GENETIC DIVERGENCE IN LOBSTERS (CRUSTACEA: PALINURIDAE AND SCYLLARIDAE) FROM THE INDIAN EEZ

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GENETIC DIVERGENCE IN LOBSTERS (CRUSTACEA: PALINURIDAE AND SCYLLARIDAE) FROM THE INDIAN EEZ

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Doctor of Philosophy

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DECLARATION

I do hereby declare that the thesis entitled, **"Genetic divergence in lobsters (Crustacea: Palinuridae and Scyllaridae) from the Indian EEZ"** is the authentic and bonafide record of the research work carried out by me under the guidance of Dr. A. Gopalakrishnan, Principal Scientist and OIC, National Bureau of Fish Genetic Resources (NBFGR) Cochin Unit, Central Marine Fisheries Research Institute, Cochin in partial fulfillment for the award of Ph. D. degree under the Faculty of Marine Sciences of Cochin University of Science and Technology, Cochin and no part thereof has been previously formed the basis for the award of any degree, diploma, associateship, fellowship, or other similar titles or recognition.

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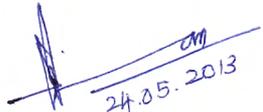
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CERTIFICATE

This is to certify that this thesis entitled, **"Genetic divergence in lobsters (Crustacea: Palinuridae and Scyllaridae) from the Indian EEZ"** is an authentic record of original and bonafide research work carried out by Ms**. Jeena. N.S.   (Reg. No. 3022)** at National Bureau of Fish Genetic Resources (NBFGR) Cochin Unit, Central Marine Fisheries Research Institute, under my supervision and guidance in partial fulfillment of the requirement for the award of the degree of **Doctor of Philosophy** in the Faculty of Marine Sciences, **Cochin University of Science and Technology, Cochin,** Kerala. It is also certified that no part of the work presented in this thesis has been submitted earlier for the award of any degree, diploma or any other similar title.

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(A. Gopalakrishnan)

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CONTENTS

List of Abbreviations

μg	:	Micro grams
μl	:	Micro litre
12SrRNA/12S	:	12S ribosomal ribonucleic acid
16SrRNA/16S	:	16S ribosomal ribonucleic acid
18SrRNA/18S		18S ribosomal ribonucleic acid
A	:	Adenine
BIOEDIT	:	Biological Sequence Alignment Editor
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base pairs
C	:	Cytosine
CHE	:	Chennai
CMFRI	:	Central Marine Fisheries Research Institute
COI	:	Cytochrome-c-oxidase subunit I
Cyt *b*	:	Cytochrome *b*
DNA	:	Deoxyribo Nucleic Acid
dNTPs	:	Deoxynucleoside tri phosphates
EDTA	:	Ethylene Diamine Tetra Acetic acid
FAO	:	Food and Agriculture Organization
F_{ST}	:	Fixation Index
G	:	Guanine
GD	:	Genetic distance
G_{ST}/F_{ST}	:	Coefficient of genetic differentiation
Hap	:	Haplotype
IUCN	:	International Union for Conservation of Nature and Natural Resources
K2P	:	Kimura-2-Parameter
MEGA	:	Molecular Evolutionary Genetics Analysis
MP	:	Maximum Parsimony method
MT	:	Metric Tonnes
mtDNA	:	Mitochondrial deoxyribonucleic acid
NBFGR	:	National Bureau of Fish Genetic Resources
NCBI	:	National Centre for Biotechnology Information
Ne	:	Effective population size
ng	:	Nano grams
NJ	:	Neighbour Joining algorithm
Nm	:	Rate of Gene flow
nucDNA	:	Nuclear DNA

P. homarus homarus	:	*Panulirus homarus homarus*
PCR	:	PCR Polymerase Chain Reaction
QLN	:	Kollam
RAPD	:	Random Amplified Polymorphic DNA
RFLP	:	Restriction Fragment Length Polymorphism
rpm	:	Revolutions per minute
SDS	:	Sodium Dodecyl Sulphate
T	:	Thymine
T. unimaculatus	:	*Thenus unimaculatus*
TN	:	Tamura-Nei
Ts	:	Transition
Tv	:	Transversion
VNTRs	:	Variable Number of Tandem Repeats
VRL	:	Veraval
VSK	:	Visakhapatnam

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Chapter 1

INTRODUCTION

1.1. Background and scope of the study

The invertebrate order Decapoda, which include freshwater prawns, shrimps, crabs, cray fishes, lobsters etc. forms one of the most diverse groups of the Class Crustacea. The ecological and morphological diversity of decapods, together with their economic importance, make them the most studied of all crustaceans (Martin and Davis, 2001). Lobsters include some of the most individually valuable and popularly fished crustacean species that have been in great demand for many years on world markets. They fall into several taxonomically distinct groups: the clawed lobsters (Nephropidae), spiny lobsters (Palinuridae), slipper lobsters (Scyllaridae) and the coral lobsters (Synaxidae). Lobsters play important roles in the ecosystems in which they are found, and virtually all the abundant species of them are subject to intense and similarly applied fishing pressure (Cobb and Phillips, 1980). The world catch of lobsters recorded in 2010 exceeded 2,79,000 metric tones (MT), of which 1,88,248 MT corresponded to true lobsters (Family Nephropidae), 78,518 MT to spiny lobsters (Family Palinuridae) and 10,310 MT to slipper lobsters (Family Scyllaridae). The genera that contributed to the highest in fishery were *Homarus* (1, 20, 000 MT) and *Nephrops* (66500 MT) of Nephropidae followed by *Jasus* (about 11,679 MT) and *Panulirus* (about 64,000 MT) of family Palinuridae (FAO, 2010). Worldwide, the market price for lobsters tended to rise in response to supply and demand rather than the costs involved in the production (Khan, 2006). Although the greatest number of commercial species occurs in tropical waters, the largest lobster catches come from cold-temperate regions like the northwest Atlantic (Fishing Area 21), and northeast Atlantic (Fishing Area 27). There are currently recognized six families, 55 genera and 248 species (with four subspecies) of living marine lobsters (Chan, 2010).

The lobster fishery is low volume but valuable and highly priced, which is estimated to constitute 1852 MT (0.34%) of total marine crustacean (5, 38,163 MT) landing in India during 2011 (CMFRI, 2012). Even though they

constituted only 0.058% of total marine landings in India in 2009, they contributed 0.25% in quantity and 1.0% in value (USD 20 million) of marine exports from the country (MPEDA, 2009). The lobster fishery in India is considered to be multi-species, comprising 14 species of littoral and six species of deep sea forms among which four littoral and one deep sea form are significant in commercial fishery (Radhakrishnan and Manisseri, 2003). The commercially important lobster species from Indian coast belong to two families, Palinuridae and Scyllaridae. Though distributed widely all along the Indian coast, major lobster fisheries are located on the northwest, southwest, and southeast coasts (Radhakrishnan and Manisseri, 2003). The northwest coast is particularly rich in lobster resources, contributing to nearly three quarters of the total lobster landing in India (Kagwade *et al*., 1991; Radhakrishnan, 1995). The annual landing of the lobsters in the country is on the decline as evident from catch data over the years from a peak of 4075 MT in 1985 to 1852 MT in 2011 (Radhakrishnan *et al*., 2005; CMFRI, 2002-2012) as depicted in the graph below (Fig. 1). The recent trends indicate that there will not be any significant increase in the landing from the presently exploited regions.
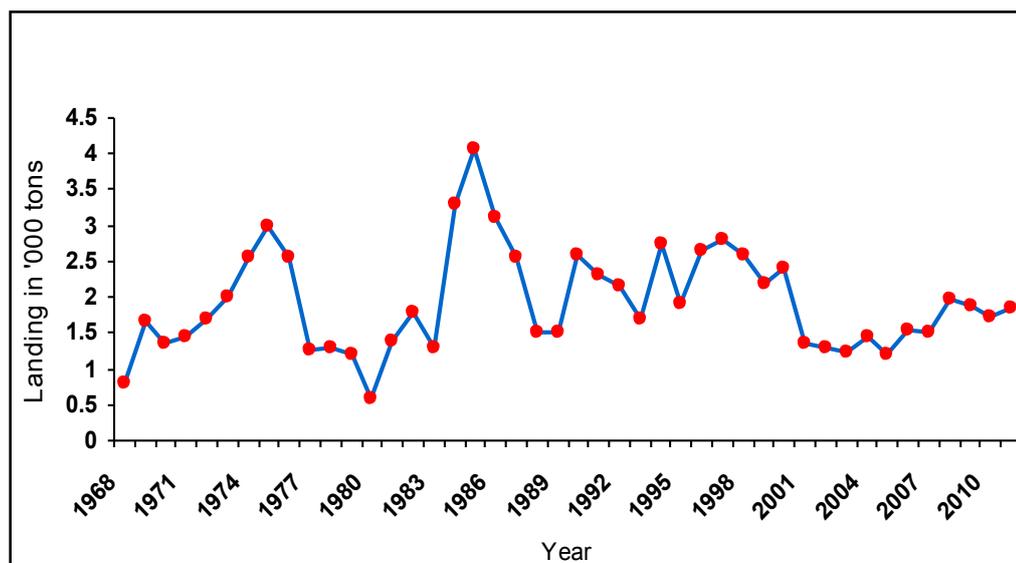
Fig. 1. Annual lobster landings in India during 1968-2012

Data adapted from: Radhakrishnan *et al.,* 2005 and CMFRI annual reports 2002-2012.

The slipper lobster *Thenus unimaculatus* Burton and Davie, 2007 and scalloped spiny lobster, *Panulirus homarus* (Linnaeus, 1758) were the most important species that contributed to the lobster fishery in India (CMFRI, 2011). The northwest coast fishery is mainly constituted by the spiny lobster *Panulirus polyphagus* and the slipper lobster *Thenus* spp. The shallow water *P. homarus homarus* is the most dominant species along the southwest coast, whereas *Panulirus ornatus*, *P. homarus* and *Thenus* spp. contribute to the fishery on the southeast coast. Small quantities of *Panulirus versicolor* are also landed along the Trivandrum and Chennai coasts. *Panulirus penicillatus* and *Panulirus longipes* are the two other species. The spiny lobster *Puerulus sewelli* is a deep-sea resident occupying the upper continental slope between 175-200 m depth off the south-west and south-east coasts from where they are fished by trawlers. *Linuparus somniosus* is another species of spiny lobster recorded from the Andaman waters (Radhakrishnan and Manisseri, 2003).

