai and Orissa were positive. Similarly, empirical analysis was conducted for the other two target species with 96 numbers of the respective target species DNA. All the tests produced positive results indicating the high level of sequence conservation of the primer binding region between different populations collected from Indian waters.

The SSPCR primers designed for the three target species produced PCR amplicons of the expected size (274 bp for *P. viridis*, 291 bp for *P. indica* and 294 bp for *C. madrasensis*). The smaller PCR product size requires lesser time duration for PCR amplification and this shall make the larval identification into a much faster process. Smaller product size by the SSPCR primers makes them equally suitable to be used in realtime PCR. Though, application of realtime PCR was not utilized in the present research work the possibilities for the same shall be explored in future using the SSPCR primers designed here.

5.4. Sensitivity of the larval detection system (SS*n*PCR)

Sensitivity of the detection system is of utmost importance since the field collected plankton samples will have varying numbers of the larvae of the target species to the total number of planktonic organisms. Dilution of the DNA isolated from the plankton sample for PCR will significantly dilute the target species DNA, and very low template concentrations in PCR will generate random fluctuations in the priming efficiency (Chandler *et al*. 2003). In order to achieve the required sensitivity, the nested PCR method (SS*n*PCR) was developed and tested for sensitivity in amplifying the target species DNA even at pico gram concentrations. Since the primers used in the first step SS*n*PCR were universal in nature, all the DNA templates belonging to different planktonic organisms having homology to the priming site got amplified along with that of the target species DNA. This step facilitates the enrichment of target DNA in the samples having lesser number of veliger larvae. On the other hand, since the second step SS*n*PCR is specific, only the samples having target species larvae were amplified (Table 16 & 18).

The sensitivity of SS*n*PCR adjudged in the present work is extremely high. A minimum of 0.1 ng/µl (100 pg/µl) of target DNA out of 250 ng/µl of plankton DNA, and also a single veliger larva of target bivalve species from a plankton biomass of approximately 40mg, could be successfully detected in the sensitivity trials. This is in line with that of Santaclara *et al*. (2007) who estimated the detection limit of the species specific PCR primers (designed for identifying *Mytilus galloprovincialis* and *Xenostrobus secures* larvae in plankton samples) from a series of experimental DNA mix containing varying

concentrations of target DNA. Thus it is established from the present study that the SS*n*PCR could be successfully employed to screen the plankton samples having very less numbers of target species bivalve larvae. As the preceding and succeeding periods of peak spawning seasons are usually characterized with lower numbers of larvae in the water (Pestana *et al.* 2008), a positive result with the SS*n*PCR prior to the peak spawning season of the target bivalves could be considered as the initiation of breeding season.

In the case of SSPCR, it was possible to detect a minimum of 2 ng/µl of target DNA out of 250 ng/µl of plankton DNA corresponding to a minimum of 20 veliger larvae of target species from a plankton biomass of 40 mg. Each 100 ml plankton sample in the present work was collected by filtering about 12.5 tons of water (sub section 3.2.2. in Materials and Methods). Then, 1.5 ml homogeneously mixed sub sample was collected from this100 ml preserved plankton sample in order to get approximately 40 mg of plankton biomass, and then used for SSPCR. Therefore, a positive result in SSPCR is an indication of presence of approximately 1330 larvae in 100 ml ([100/1.5] ×20) and for 12.5 tons of water filtered the larval density will be around 106 numbers per 1000 liters (1330/12.5). Booth (1983) has categorized the availability of bivalve larvae in the water body based on the larval density. He suggested that the bivalve larval availability could be regarded as **abundant** (if the larval density is = or > 100 / 1000 liters), **common** (if the density is = 30 but <100), **frequent** (if the density is = 3 but <10) and **occasional** (if the density is <1). Therefore, according to this categorization, a positive result in SSPCR with the field collected plankton sample represents the **abundant** availability of the target species bivalve larvae in the natural water body.