Spiny lobsters (Palinuridae) are one of the most commercially important groups of decapod crustaceans (Phillips, 2006; Palero and Abelló, 2007; Follesa *et al*., 2007) that are usually inhabitants of hard substrates associated with coral reefs, rocky shores and boulder-strewn bottoms. They are common throughout tropical and subtropical seas (Holthuis, 1991) and form some of the most important commercial fisheries of the world. There are eleven extant genera of spiny lobsters. Their biology, ecology and population genetics have therefore been the subject of intensive research for aquaculture and fishery management purposes. Among the invertebrate taxa, the longest pelagic larval duration (PLD) extreme are in spiny lobsters whose larval periods are typically 4 to 12 months with some as long as 24 months (Phillips *et al*., 2006). Out of the four commercially exploited species of spiny lobsters distributed along the Indian coast, the scalloped spiny lobster *Panulirus homarus* (Linnaeus, 1758), which has a wide distribution in the Indo-West Pacific region is the most dominant species along the southwest and southeast coasts of India. *P. homarus* is having three recognized sub-species (Berry, 1974; FAO, 1991). They are *P. homarus homarus* (Linnaeus, 1758) (Plate I-1), *P. homarus megasculptus* (Pesta, 1915) and *P. homarus rubellus* Berry, 1974. One more

sub-species *P. homarus* "Brown" endemic to Marquesas Islands was identified by George (2006a). Among the four subspecies, *P. homarus megasculptus* and *P. homarus rubellus* have large, well-developed scallops along the abdominal transverse grooves (the '*megasculpta*' form) and the other two, *P. homarus homarus* and *P. homarus* "Brown" possess low scallops along these grooves (the '*microsculpta*' form) (Plate II- C). Berry (1974) referred the *P. homarus megasculptus* as the 'Northern megasculpta form' and *P. homarus rubellus* as the 'Southern megasculpta form' (Plate II- A, B).

It is reported that all the three recognized subspecies of *Panulirus homarus* (*P. homarus homarus*, *P. homarus rubellus* and *P. homarus megasculptus)* are recorded in the Western Indian Ocean or Fishing area 51 (FAO, 1991). The nominotypical form (*P. homarus homarus*) is found throughout the range of the species. The FAO identification sheets (1991) and Berry(1974) reported occurrence of *P. homarus megasculptus* subspecies in the west coast of India along with other places of distribution like the south coast of Arabian Peninsula and Socotra, which is not confirmed by scientific studies. Major works in India were focussed on fishery assessment, general biology, breeding and culture of the resource. Attempts at rearing phyllosoma larvae of spiny lobsters through their entire life cycle have been unsuccessful due to difficulties in providing suitable diets in the later stages of development. Few molecular works has been carried out on spiny lobsters in India. For a commercially important species like *P. homarus*, whose hatchery technology has not been perfected anywhere in the world to date, the only way to conserve the stock is through proper management for which stock identification is a prime requisite or else the fishery will not be sustainable at the present level of exploitation.

Slipper or shovel-nosed lobsters belong to a fascinating family (Scyllaridae) within the order Decapoda, which are being targeted as a saleable by-product of spiny lobster or shrimp fisheries and are the focus of directed fisheries in some regions of the world like India, Hawai and Australia (Lavalli and Spanier, 2007; Vijayakumaran and Radhakrishnan, 2011). Altogether there are four subfamilies, 20 extant genera and 89 extant species

known to date in the family Scyllaridae (Yang *et al*., 2012). The family has a mainly warm-water distribution mainly between 30˚N and 30˚S (Webber and Booth, 2007). *Thenus* (Leach, 1815) is the most commercially significant of the seven scyllarid genera (Jones 1990, 1993) with many common names such as shovel nosed lobsters, slipper lobsters, flathead lobster and Moreton Bay Bug or bay lobster in Australia. They are bottom-dwellers and inhabit sand and mud from 10 to 50 m depth. The shovel-nosed lobster genus *Thenus* Leach, 1815, long considered monotypic with *Thenus orientalis* (Lund, 1793), was revised by Burton and Davie (2007). They resurrected *T. indicus* Leach, 1815 from the synonymy of *T. orientalis* and described three new additional species *T. australiensis*, *T. unimaculatus* and *T. parindicus*. *Thenus* was long considered to contain only *Thenus orientalis* and *Thenus indicus*. Earlier studies and reports of shovel nosed lobsters of the genus *Thenus* in India were based on the single species– *Thenus orientalis* (Prasad and Tampi, 1957; Chacko, 1967; Rahman and Subramoniam, 1989; Kagwade and Kabli, 1996; Deshmukh, 2001; Subramanian, 2004; Kizhakudan *et al*., 2004 (a, b); Radhakrishnan *et al*., 2005; Radhakrishnan *et al*., 2007; Vijayakumaran and Radhakrishnan, 2011). The annual landing of *Thenus* spp. resource has also fallen drastically from about 600 MT to about 130 MT over a span of a decade (1991 - 2001) (Kizhakudan, 2006a). In Mumbai, the slipper lobster *T. orientalis* disappeared from the fishery by 1994 (Deshmukh, 2001) due to recruitment overfishing (Radhakrishnan *et al*., 2007). At Veraval, there was a drastic decline in lobster fishery from an average of 97.7 MT (1991-2000) to 6 MT in 2004 (Radhakrishnan *et al*., 2007). Even though the seed production techniques of *Thenus* spp*.* has been standardized in India at CMFRI (Kizhakudan *et al*., 2004a), it has been not been taken up to a commercial level. In view of the species revision of the previously believed monotypic *Thenus* spp., and the lack of information on species composition and also at intra-species level of shovel-nosed lobsters, there is a need to carry out in-depth analysis on these lines for accurate documentation of lobster diversity in Indian seas. The genetic identity of *Thenus* widely distributed along the coast of India was confirmed to be *Thenus unimaculatus* Burton and Davie, 2007 in this study (Plate VI-1).

A pre-requisite for the management of commercially exploited fish and shellfish resources is to define how the resource is partitioned spatially (geographically) and temporally, i.e., to identify stock units (Ungfors *et al*., 2009) so that individual stocks can be managed to better ensure their long–term sustainability. Failure to recognize stock structure of an exploited species can lead to over fishing and depletion of less productive stocks. Much of the difficulty in successfully managing marine species arises from the lack of knowledge of population connectivity in organisms with a pelagic larval stage (Carr *et al*., 2003). Evidence for marine geographical speciation must be evaluated through geographical studies of genetic and morphological differences among populations and between species (McCartney *et al*., 2000). By characterizing the distribution of genetic variation, population sub structuring can be detected and the degree of connectivity among populations estimated (Nesbo *et al*., 2000; Hutchinson *et al*., 2001). Efforts to establish effective marine protected areas require detailed information regarding connectivity among disjunct populations of species (Halpern and Warner, 2003; Cowen *et al*., 2006). The lobsters also belong to the highly migratory group with a lengthy pelagic larval life and hence wide larval dispersal. Unlike other lobster fishing countries like Australia where the fishery appears to be sustainable, the fished populations in India appear to be overexploited. Although Ministry of Commerce and Industry, Government of India promulgated Minimum Legal Size for export of lobsters (Notification No. 16 (RE 2003)/2002-07 dated 17 July, 2003) and participatory management approach project has been formulated and implemented (Radhakrishnan and Thangaraja, 2008), the connectivity pattern or the population sub-structuring of the lobster species has not been assessed from Indian coastline without which marine protected areas cannot be designed.

Population genetics offers a useful technique for studying the population structure of marine organisms and has relevance to both systematics and the conservation of biodiversity. The genetic makeup of a species is variable between populations of a species within its geographic range. Loss of a population results in a loss of genetic diversity for that species and a reduction

of total biological diversity for the region. This level of biodiversity is critical in order for a species to adapt to changing conditions and to continue to evolve in the most advantageous direction for that species.

Molecular genetic markers are powerful tools to detect genetic uniqueness of individuals, populations or species and are powerful tools for describing stock structure **(**Utter, 1991; Avise, 1994; Linda and Paul, 1995). It is theoretically possible to observe and exploit genetic variation in the entire genome of organisms with DNA markers. Both genomic and mitochondrial DNA is used for varied applications. The commonly used technique are allozyme analysis, types of restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellite typing, single nucleotide polymorphism (SNP), and expressed sequence tag (EST) markers, etc. Although to date marine invertebrate fisheries have not received the same level of attention from geneticists as finfish fisheries, it is clear that for invertebrate fisheries it is relatively far more important to have genetic data if a fishery is to be exploited without being endangered (Thorpe *et al*., 2000).

RAPD (Random Amplified Polymorphic DNA) technique (Welsh and McClelland, 1990) has been proved a quick and effective method for the detection of intra- and interspecific genetic polymorphism in Crustacea (Baratti *et al*., 2003). Mitochondrial DNA (mtDNA) can assist in determining the taxonomic distinctiveness of individual populations and therefore aid in setting priorities for future management and conservation programmes (Moritz, 1994; Stamatis *et al*., 2004). The mt DNA COI gene has been extensively used in population genetics studies of a wide variety of marine invertebrates (e.g., Kelly and Palumbi, 2010; Krakau *et al*., 2012; da Silva *et al*., 2011; Naro-maciel *et al*., 2011) and is considered as an ideal molecular marker to identify genetic variation in natural populations. Even though mtDNA phylogenies can provide unique insights into population history (Avise, 1994), mtDNA must be used in conjunction with nuclear markers to identify evolutionary distinct populations for conservation (Cronin, 1993). Hence in this study, a combination of RAPD, a type II nuclear marker and partial sequences of hypervariable region of mtDNA

COI gene are used to analyse the stock structure of *P. homarus homarus* and *T. unimaculatus* populations along the Indian coast.

A solid taxonomy is fundamental to all biology, and phylogenies provide a sound foundation for establishing taxonomy (Chen *et al*., 2004). Molecular genetic data have become a standard tool for understanding the evolutionary history and relationships among species (Avise, 1994). Mitochondrial cytochrome oxidase I gene (COI), was recently elected as the standardized tool for molecular taxonomy and identification (Ratnasingham and Hebert, 2007). DNA barcoding (Hebert *et al*., 2003a) using the COI gene to identify species, has helped to rejuvenate taxonomic research. However, other genes are also required to evaluate the evolution or phylogenetic information contained in the barcode region of mtCOI (DeSalle *et al.*, 2005; da Silva *et al*., 2011). In the present study species-specific signatures for 11 commercially important lobster species along the Indian coast *viz. P. homarus homarus*, *P. versicolor*, *P. ornatus*, *P. longipes longipes, P. polyphagus, P. penicillatus, Puerulus sewelli* and *Linuparus somniosus* of family Palinuridae and *T. unimaculatus, T. indicus* and *Petrarctus rugosus* of family Scyllaridae were generated using COI and additional mitochondrial (mtDNA) genes like 16SrRNA, 12SrRNA as well as by the nuclear 18SrRNA gene.

1.2. Objectives of the present study

A. To assess the genetic stock structure of the scalloped spiny lobster *Panulirus homarus* (Linnaeus, 1758) and the shovel-nosed lobster *Thenus unimaculatus* Burton and Davie, 2007 from Indian coast using RAPD and hypervariable region of mt-DNA Cytochrome Oxidase I gene.