5.5. Screening of the plankton samples for bivalve larvae

The main objective of the present work is to identify the presence of the target bivalve species larvae in the natural water bodies where a large number of larval forms of different organisms are present. The SSPCR and SS*n*PCR tests indicated the overlapping of the spawning period of *P. viridis* and *P. indica* (Table 16, Fig. 21 & 23). Presence of the larvae of these two species could be noticed during the months of May to July. This is a peculiarity of the sampling site where both of these two species co-occupy the substratum. The peak spawning season of *P. viridis* has been reported to be between June and August at different regions along the Kerala coast (Appukuttan *et al.,* 2001 and Alagarswami, 1980). The current study shows the presence of *P. viridis* larvae in Thankassery Bay between the middle of May and up to the end of June. Extend of peak spawning season is slightly variable and it is associated with the variations in the seasonal distribution of temperature and onset of monsoon (Rajagopal *et al.,* 1998a). There are a few reports on the high intensity short spells of rain in Kerala (Khole *et al.,* 2011 and Vakily, 1989) prior to the normal monsoon season (starts by June) which triggers the mussel spawning (Laxmilatha and Sivadasan, 2007). It is assumed that the same reason could have initiated mussel spawning at the

sampling location early in the month of May and most of the mature mussels must have been spent by the end of June. The SSPCR conducted for the same plankton samples showed the abundant occurrence of the larvae during May to June and that could be regarded as the peak spawning period in 2011 and 2012. SS*n*PCR analysis of the plankton samples collected during 2011 and 2012 from Azhikode Estuary showed the intermittent presence of *C. madrasensis* larvae from Sepetember to December with a continuous occurrence during the month of October. The spawning frequency of the bivalves in an estuary can fluctuate even during a breeding season as the estuary is highly susceptible to the variations in salinity, temperature and the primary productivity. The SSPCR tests conducted with the same plankton samples also showed the abundant occurrence of the *C. madrasensis* larvae during October and hence, this period could be considered as the peak spawning period of *C. madrasensis* in the Azhikode Estuary in 2011 and 2012.

5.6. Reliability of the Spat-fall prediction

Prediction of spat-fall for the three target bivalve species was one of the major objectives of the present research work. The SS*n*PCR method is highly sensitive and it could detect even a single veliger larva from the plankton samples, whereas, the positive SSPCR indicate the abundance of bivalve larvae in the water. A regular screening of the plankton samples collected periodically from a sampling site using both the SS*n*PCR and SSPCR would be helpful to predict the spat-fall. The SS*n*PCR method could be used to find out the exact time of beginning of bivalve spawning. The presence of stray numbers of veliger larvae detected by the SS*n*PCR method indicated the presence of ripe individuals in the locality. This could be considered as an indication of the beginning of a breeding season. Continuous larval detection through SS*n*PCR in the successive plankton samples showed the larval availability over a span of approximately eight weeks in the water column. Massive spat settlement could be expected during this period of continuous larval detection. Similarly, the continuous positive results with the SSPCR indicate abundance of the target species larvae. Hence, the period in which all of the plankton samples produced positive results both with the SS*n*PCR and SSPCR would be ideal to be considered as the period of spat-fall. In the present study, the continuous occurrence of the larvae of *P. viridis* and *P. indica* at a considerable numerical density at Thankassery Bay was observed during the month of June, until the last week (Table 16 & 17; Fig. 21-28). As this period would be observed with high numerical density of bivalve larvae in the water column, the probability of spat-fall would also be high. Results of SS*n*PCR and SSPCR for the detection of *C. madrasensis* larvae in the plankton samples collected from Azhikode Estuary showed the continuous occurrence of the larvae at considerable numerical density during the month of October (Table 18 & 19; Fig. 29-32). This would be the ideal period for keeping the spat collectors as there is a higher probability for massive spat-fall to occur. Based on this conclusion the spat collectors in the form of oyster rens were kept in the Azhikode Estuary by the end of October 2011.