B. To develop species-specific molecular signatures and derive phylogenetic relationship of 11 commercially important lobster species using partial sequence information of mitochondrial COI, 16SrRNA, 12SrRNA and nuclear 18SrRNA genes.

The ultimate outcome will be

1) The genetic stock structure of *P. homarus homarus* and *Thenus unimaculatus* using molecular markers, which will be helpful in

    a) determination of genetic variation in natural population of these fast declining resources that would reveal the extent of genetic base restriction that has taken place.

    b) Conservation and management of natural resources of lobsters in Indian waters.

2) Generate species-specific markers

    a) for accurate species identification of lobsters at phyllosoma/ puerulii/ adult phase.

    b) reconstruction of phylogeny based on the above data to understand the evolutionary relationships among species.

1.3. DESCRIPTION OF THE SPECIES

Key for identification of family, genera and species

All the eleven species included in the present study were identified as per FAO (1991) and Burton and Davie (2007). Identifying features are listed below and figures are given in plates. Comparatively longer descriptions are provided for *P. homarus homarus* and *T. unimaculatus* as they are selected for population structure analysis. The figures 1A, 1B, 2A and 2B of Plates I, III and IV; 1A and 2A of Plate V; 2A of Plate VII are adapted from FAO (1991) for comparison.

1.3.1. FAMILY PALINURIDAE Latreille, 1802

Lobsters of this family are commonly known as spiny lobsters. They are moderate to large-sized crustaceans with a carapace subcylindrical in section, without a distinct median rostrum, ornamented with spines and granules of

various sizes; each eye protected by a strong, spiny frontal projection of the carapace (frontal horns). The antennae are long and whip-like, antennules slender, each consisting of a segmented peduncle and two long or short flagella. In some genera, the bases of antennae are separated by a broad antennular plate usually bearing 1 or 2 pairs of spines, but spineless in some species; a projection from the base of each antenna forms with the rim of the antennal plate a stridulating organ, through which the animal by movement of the antenna can produce a grating/ stridulating sound (the stridentes lineage). Tail powerful, with a well developed fan; abdominal segments either smooth or with one or more transverse grooves. Legs without true pincers or chelae (except the fifth pair of legs of the female, which ends in a very small pincer), the first pair usually not greatly enlarged. Most species are brightly coloured and patterned with bands or spots, others uniform.

Taxonomic status

Phylum	:	Arthropoda
Subphylum	:	Crustacea
class	:	Malacostraca
Subclass	:	Eumalacostraca
Superorder	:	Eucarida
Order	:	Decapoda
Suborder		Macrura Reptantia
Infraorder	:	Achelata
Family		Palinuridae

Key to genera occurring in the area:

Two distinct, widely separated tooth-like frontal horns, between which the anterior margin of the carapace is visible; antennal flagella quite flexible. Flagella of antennulae long, whiplike, longer than peduncle of antennule, antennular plate and stridulating organ present..................*Panulirus*

Frontal horns with a single tooth on anterior margin; pleura of second to fifth abdominal segments ending in two about equally strong teeth, antennular plate and stridulating organ present; carapace strongly ridged…..... *Puerulus*

Frontal horns fused to a quadrangular median process, with 2 points placed over bases of eyes; antennal flagella straight, inflexible....................*Linuparus*

A. GENUS *PANULIRUS*

A.1. *Panulirus homarus* (Linnaeus, 1758)

This palinurid lobster species was first described by Linnaeus in 1758 as *Cancer homarus* (Systema Naturae, (ed. 10)1: 633) and the type locality is Amboina, Moluccas, Indonesia. The species is having the vernacular name 'Scalloped spiny lobster'**.**

Diagnosis: The species has a tubular body; carapace without a rostrum, legs 1-4 without true pincers; first pair not enlarged, Antennae enlarged, cylindrical, longer than body. Carapace (or "head") rounded, without a distinct median rostrum, ornamented with spines and granules of various sizes; each eye protected by a strong, spiny frontal projection of the carapace (frontal horns). Anterior margin of carapace between frontal horns with about 10 small, sharp teeth; pleura of second to fifth abdominal segments ending in a strong tooth with denticles on posterior margin. Antennae long and whip-like, antennules slender, each consisting of a segmented peduncle and two long or short flagella. Flagella of antennules long, whiplike, longer than peduncle of antennule, a projection from the base of each antenna forms with the rim of the antennal plate a stridulating organ, through which the animal by movement of the antenna can produce a grating sound. Bases of antennae separated by a broad antennular plate bearing two equal, well separated pairs of principal spines and scattered smaller spines in between (Plate I-1A).

Each abdominal segment with a transverse groove, sometimes interrupted in the middle, its anterior margins formed into shallow scallops. The scalloped anterior margin of the transverse groove of the abdominal segments

distinguishes *P. homarus* from all other species of the family Palinuridae (Plate I-1B). Tail powerful, with a well developed fan; abdominal segments either smooth or with one or more transverse grooves.

P. homarus homarus (Linnaeus, 1758) with the scallops of the abdominal grooves small and indistinct, especially in the median part of the groove, which is often interrupted there, colour dark greenish to blackish with numerous, very small white spots (especially distinct on posterior half of abdomen), without transverse bands, antennules banded with white and greenish, legs with indistinct spots and stripes of white, the squamae of the abdominal grooves range from being at best poorly developed, truncate and irregular in size, to so minute as to be virtually indistinguishable . When present these squamae are best developed laterally and become reduced in size and usually disappear medially where the abdominal grooves are often interrupted. This median interruption is normally present in at least one segment and sometimes in up to four. Specimens with this morphology, which will be referred to as belonging to the "microsculpta form", are always dark green in overall colour (Berry, 1974) (Plate I-1). According to Holthuis (1946) this is the original figure on which Linnaeus (1758) based the name Cancer homarus. Hence the name *P. homarus homarus* (Linnaeus, 1758) is used for the microsculpta form (Berry, 1974) (Plate II-C).

Distribution: Indo-West Pacific region: East Africa to Japan, Indonesia, Australia, New Caledonia and probably the Marquesas Archipelago**.** It is described from FAO Fishing Area 51 (Western Indian Ocean). The nominotypical form (*P. homrus homarus*) is found throughout the range of the species. It is the most widely distributed among the three subspecies of *P. homarus* and is found throughout the Indo-Pacific region with centers of high concentrations in East Africa and Indonesia (Berry, 1974; Pollock, 1993).

Habitat and Biology: Found in rocky areas, often in the surf zone, sometimes in somewhat turbid water. The species inhabits shallow waters between 1 and 90 m depth, mostly between 1 and 10 m. It is gregarious and nocturnal. It

attains a maximum size of 320 mm, carapace length of 120 mm, average total body length is 20 to 25 cm, grows to a max of 1.5 kg and attains sexual maturity at 55 mm carapace length around 175 g.

A.2. *Panulirus versicolor* **(Latreille, 1804)**

Commonly known as 'painted spiny lobster'. Carapace rounded, covered with numerous spines of varying size; bases of antennae separated by a broad antennular plate bearing two pairs of unequal and separated principal spines (Plate I-2A). Abdominal segments without transverse grooves. Colour: green-blue with a distinctive pattern of blue-black patches and white lines on carapace; a transverse band of white, bordered by two black lines, across each abdominal segment (Plate I-2B). The bright colour pattern of this species clearly separates it from all other lobsters, legs and antennules longitudinally striped; bases of antennae bright pink not extending on to antennular plate (Plate I-2).

A.3. *Panulirus ornatus* (Fabricius, 1798)

'The ornate spiny lobster' has a broad antennular plate bearing one pair of principal spines anteriorly and a second pair, half the size of the first, in middle of the plate (Plate III-1A). Each abdominal segment smooth, without a transverse groove (Plate III-1B). Colour: abdomen with a broad, dark transverse band over the middle of the segments, legs with distinct, sharply defined dark and pale blotches. The presence of only two spots on either side of the second to fourth abdominal segments, and the presence of vermicular markings on and near the bases of frontal horns, distinguishes this species from all other *Panulirus* species in the area   (Plate III-1).

A.4. *Panulirus longipes longipes* (A. Milne Edwards, 1868)

This species with a vernacular name 'Longlegged spiny lobster' has a rounded carapace covered with numerous spines of varying size. The antennular plate having one pair of principal spines followed by some scattered minor spines

(Plate III-2A). Each abdominal segment with a complete transverse groove joining the pleural groove (Plate III-2B).

Colour: variable from brown through blue to indigo; carapace and tail covered with numerous medium-sized pale spots, and a central darker region on the carapace; crossbanded antennal and antennular flagella. The subspecies *P. longipes longipes* is characterized by spotted legs and lines of yellow in between which distinguishes it from the *P. longipes femoristriga* which has visibly banded legs (Plate III-2).

A.5. *Panulirus polyphagus* (Herbst, 1793)

The 'Mud spiny lobster' *P. polyphagus* has a rounded carapace, antennular plate bearing a single pair of principal spines (Plate IV-1A); antennules very long, about 1½ times the total body length; abdominal segments without transverse grooves. Colour: dull greenish, abdominal segments each with a distinct transverse band of white (not black -edged) across posterior margin (Plate IV-1B). Antennules broad-banded; legs irregularly blotched creamy white. No other spiny lobster has such long antennules nor the conspicuous plain white crossbands near hind margins of abdominal segments (Plate IV-1).

A.6. *Panulirus penicillatus* (Olivier, 1791)

Commonly known as the 'Pronghorn spiny lobster' which has an antennular plate bearing two pairs of almost equal principal spines joined at their bases, their tips diverging (Plate IV-2A). Each abdominal segment with a transverse groove not joining the pleural groove (Plate IV-2B). Colour: ground colour in a wide range with many cream spots on upper surface of carapace, and many tiny pale spots on abdomen; antennular flagella uniform green or brown; legs with fine or broader longitudinal white to yellow stripes. Males are usually darker than females in any one area. No other spiny lobster has two pairs of almost equal spines joined at their bases on the antennular plate (Plate IV-2).

B. GENUS *PUERULUS*

B.1. *Puerulus sewelli* Ramadan, 1938

The Arabian whip lobster, *P. sewelli* has an angular carapace (unlike the rounded carapace of species 1-6), with a median arid two lateral tuberculate longitudinal ridges behind the transverse cervical groove, and three pairs of ridges in front (the first pair submedian, converging anteriorly and posteriorly; the second originating behind the frontal horns and the third behind the antennal bases); median postcervical ridge with eight small teeth; frontal horns compressed and sharply pointed, with a single, small, sharp tooth on basal part of anterior margin; surface of carapace covered with scattered granules, and larger tubercles or teeth on the ridges (Plate V-1A). Antennules slightly over reaching antennal peduncle, with two short flagella; antennular plate present, without spines, forming stridulating organs with the antennal peduncle; basal part of antennal peduncle with a large, rounded, ciliated lobe on inner margin. Tail powerful, segments one to three with a low, tuberculate median longitudinal ridge, sixth segment with two sub median, tuberculate ridges. Surface of abdominal segments with some sculpturation, and with at most two transverse grooves; pleura ending in one or two sharp teeth. Legs one to four without pincers. None of the other lobster species of this family have the six precervical and three postcervical ridges on the carapace typical of *Puerulus* (Plate V-1).

C. GENUS *LINUPARUS*

.C.1. *Linuparus somniosus* Berry and George, 1972

The species commonly known as 'African Spear lobster' has a carapace which is angular dorsally, with ore median and two lateral longitudinal crests behind the cervical groove, the two frontal horns are moved to the central part of the anterior margin and fused to a single broad two- or four-pointed lobe between the eyes; antennae long, flagella long and stiff, slightly flattered and rigid; bases of antennae touching each other, antennular plate very small, covered

by a stridulating organ (Plate V-2A). Tail powerful; each abdominal segment with at most one transverse groove; and, on each side, a longitudinal, tuberculate crest over the bases of the pleura; first five segments with a median crest with that of sixth segment double. Colour: reddish brown dorsally; laterally and ventrally mostly whitish; antennal flagella dirty white. All other species of Palinuridae except *L.somniosus* has widely separated frontal horns (not fused); cylindrical abdomen and without a longitudinal crest over bases of pleura (Plate V-2).

1.3.2. FAMILY SCYLLARIDAE

Phylum	:	Arthropoda
Subphylum	:	Crustacea
class	:	Malacostraca
Subclass	:	Eumalacostraca
Superorder	:	Eucarida
Order	:	Decapoda
Suborder		Macrura Reptantia
Infraorder	:	Achelata
Family	:	Scyllaridae

The lobsters belonging to this family are commonly called as the 'Slipper lobsters'. They are small to large crustaceans (total length between 2 and 40 cm) with a more distinctly flattened body than in any other group of lobsters. Carapace usually granular, sometimes with teeth, spines and ridges; eyes movable but recessed into anterior margin of carapace. Antennae short and broad, plate-like, lacking flagella; antennules short and slender, with two short flagella. Tail broad and powerful, with a well developed tail fan. All legs without pincers (except the fifth leg of the female which in most species ends in a small pincer); all legs of about same size. No other family of lobsters has such a flattened body or plate-like antennae without flagella.

Key to genera used in the study:

Eyes placed at the anterolateral corners of carapace; carapace flat, triangular, narrowing posteriorly; posterior lateral margin without teeth................... *Thenus*

Small-sized lobsters (adults less than 10 cm in total length); margin of distal segment of antenna with few (less than 10) distinct wide teeth; abdominal segments either with a transverse groove or with arborescentnarrow grooves, without elevated crenulated structures...................................... *Scyllarus*

A. GENUS *THENUS*

Previously the shovel-nosed lobster genus *Thenus* Leach, 1815, long considered to contain only *Thenus orientalis* (Lund, 1793) was revised by Burton and Davie, 2007. Three new species *T. australiensis*, *T. unimaculatus* and *T. parindicus* are diagnosed along with the already described *Thenus indicus* and *Thenus orientalis* species. The collected *Thenus* spp. from Indian coast was identified as per Burton and Davie, 2007. The identifying characters of the most abundant *Thenus unimaculatus* and sparingly caught *Thenus indicus* species are given below.

A.1. *Thenus unimaculatus* Burton and Davie, 2007

This is a scyllarid lobster described by Burton and Davie in 2007 from Phuket, Thailand (Plate VI- 1).The vernacular names for the species are shovel-nosed lobster, slipper lobster, sand lobster or flathead lobster. It belongs to subfamily Theninae of Scyllaridae.

Distinguishing Characters

Diagnosis: Purple to black pigmentation blotch on inner face of on the propodus of first, second sometimes the third pereiopods (Plate VI-1C, 1D), usually large but variable in extent and may be reduced to a narrow streak; purple pigmentation occasionally surrounding eye socket on carapace; outer face of propodus of second pereipod having upper-most longitudinal groove

bearing obvious setae over at least proximal half. Merus of third maxilliped with a small spine proximally on inner ventral margin; inner margin of ischium prominently dentate along entire length. No single morphometric ratio has been isolated that will exclusively identify this species, but only *T. unimaculatus* can have ratios that fall outside the following maximum and minimum values: carapace width (CW1) greater than 1.29 times carapace length (CL); length of propodus of first pereiopod (PL1) less than 0.23 times carapace length (CL); length of propodus of second pereiopod (PL2) greater than 0.39 times carapace length (CL); width of propodus of first pereiopod (PW1) greater than 0.35 times length (PL1).

Distribution: The species is apparently confined to the Indian Ocean and is known only from a few specimens from Thailand, United Arab Emirates, and Mozambique and its exact distribution is unknown. The data on the distribution of species is deficient in the IUCN Red List of Threatened Species (Version 2011.2). Furthermore data are lacking on population, habitat and threats to this species. Further research is required before a more accurate conservation assessment can be made.

A.2. *Thenus indicus* Leach, 1815

Diagnosis: No spotting on pereiopods; outer face of propodus of second pereiopod having upper-most longitudinal groove bearing obvious setae over at least proximal half (Plate VII-1A). Merus of third maxilliped with a small spine proximally on inner ventral margin; inner margin of ischium prominently dentate along entire length. No single morphometric ratio has been isolated that will exclusively identify this species, but only *T. indicus* can have ratios that fall outside the following maximum and minimum values: merus width (MW1) less than 0.07 carapace length (CL); merus length (ML3) more than 0.48 carapace length (CL) (Iamsuwansuk *et al*., 2012) (Plate VII-1).

B. GENUS *PETRARCTUS*

B.1. *Petrarctus rugosus* (H. Milne Edwards, 1837)/ *Scyllarus rugosus* H. Milne Edwards, 1837

The species is commonly known as 'Hunchback locust lobster' which belong to subfamily Scyllarinae. The carapace has the median teeth before the cervical groove blunt and inconspicuous: the rostral tooth is reduced to a tubercle, the pregastric tooth is replaced by a double row of one or two tubercles and a few inconspicuous median tubercles. The gastric tooth is the most conspicuous, it is broad and blunt and bears a double row of tubercles. The surface of the carapace is very uneven and the tubercles are high (Plate VII-2A). The abdomen shows a distinct median longitudinal carina on somites two to five, that of somite three is by far the highest, and (like the one of somite four) bears numerous tubercles laterally. In each somite there is a wide transverse groove there. In second somite, both before and behind this groove there is a perfectly smooth broad ridge, a character in which the species differs from most others. The fourth antennal segment has a sharp and high oblique median carina. Outside the carina on the upper surface of the fourth antennal segment,a row of tubercles is present. The outer margin of the segment bears four or five teeth (apical tooth of the segment not included), the inner margin has five to seven teeth of irregular size. The anterior margin of the thoracic sternum is deeply U-shapedly incised. Each of the thoracic sternites bears a rounded median tubercle. The dactyli of pereiopods three to five show two short fringes of hair each. The dorsal surface of the body is greyish or purplish brown with darker spots. The distal segment of the antenna is often lighter. The first abdominal somite shows dorsally often a dark blue colour (Plate VII-2).
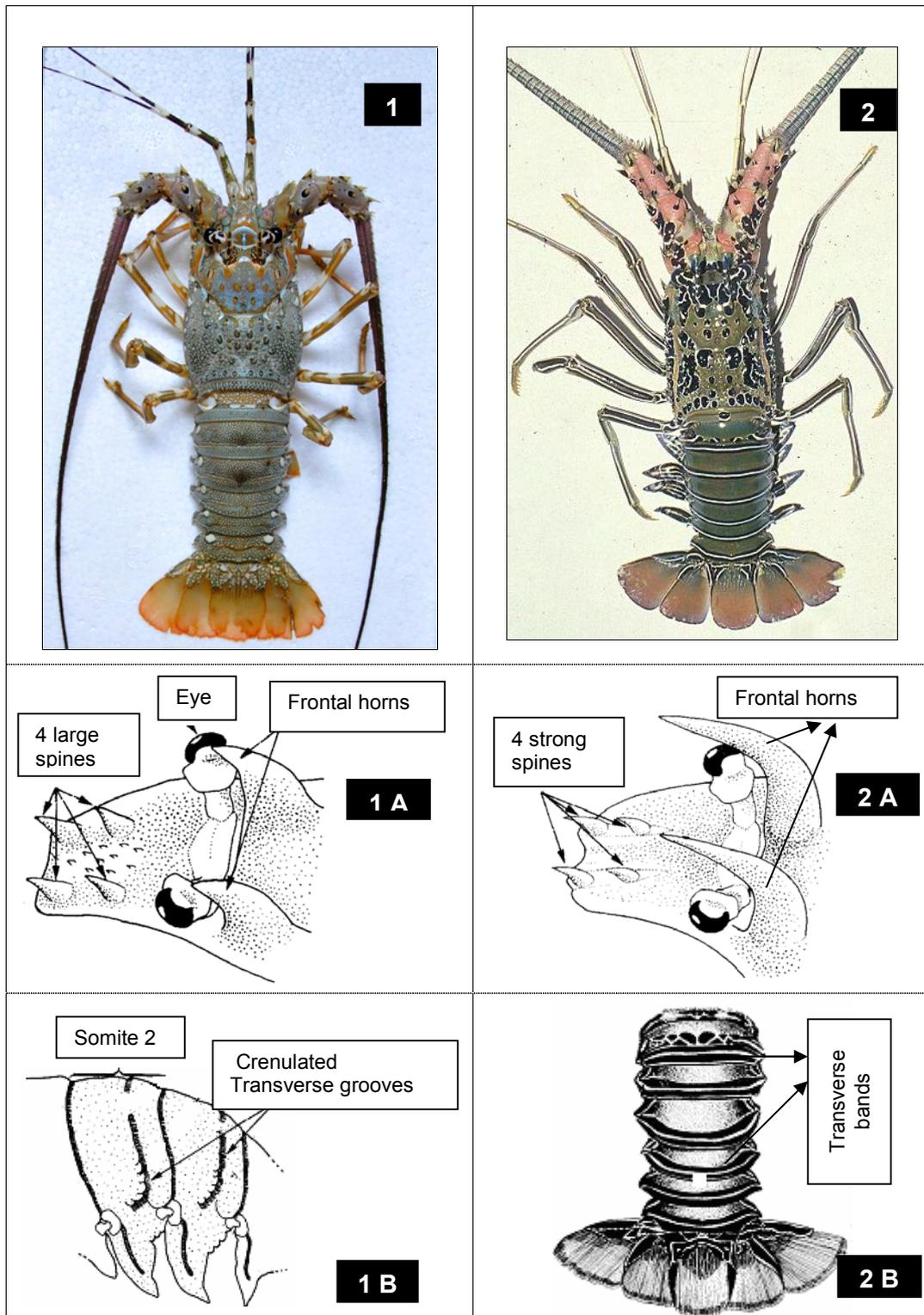
PLATE- I