Field observation conducted at the study areas for the spat settlement of mussels and oysters showed a positive correlation with the prediction of spat-fall. The massive spat-fall of *P. viridis* and *P. indica* were predicted to occur at Thankassery Bay by the end of June. The bivalve spats take almost a month to reach a size of around 5 mm that could be seen without any visual aids. Accordingly, as expected, the spat-settlements could be observed by the end of July and in the beginning of August in the corresponding years. Therefore, from analyzing the lab results and correlating the field observations, it may be concluded that large numbers of *P. viridis* and *P. indica* spats could be collected by installing the spat collectors by the month of June at Thankassery Bay. In a similar way, a positive correlation could be seen in the prediction of spat-fall for *C. madrasensis* at Azhikode Estuary. The spat-fall of *C. madrasensis* was expected to occur at Azhikode Estuary by the end of October in the years 2011 and 2012. The spat collectors (oyster rens) installed in Azhikode Estuary during October 2011 as per the prediction were observed to have the settlement of *C. madrasensis* spats by the end of November and in the beginning of December in the same year, as expected. Therefore, screening of the plankton samples collected from the study area using the SS*n*PCR and SSPCR developed in the present work was found to be a promising tool to predict the impending spat-fall of *C. madrasensis*. Hence, initiation of the screening in the water body for bivalve larvae a month before the anticipated beginning of the spawning period, and installation of the spat collectors based on the results of plankton screening tests would be a proper strategy for collecting the bivalve spats of interest from a location.

5.7. Relevance of the outcome

The research outcome of this study can serve as an effective management tool in spat collection using cultch materials. The species specific molecular identification of the bivalve larvae and the spat-fall prediction method developed in this research work can overcome the pitfalls in the conventional methods of larval identification. It shall make the spat collection process into a successful venture. It can therefore, augment the culture production of bivalve species through mariculture. It can help to reduce the indiscriminative exploitation of natural bivalve spat resources for culture purposes.

The species specific DNA markers developed in the present work could also be used for applications other than spat-collection such as the studies of larval dispersal and larval ecology; gut content analysis of fishes; invasive species detection and management; environmental monitoring for the larvae of sedentary species in the cooling systems of thermal power plants; adulterations in canned food products etc., with further optimization.

CONCLUSION

Short supply of spats is the major constraint to the Indian bivalve mariculture industry. Though, hatchery technologies have been developed for *P. viridis*, *P. indica* and *C. madrasensis*, the commercial ventures for the spat production are not yet available in India. Spat availability from the natural resources is also under decline. Limited and patchy distributions of the bivalve resources and less availability of suitable substratum for bivalve larval attachment are the reasons for this. The ever increasing demand for the bivalve meat and the limited availability of natural spat resources necessitate the development of alternative means for enhancing spat collection efficiency. Bivalve farmers still depend mainly on natural spat resources, and most of the bivalve farmers resort to the method of spat collection using cultch materials. The variations in bivalve larval recruitment due to the fluctuations of spawning period, overlapping spawning periods of different species of bivalves found in the same habitat and occurrence of the larval forms of bio-fouling organisms in water column makes the process of cultch based spat collection into a less efficient activity. It was felt that accurate identification of the larvae of desired bivalve species, assessment of its abundance in water, and prediction of the probable days of spat settlement, using modern techniques, would be a proper management approach to improve the efficiency of spat collection using cultch materials. Therefore, specific DNA markers in the form of species specific PCR (SSPCR) primers were developed in the present study for accurately identifying the veliger larvae of the three target bivalve species. These species identification DNA markers could be used to predict the spat-fall and thereby it would enable the farmers to collect the sufficient seed material of the candidate bivalve species for mariculture.