1. ***Panulirus homarus*** (dorsal view); **1 A**- Antennular plate; **1 B**- Abdominal somites (lateral view)

2. ***Panulirus versicolor*** (dorsal view); **2 A**- Antennular plate; **2 B**- Abdominal somites (lateral view)

I) *Panulirus homarus* **megasculpta form**

Panulirus homarus megasculptus
(Northern megasculpta form
- from Oman)

Panulirus homarus rubellus
(Southern megasculpta form
-from South Africa)

Deep and large scallops and no median interruption

(Courtesy: *Dr.Johan Groenfield,ORI,
South Africa*)

(Courtesy*: Dr.Peter Fielding, FieldWork,
South Africa*)

II) *Panulirus homarus* **microsculpta form –**
Panulirus homarus homarus- (from the Indian coast)
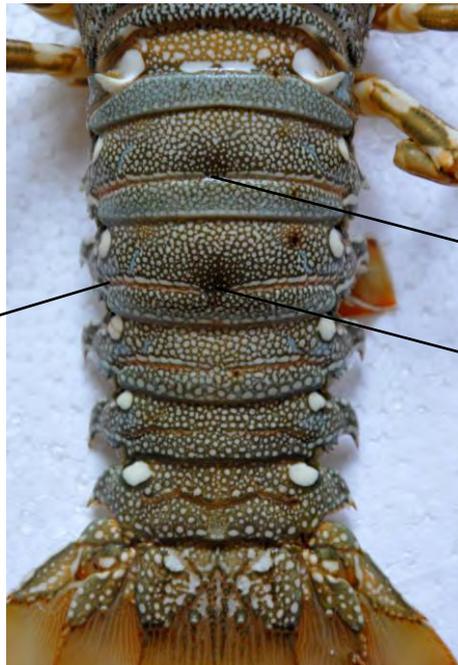
Shallow scallops

Prominent median interruption in the transverse abdominal grooves

PLATE- II - Subspecies of *Panulirus homarus*

21
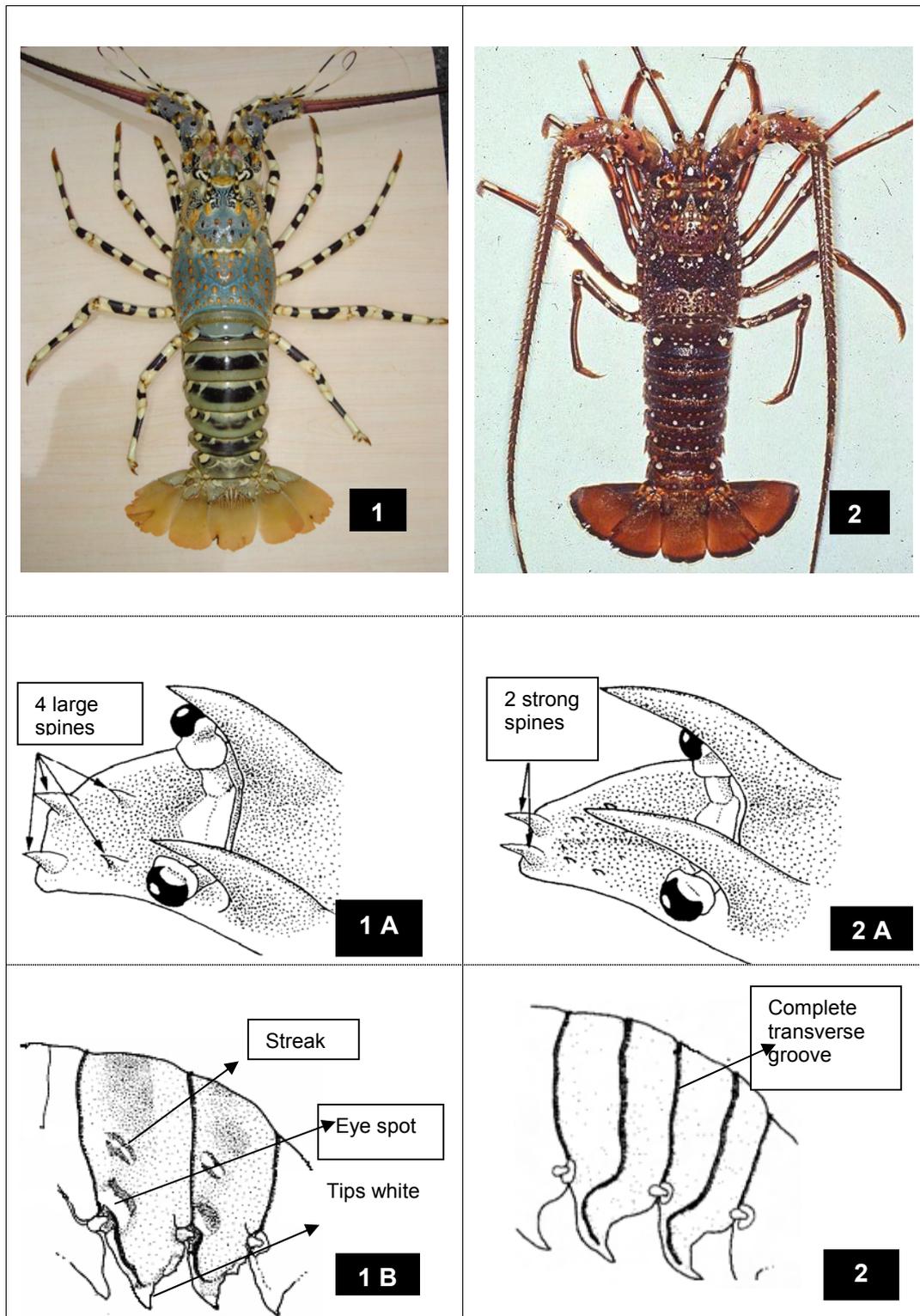
PLATE- III

1. ***Panulirus ornatus*** (dorsal view); **1 A**- Antennular plate; **1 B**- Abdominal somites (lateral view)

2. ***Panulirus longipes longipes*** (dorsal view); **2 A**- Antennular plate; **2 B**- Abdominal somites (lateral view)
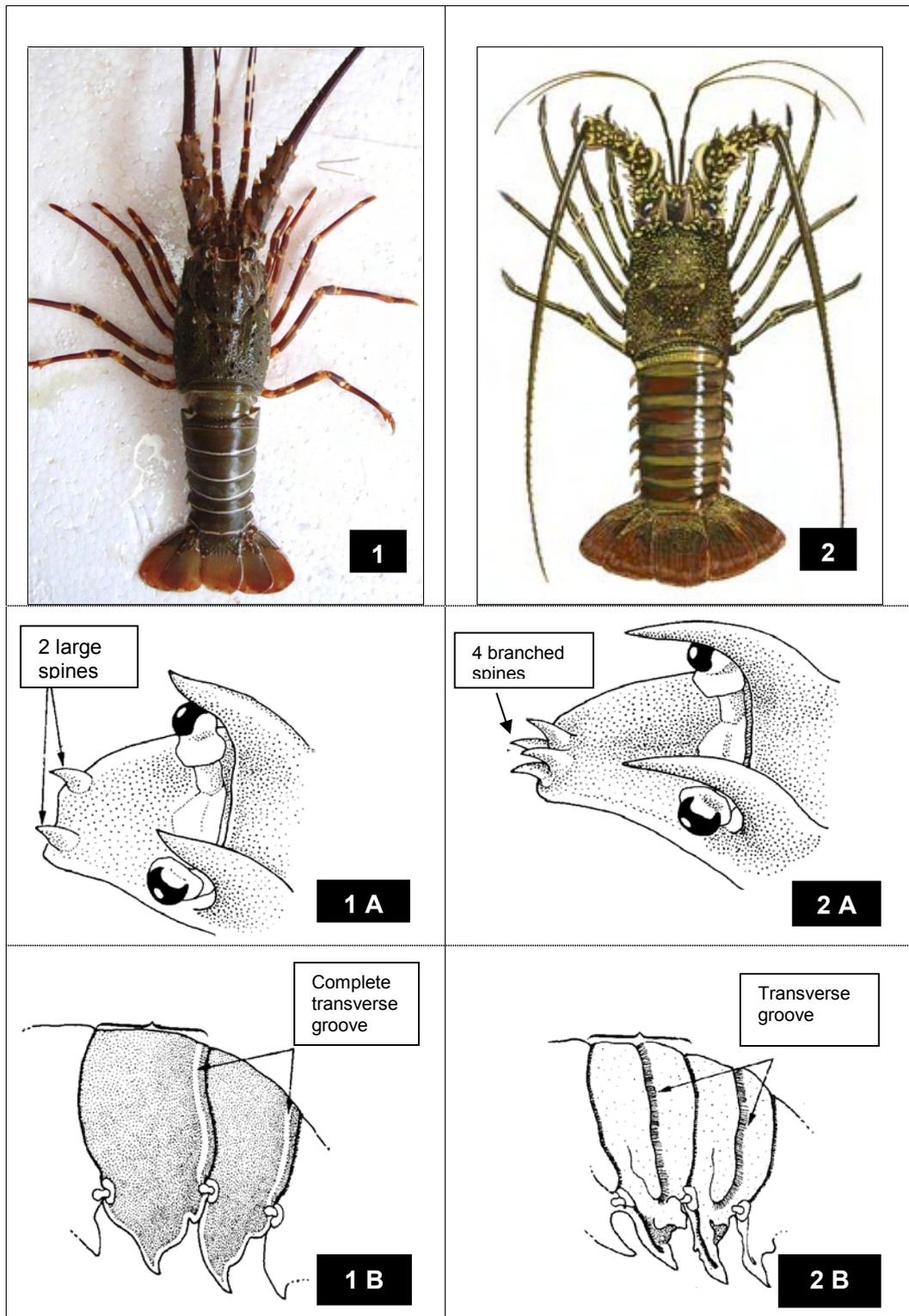
PLATE- IV

1. *Panulirus polyphagus* (dorsal view); **1 A**- Antennular plate; **1 B**- Abdominal somites (lateral view)

2. *Panulirus penicillatus* (dorsal view); **2 A**- Antennular plate; **2 B**- Abdominal somites (lateral view)
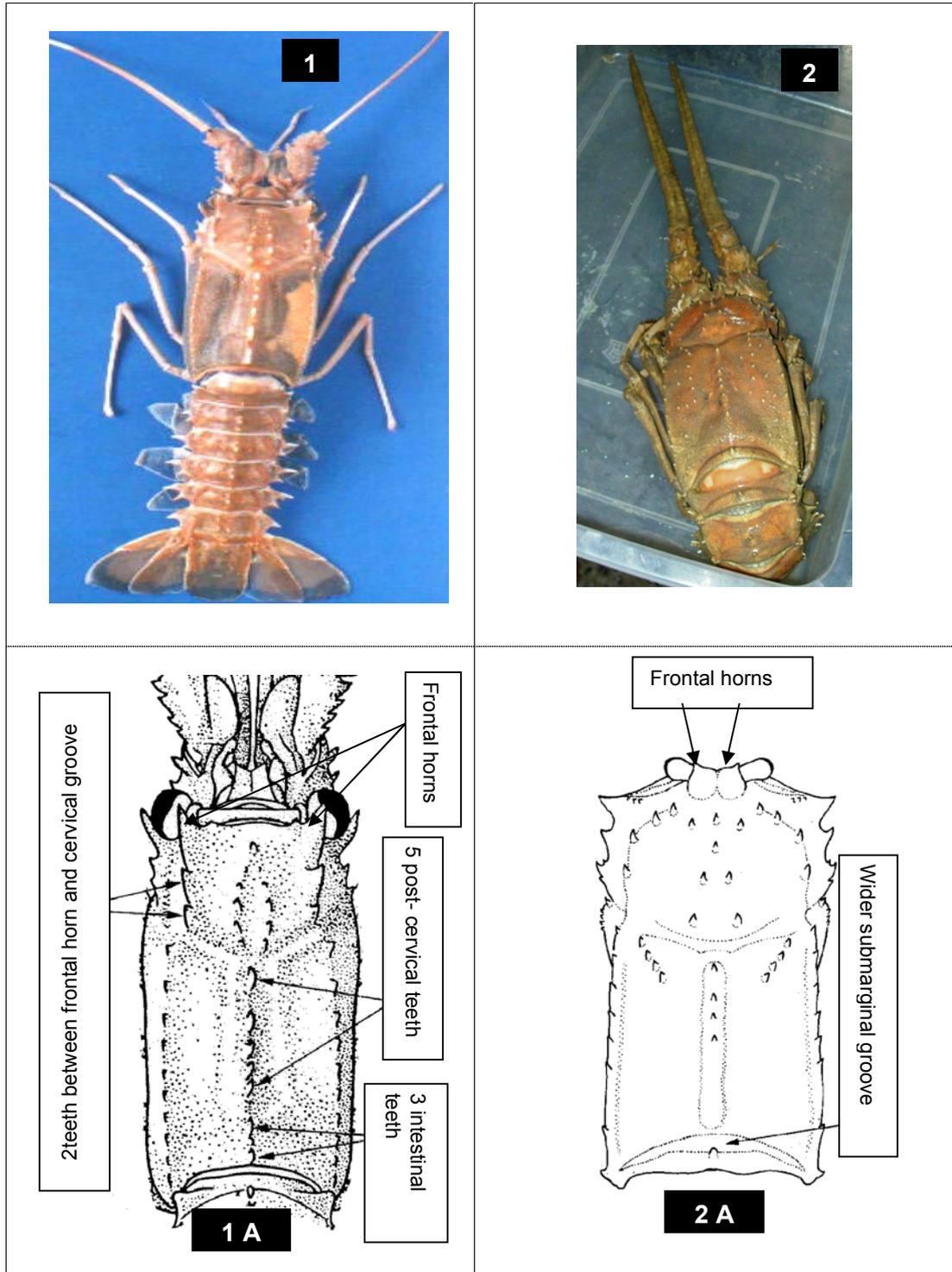
PLATE- V
1. *Puerulus sewelli* (dorsal view); **1 A**- Antennular plate.
2. *Linuparus somniosus* (dorsal view); **2 A**- Antennular plate.
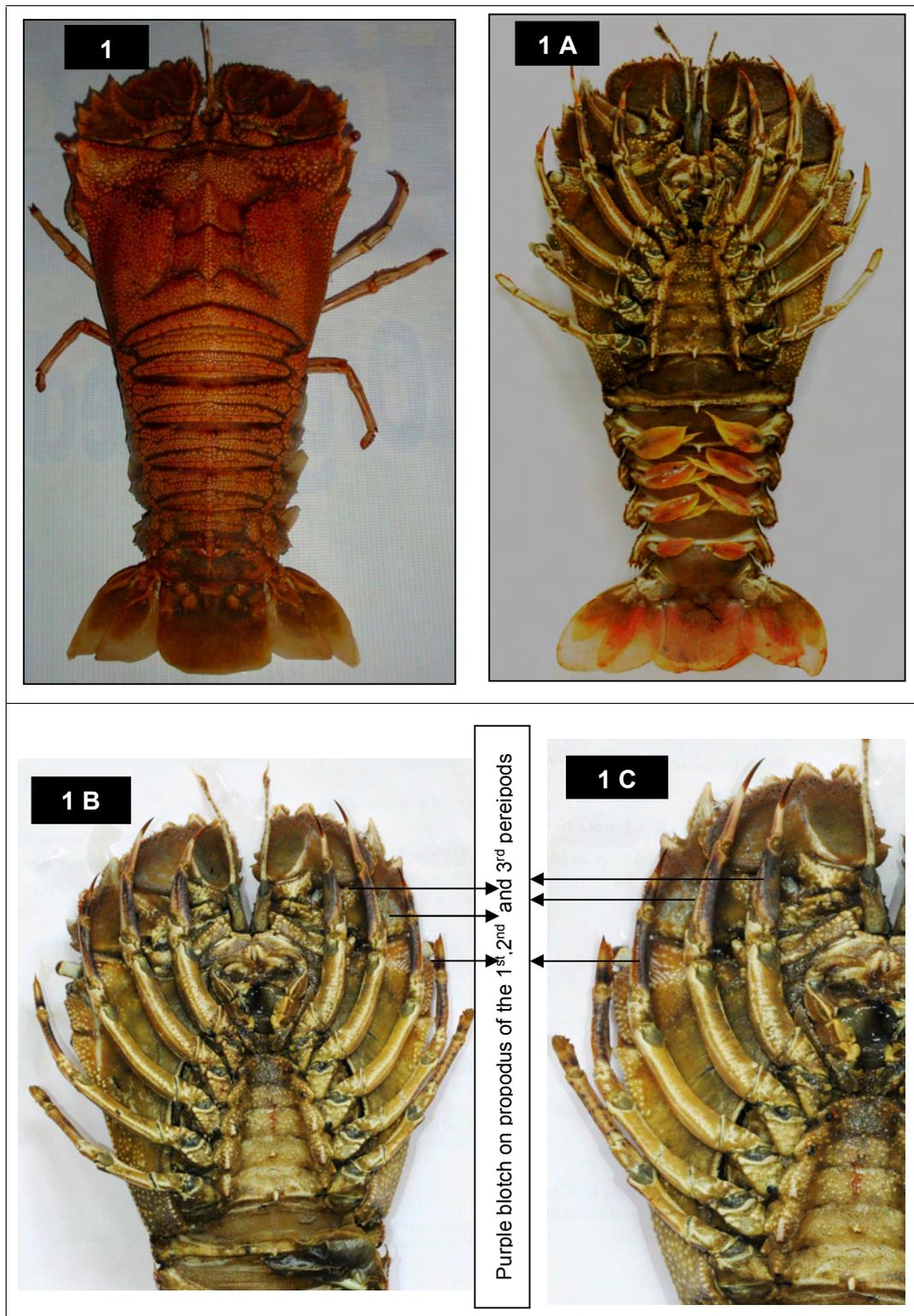
PLATE- VI

1. ***Thenus unimaculatus*** (dorsal view); **1 A-** Ventral view; **1 B-** Carapace (ventral view)

1 C. Carapace (ventral half view to mark the purple blotch)
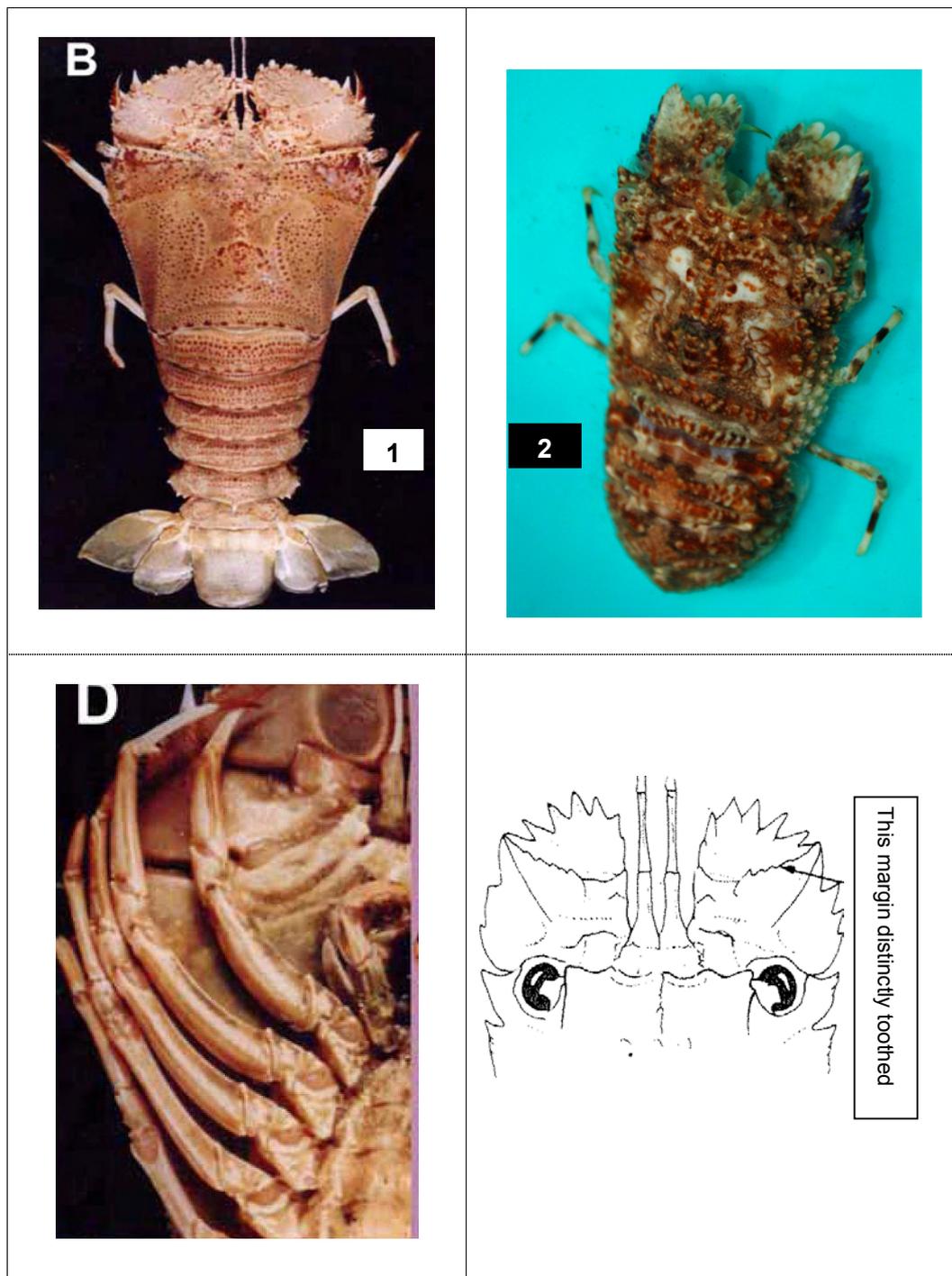
PLATE- VII

1. *Thenus indicus* (dorsal view); **1 A.** Half view of carapace ventral side
   (*Courtesy*: Burton and Davie, 2007).
2. *Petrarctus rugosus* (dorsal view); **2 A.** Antennular plate

Chapter 2
REVIEW OF LITERATURE

SECTION- A

LOBSTERS – THE PRESENT PROFILE

2A.1. Lobsters of the world: Genera, species, classification and time of evolution

The order Decapoda Latreille, 1802 forms one of the many orders of the Class Crustacea, Brünnich 1772. There are four suborders in the Decapoda. They are Macrura Natantia (shrimps), Anomura (hermit crabs, etc.), Brachyura (crabs) and Macrura Reptantia (lobsters, crayfishes etc.). The marine lobsters were first described by Linnaeus in 1758. The marine lobsters are considered to form part of the suborder Macrura Reptantia Bouvier, 1917. In several handbooks (e.g. Bowman and Abele, 1982), the Decapoda are divided into two suborders, the Dendrobranchiata (containing the Penaeidea-penaeid and sergestid shrimps) and the Pleocyemata (containing all the other Decapoda).