Around 495 numbers of *P. viridis*, 105 numbers of *P. indica* and 125 numbers of *C. madrasensis* specimens belonging to different populations from Indian peninsula were collected and preserved in ethanol as part of this study. The species *P. viridis* has widely distributed populations all over world than the other two candidate species namely, *P. indica* and *C. madrasensis* which are confined to Indian peninsula. Therefore, the DNA samples of *P. viridis* from different populations were used for the initial amplification of the genes considered for developing DNA markers. The amplified genes were sequenced and the level of sequence divergence among them was compared. The gene selected after the sequence divergence analysis was then used for designing SSPCR primers in the target species.

The prospective genes of *P. viridis* such as Cytochrome Oxidase subunit 1 (CO1), 16S ribosomal RNA (16SrRNA), 18S ribosomal RNA (18SrRNA) and Internal Transcribed Spacer (ITS) were amplified using the universal PCR primers and sequence characterized. The characterized gene sequences of *P. viridis* were compared with that of *P. perna* and *P. canaliculus* in order to measure the level of inter-specific sequence variation and intra-specific sequence conservation. As a result, CO1 gene was found to be having highest percentage of inter-specific sequence variation (24.6% between *P. viridis* and *P. canaliculus*; 23.4% between *P. viridis* and *P. perna*) and also having highest percentage of intra-specific

sequence conservation (highest percentage of variation between two of its populations, Chennai and Venezuela is only 1.5%), and thus forms an ideal gene to design species specific DNA markers. Therefore, CO1 gene was selected for designing SSPCR primers for the three target species using the Primer-BLAST application in NCBI.

The SSPCR primers, thus designed, were subjected to specificity analysis in which the SSPCR primers were used to amplify the DNA of the target species and other closely related species that could be seen in the same habitat. This has helped to reveal the possibility of non-specific amplification by the SSPCR primers. The primer pair that specifically amplifies the target species DNA alone was selected as the SSPCR primer for respective species, and further used in the plankton screening tests. The PCR primer pairs found to be species specific (SSPCR) were, PVCO1F265 & PVCO1R539 for *P. viridis*; PICO1F284 & PICO1R575 for *P. indica*; and CMCO1F66 & CMCO1R315 for *C. madrasensis*.

Methods were designed and standardized for the sensitive identification of the target species larvae from the plankton samples collected from natural water bodies. A nested PCR technique (SS*n*PCR) consisting of two steps was designed in order to detect the presence of very low numbers of target species larvae in the plankton samples. In the nested PCR technique the universal primers of CO1 gene was used for the first step PCR, wherein, the low number of copies of target DNA in the plankton samples could be multiplied. The SSPCR primers designed for each target species were used for the second step nested PCR, so that the presence of target species could be specifically detected. Standardization of the SS*n*PCR for each target species was carried out with the experimental DNA mix containing plankton DNA and target species DNA at varying proportions, and also the experimental plankton samples containing known numbers of target species larvae. In this way, it was possible to detect the presence of a minimum of 0.1 ng/µl of target DNA in a plankton DNA of 250 ng/µl, and even a single veliger larva of the target species in a plankton sample of approximately 40 mg wet weight. The sensitivity of SSPCR was also standardized using the experimental DNA mix and the experimental plankton samples. It was possible to detect a minimum of 2 ng/µl of target DNA in a plankton DNA of 250 ng/µl and 20 numbers of veliger larvae of the target species in a plankton sample of approximately 40 mg wet weight.

The ethanol preserved plankton samples were subjected to screening for the presence of larvae of the target species through SS*n*PCR and SSPCR. The veliger larvae sorted from the plankton samples were species identified using SSPCR. The whole plankton samples were also subjected to larval screening using SS*n*PCR and SSPCR. In order to get results with minimum error, SS*n*PCR and SSPCR tests were conducted with five different aliquots from every plankton sample. The number of positive results obtained from five different aliquots shows the percentage representation of larvae in a unit volume of preserved plankton sample. In most of the cases of SS*n*PCR, the first step PCR is positive as the primer used in first step PCR is universal and it amplifies the DNA of multiple bivalve species. In the case of second step nested PCR, only the sample having the target DNA produce a positive result. In the case of a

few plankton samples the second step PCR was positive even though the first step PCR was negative. This was mainly because of the presence of very less numbers of bivalve larvae and, in spite of amplification, product of the first step PCR could not be visualized in agarose gel because of its low concentration.