The taxonomy of marine lobsters has remained fairly stable over many years and some authors, such as Burukovsky (1983) and Phillips *et al*. (1980) had complied lists of all the valid species of extant marine lobsters known in the world, at that time. In the monumental work of Holthuis (1991), a detailed account was provided for almost all the living marine lobster species up to 1991. Still, the discovery rate of marine lobsters remains high to this day (Chan, 2010). There are currently recognized six families, 55 genera and 248 species (with four subspecies) of living marine lobsters (Chan, 2010). The six families of extant lobsters are Enoplometopidae, Nephropidae, Glypheidae, Palinuridae, Scyllaridae and Polychelidae.

2A.1a. Family Palinuridae and Scyllaridae

The subsuborder Macrura Reptantia has four infraorders: Astacidea, Glypheidea, Achelata and Polychelida (Chan, 2010). The infraorder Achelata contains three main families, namely Palinuridae (spiny lobsters),

Scyllaridae (slipper lobsters), and Synaxidae (furry or coral lobsters) (Palero *et al*., 2009a). These families share a unique larval phase called phyllosoma and lack chelae on their first pair of pereiopods (Scholtz and Richter, 1995; Dixon *et al*., 2003). In the two most recent molecular phylogenetic analyses of Achelata and Palinuridae (Palero *et al*., 2009a; Tsang *et al*., 2009), the family Synaxidae Bate, 1881 was proven to be polyphyletic and the authors suggested to treat this family as a junior synonym of Palinuridae (Tsang *et al*., 2009; Chan, 2010).

The Palinuridae arose in the Atlantic-European region during early Mesozoic (250 million years ago) at a period of high sea level in the vast; warm 'Super Tethys' Ocean (George, 2006b). The spiny lobster family Palinuridae contain eleven genera. They are *Jasus*, *Justitia*, *Linuparus*, *Nupalirus*, *Palibythus*, *Palinurellus*, *Palinurus*, *Palinustus*, *Panulirus*, *Projasus*, *Puerulus* and *Sagmariasus*. Palinurid genera are commonly divided into two major lineages (i) spiny lobsters with a stridulating organ or Stridentes and (ii) without a stridulating organ or Silentes (Parker, 1884; George and Main, 1967). It was in the Jurassic that the family diverged into the Stridentes group, seven genera (*Linuparus*, *Palinustus*, *Puerulus*, *Palinurus*, *Panulirus*, *Nupalirus* and *Justitia*) of which survive today in the low-latitude equatorial zone, and the Silentes group, three genera (*Jasus*, *Sagmariasus* and Projasus) of which live today in the mid-latitude temperate zone of the Southern Ocean and one genus (*Palinurellus*) in the equatorial zone (George 2006b; Tsang *et al*., 2009). The Silentes genera (*Jasus*, *Projasus* and *Sagmariasus*) are shown to be the basal spiny lobsters (George 2006a; Tsang *et al*., 2009). As the three genera are restricted to the southern high latitudes constitute the basal lineages, Tsang *et al*. (2009) suggested a Southern Hemisphere origin for the Palinurid group. In the family Palinuridae, *Puerulus* was considered to be the oldest living genus of the Stridentes (George, 2006b). The relatively shallow water genus *Panulirus* was acknowledged as the most recently evolved (George and Main, 1967; Pollock, 1992; Baisre, 1994; McWilliam, 1995; George, 1997, 2006a). But Tsang *et al*. (2009) inferred that *Panulirus* diverged in the early stage of Stridentes evolution instead of being the most recently derived, as long believed. Moreover, the two deep-sea genera (generally found in depths >200 m), considered to be the most primitive, *Puerulus* and *Linuparus* (George

and Main, 1967; Baisre, 1994; George, 2006b), were relatively derived in their gene tree and they suggested that spiny lobsters invaded deep-sea habitats from the shallower water rocky reefs and then radiated.

Spiny lobsters have received great attention during recent years, including numerous studies on their ecology, phylogeography, and molecular phylogeny (Díaz *et al*., 2001; Patek and Oakley, 2003; Palero *et al*., 2008a). The spiny lobster genus *Panulirus* White, 1847, has long been of interest to evolutionary biologists because of its high level of species diversity, its wide geographic distribution, and the importance of many species to commercial fisheries. Among genera in the family Palinuridae, *Panulirus* has been the most successful in terms of species diversity; 19 species have been described to date, three of which are divided into seven recognized subspecies (Holthuis 1991; George 1997; Sarver *et al*., 1998; Ptacek *et al*., 2001). Recently Chan (2010) in his annotated checklist of lobsters revised it to 21 taxa (with three subspecies).

The slipper lobsters of the family Scyllaridae are a unique group of decapod crustaceans characterized by the flattened antennal flagellum (Spanier and Weihs, 1990) and some members with a ventrally-flattened body to a plate (Lavalli *et al*., 2007; Jones, 2007). However, the slipper and coral lobsters have been the subject of much less research, probably because they do not include many species of commercial interest (Holthuis, 1991; Lavalli and Spanier, 2007). Slipper lobsters are distributed world-wide throughout warm waters with a vertical range from very shallow to more than 800 m deep (Webber and Booth, 2007). Scyllarid lobsters are found in coast along continental shelf and upper slope areas across the Equator, at low latitudes, and in temperate latitudes influenced by warm water currents (Webber and Booth, 2007). The family has a mainly warm-water distribution mainly between 30˚N and 30˚S. Many large species are fished commercially (Duarte *et al*., 2010) although the highest taxonomic diversity is among the smaller species (Holthuis, 1991; Chan, 2010). Based on the different carapace shapes as well as the morphology of the maxilliped exopods and mandibular palp, four subfamilies were proposed by namely Arctidinae, Ibacinae, Scyllarinae, and

Theninae (Holthuis, 1985, 1991, 2002). Altogether there are four subfamilies, 20 extant genera and 89 extant species known to date in the family Scyllaridae (Arctidinae = 17 species, Ibacinae = 15 species, Theninae = 5 species, Scyllarinae = 52 species) (Yang *et al.*, 2012).

2A.1b. Offshore shift and diversification

There are two hypotheses that postulated evolution of lobsters. They are contradictory in that the first one suggests origin of lobsters from a deep-sea ancestral stock to the shallow-water genera and the second, vice versa. It has been hypothesized that radiation in the spiny lobsters occurred when the deep-water ancestral stock of high-latitude areas invaded the shallow warm water seas in lower latitudes, with subsequent specialization and diversification (George and Main, 1967; Baisre, 1994; George, 2005, 2006b). Earlier studies based on adult similarity and a larval cladistic analysis (e.g., George and Main, 1967; Baisre, 1994; George, 2005, 2006a, b) proposed a hypothesis of deep water to shallow water evolution. Contrary to this view, Davie (1990) proposed that the ancestral form of the family initially inhabited shallower waters and then retreated into the deeper region and not the other way round. The recent studies in Palinuridae supported this view of a general onshore (shallow-water) reef origin of the spiny lobsters, which then dispersed into offsore (deeper) reefs and eventually adapted to the typical soft deep-sea bottoms (Chan *et al*., 2009; Tsang *et al*., 2009; Tsoi *et al*., 2011). Past researchers have suggested a deep-water origin of the Scyllaridae too based on larval and adult characteristics (e.g., George and Main, 1967; Baisre, 1994; George, 2005, 2006a, b), while recent molecular analyses suggest the opposite trend (Chan *et al*., 2009; Tsang *et al*., 2009; Yang *et al*., 2012).

2A.2. Commercial importance of lobsters- catch, fishing areas, species and aquaculture

Lobsters support commercially valuable fisheries in many parts of the world and in some regions the most economically important one. The world catch of lobsters recorded in 2010 exceeded 2,79,000 MT, of which 1,88,248 MT corresponded to true lobsters (Nephropidae) 78,518 MT to spiny lobsters

(Palinuridae) and 10,310 MT to slipper lobsters (Scyllaridae) (FAO, 2010). Although the greatest number of commercial species occurs in tropical waters, the largest lobster catches come from cold-temperate regions like the northwestern Atlantic (Fishing Area 21) and the northeastern Atlantic (Fishing Area 27). Species of Nephropid lobster genera like *Homarus* (about 1,20,000 MT), *Nephrops* (66500 MT) and Palinurid genera like *Jasus* (about 11,679 MT) and *Panulirus* (about 64,000 MT) form the subject of specialized fisheries and are the basis for important industries (FAO, 2010). Overall, the global supply of lobsters from wild fisheries for all species combined appears to be at, or close to its maximum (Jeffs, 2010).

Spiny lobsters (Decapoda: Palinuridae) are one of the most commercially important types of marine animal (Phillips, 2006). They inhabit temperate and tropical seas, but most species and the highest abundances are found in the tropics (Holthuis, 1991). Spiny lobsters are captured and marketed in more than 90 countries and sustain major commercial fisheries while simultaneously supporting local, small-scale fisheries in remote coastal locations and islands. Many form the basis for specialized fisheries such as *Panulirus argus* in the Carribean, *Panulirus cygnus* in Western Australia and *Jasus* in New Zealand. The principal producing countries are Australia, New Zealand, South Africa, Cuba, Brazil, Mexico and the USA with over 70% of the spiny lobster catch coming from the Carribean and South-east Atlantic region and the Eastern Indian Ocean (Phillips and Kittaka, 2000). They are highly valued sea food, and the wild stocks support some of the most valuable commercial fisheries in the world's major oceans (Booth and Phillips, 1994; Kittaka and Booth, 2000). The genus *Panulirus*, comprising 19 or more species, is the largest group in the family Palinuridae (George and Main, 1967; Holthuis, 1991; McWilliam, 1995), and all species are highly prized in many countries. More attention was given to the spiny and clawed lobsters as their fisheries became more profitable in the early 1990s with emphasis on potential of aquaculture. Valuable literature narrating various aspects like biology, management, fisheries ,culture etc of spiny lobsters were written by Cobb and Phillips,1980; Factor,1995; Phillips and Kittaka, 2000; Phillips, 2006).