Analysis of the results of SS*n*PCR with plankton samples collected from Thankassery Bay showed the presence of *P. viridis* larvae from May second week to July first week in 2011 and 2012. Similarly, the presence of *Perna indica* could be noticed in the water from the first week of May until the first week of July in 2011 and from the last week of May to the second week of June in 2012. Analysis of the results of SS*n*PCR with plankton samples collected from Azhikode Estuary showed the occurrence of *C. madrasensis* larvae from the third week of September until the second week of December 2011. But, the continuous larval detection in all of the plankton samples is evident only in the plankton samples collected during the first three weeks of October 2011. After this period the larval detection in the plankton samples becomes irregular, indicating the decreased or the lower numerical density of *C. madrsensis* larvae in the water body. SS*n*PCR with the plankton samples collected during 2012 shows the presence of *C. madrsensis* larvae from the second week of September until the second week of December with a similar trend of occurrence as in the previous year.

SSPCR was conducted with the wild collected plankton samples which contain random numbers of target species larvae. The SSPCR results could provide an idea of numerical density of the target bivalve larvae in the water body. Positive results of SSPCR with all five aliquots of subsamples taken for analysis from the preserved plankton sample (collected by filtering 12.5 tons of water) indicated an approximate numerical density of 106 numbers of target larvae in each 1000 liters of water filtered for sample collection. Analysis of the results of SSPCR with plankton samples collected from Thankassery Bay showed the presence of *P. viridis* larvae during the initial three weeks of June in 2011 and 2012. Similarly, the presence of *Perna indica* could be noticed in the water from the last week of May until the third week of June in 2011 and from the last week of May to the second week of June in 2012. Analysis of the results of SSPCR with whole plankton samples collected from Azhikode Estuary showed the presence of *C. madrasensis* larvae during the initial three weeks of October in 2011 and 2012.

Continuous larval detection through SS*n*PCR and positive results in SSPCR with the successive plankton samples are the indicators for the impending massive spat-fall of the target bivalve species. In this study, the continuous occurrence of the larvae of *P. viridis* and *P. indica* at a considerable numerical density at Thankassery Bay was observed during the month of June until the last week. The mussel spats of around 5 mm size were found to appear on the rocky break water of Thankassery Bay at the end of July and in the beginning of August in the same year. This shows the positive correlation of larval detection using SS*n*PCR and SSPCR with the succeeding spat-fall and spat settlement on the rocky substratum of the study area. Results of SS*n*PCR and SSPCR for the detection of *C. madrasensis* larvae in the plankton samples collected from Azhikode Estuary showed the continuous occurrence of the larvae at considerable

numerical density during the month of October. Hence, by the end of October 2011, spat collectors in the form of Rens were installed in the Azhikode Estuary in order to collect the spats of *C. madrasensis*. The spat settlement on the Rens could be noticed by the end of the November and in the beginning of December. Oyster spats were also found to appear on the rocky substratum of Azhikode Estuary during the same period.

To summarize; species identification DNA markers in the form of SSPCR primers were developed for the three target bivalve species, *P. viridis, P. indica* and *C. madrasensis*. The specificity, sensitivity and efficiency of these markers were standardized for each target species. From the analysis of lab results and correlating it with the field observations, it could be concluded that large number of spats of target species could be collected by installing spat collectors when successive positive results are obtained from both SS*n*PCR and SSPCR carried out with the plankton samples collected from the field. Initiation of plankton screening a month before the anticipated beginning of spawning period, and installation of the spat collectors based on the results of plankton screening tests would be a proper strategy for collecting the bivalve spats of interest from a location.

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