Slipper or shovel-nosed lobsters of the family Scyllaridae are found throughout the world's tropical and temperate oceans. Although they are often a desirable incidental catch in a commercial fishery, they are generally considered too small and scarce to warrant targeted harvesting (Nishikiori and Sekiguchi, 2001; Freitas and Santos, 2002; Vance *et al.*, 2004). More recently, however, a few species in a few locales have become major target species for small-to-moderate scale fisheries (Coutures and Chauvet, 2003; Molina *et al.*, 2004; Haddy *et al.*, 2005; Radhakrishnan *et al.*, 2007). The major species contributing to fishery were *Ibacus* (fishing area 61) and *Thenus* (fishing area 71) (FAO, 2010). In the Western and Central Pacific (fishing area 71), slipper lobsters contribute about 25-50% of the total lobster catch. Out of 89 species of extant scyllarid species, only 30 larger slipper lobster species are of commercial importance (Holthuis, 1991). The slipper lobster *Thenus* species has become focus of targeted fishery in India (Radhakrishnan *et al.*, 2007). Other slipper lobsters that contribute to fishery are *Thenus* spp. in Austalia, *Ibacus* spp in Australia, and *Scyllarides* spp. Some of the smaller species such as *Petrarctus rugosus* have commercial aspects in aquarium trading (Spanier and Lavalli, 2007; Kumar *et al.*, 2009).

2A.3. Biology, aquaculture importance, breeding and larval dispersal of lobsters

The distinctive phyllosoma larva is the most important characteristic in the early life history of palinurids and scyllarids. Reaching 80 mm or so in total length in some species, this flat, virtually transparent, long-lived, leaf-like larva, which is often widely dispersed in the open ocean, is adapted for passive horizontal transport assisted by vertical migration. Early development in both families comprises a short-lived embryonised 'prelarva' (naupliosoma) in some species, a larval phase (phyllosoma), and a postlarval phase (puerulus in palinurids, nisto in scyllarids) which precedes the first juvenile stage (Phillips, 2006; Phillips and Kittaka, 2000).

The life cycle of spiny lobsters is complex and includes a long oceanic larval phase varying in length between species. Indeed, planktonic

development has in many cases been shorter in culture than in nature. Most palinurids for which there are data have estimated larval durations in nature of 6–12 months (but longer for *Jasus* spp.-up to 24 months), and all disperse well offshore (Phillips, 2006). The larval life of *P. homarus* is estimated to be 5.5-8 months (Phillips and Matsuda, 2011), but has not been successful so far anywhere in the world.

But among the Scyllaridae there is a wider range of estimated larval lifespans, from 1 month to at least 9 months. Many of the small adult scyllarid species have brief, inshore development. Warm-water species tend to have shorter larval lives than cool-water ones. Like the spiny lobster, the sand lobster, too has a complex and prolonged life cycle, though not as prolonged as in the case of the former (Kizhakudan, 2006b). The larval life estimated for *Thenus* species in wild is estimated to be 27-45 days (Jones, 1988; Mikami and Greenwood, 1997; Radhakrishnan *et al*., 2007).

There is considerable interest in the aquaculture of spiny lobsters because of their consistently high demand and price, and because of the full exploitation of the natural stocks. Rock lobster aquaculture produced 1611 MT in 2010 (FAO, 2010). Successful larval development was achieved in different parts of the world in eight species (Phillips and Matsuda, 2011). Despite success of larval rearing in some species, when the prospects for spiny lobster aquaculture were reviewed by Kittaka and Booth (1994), they stated that 'the greatest hurdle in the commercial culture of spiny lobster is the difficulty in growing species through their larval stages'. The larval life cycle has been unsuccessful in lab due to difficulties in providing suitable diets in the later stages of development. The key bottleneck for lobster aquaculture is the hatchery nursery phase (Phillips, 2006). The hardy spiny lobsters with good growth rates for juveniles, but the long larval life extending over several months, with limited success in production of seeds, has discouraged its large-scale aquaculture (Kittaka and Booth, 2000). Problems and prospects of spiny lobster aquaculture in India were reviewed by Radhakrishnan and Vijayakumaran (2000).

The advantage in captive rearing of the sand lobster will be the relatively shorter span for larval metamorphosis as compared to the spiny lobsters (Robertson, 1968). Complete larval rearing has been successfully achieved in different parts of the world in scyllarid lobsters. The days of culture from phyllosoma to nisto ranged from 28 days (*Thenus* spp.) to 192 days (*Scyllarus arctus*) in laboratory conditions (Vijayakumaran and Radhakrishnan, 2011). The adult females of Ibacinae and Theninae are also relatively large (CL>70mm) and produce larger eggs that complete developments in about a month (Theninae) or in 2-4 months (Ibacinae). With a shorter larval life, high growth rates for juveniles, hardiness and a good market value, the slipper lobster is fast emerging as a new species of aquaculture interest (Mikami and Kuballa, 2007; Vijayakumaran and Radhakrishnan, 2011).

Except for a few species like *Panulirus cygnus*, *P. argus* etc. studies of larval transport of Palinurids integrating oceanic or coastal circulation patterns with larval distribution patters has not been studied in detail (Phillips and Kittaka, 1994). The lengthy larval life and the often highly dispersed larval distributions have been major obstacles to research and management.

2A.4. Lobsters of the Indian Seas: Commercial importance, fishery, species distribution and abundance along the Indian coast

Though not big in volume, lobsters are one of the most valuable and highly priced crustaceans from the Indian seas. In India, lobsters form only 0.36 % of the total marine crustacean landings in 2010 (CMFRI, 2011). But they were an important export commodity comprising 0.25% in quantity and 1% in value (MPEDA, 2009). India earned an approx USD 20 million through export of lobsters in 2009. Lobsters are exported as live lobster, frozen lobster tails, frozen whole cooked and frozen lobster tails. The live ones are considered superior in South-east Asian markets.

The lobster fishery improved from 800 MT in 1968 to 2991 MT in 1975 and attained a peak of 4075 MT in 1985. Thereafter the fishery showed a trend of decline averaging around 2200 MT for nearly 15 years, but declined to 1371 MT in 2004 (Radhakrishnan *et al*., 2005) and is estimated to be 1852 MT in

2011 (CMFRI, 2012). Twenty five species of lobsters have been reported so far from Indian coast (Modayil and Pillai, 2007). They are widely distributed along the entire coast of the country with maximum landings from the northwest coast (70%), followed by the southeast (16%) and southwest (14%) coasts (Radhakrishnan and Manisseri, 2003; Radhakrishnan and Thangaraja, 2008). The northwest coast comprising Gujarath and Maharashtra is particularly rich in lobster resources, contributing to nearly three quarters of the total lobster landing in India (Kagwade *et al.*, 1991; Radhakrishnan, 1995).

Radhakrishnan and Manisseri (2003) discussed the species distribution and fishery of lobsters in Indian seas. The southeast coast except a small region is a potential lobster fishing area. *P. ornatus*, *P. homarus* and *Thenus orientalis* are the major species exploited. Small quantities of *P. versicolor* are also landed along the Trivandrum and Chennai coasts. *P. penicillatus* and *P. longipes* are the two other species, which are not important from the fishery point of view. *Linuparus somniosus* was reported from the Andaman and Nicobar Islands, but has not been commercially exploited. In the northwest coast, 95% of lobsters are caught by trawls whereas it is traps, gill nets, trawls and trammel nets that catch most of the lobsters in southwest and southeast coasts.

Although the lobster fauna of commercial fishing grounds comprises 14 species of littoral and six species of deep sea forms, only four littoral and one deep sea form are significant in commercial fishery. Two species, the palinurid spiny lobster *Panulirus polyphagus* (Herbst) and scyllarid *Thenus orientalis* (Lund) predominate in the fishery along the northwest coast (Chhapgar and Deshmukh, 1971). The major sand lobster fishery completely collapsed by 1994 due to recruitment overfishing and there is no sign of its recovery (Deshmukh, 2001). In the southwest, *P. homarus homarus* is the dominant species in the shallow water lobster fishery (Modayil and Pillai, 2007). *Puerulus sewelli* was the only deep sea species exploited in commercial quantitites from the area. However, *P. versicolor* and *P. ornatus* are also landed in small quantities. Major fishing grounds for the deep sea lobster, *P. sewelli* were

located off Quilon in the southwest and off Tuticorin in the southeast coasts, at depths ranging from 150m to 400m.

The most important species that contributed to the lobster fishery in India in 2010 were the Slipper lobster, *Thenus unimaculatus* and spiny lobster, *Panulirus homarus* (CMFRI, 2011)*. T. orientalis* is the only slipper lobster of commercial significance among the rich diversity of scyllarid lobsters recorded from the Indian coast (Radhkrishnan *et al.*, 2007). They appear as by-catch in trawl fisheries and although catch rates are low, they constitute the most important component of the lobster fishery on the northwest, southwest and southeast coasts of India. In the northwest, along the Mumbai coast, the *Thenus* fishery collapsed in 1994 and has yet to recover, causing concern about the sustainability of the slipper lobster fishery (Deshmukh, 2001).

The research on lobsters in India are mainly focused on biology, breeding, larval nutrition and farming (e.g. Thangaraja and Radhakrishnan, 2012; Vijayakumaran *et al*., 2012; Chakraborty *et al*., 2010; Rao *et al*., 2010). The successful larval rearing was achieved for *Thenus* spp. in India other than Australia (Kizhakudan *et al.*, 2004a). The phyllosoma larvae of *P. homarus* was successfully reared up to the eighth stage (CMFRI, 2005) in India.

SECTION- B

MOLECULAR MARKERS

2B.1. Need for genetic markers

Genetic variation enhances the capability of any species to adapt to changing environment and hence necessary for survival of a species. Patterns of genetic diversity or variation among populations can provide clues to the populations' life histories and degree of evolutionary isolation. Population genetics can be defined as the science of how genetic variation is distributed among species, populations and individuals, and fundamentally, it is concerned

with how the evolutionary forces of mutation, selection, random genetic drift and migration affect the distribution of genetic variability (Hansen *et al*., 2007). Several evolutionary forces affect the amount and distribution of genetic variation among populations and thereby population differentiation (Felsenstein, 1985). Geographic distance and physical barriers enhance reproductive isolation by limiting the migration and increase genetic differentiation between populations (Ryman, 2002).

The identification of stock structure has been recognized widely as a prerequisite for sustainable management of marine fisheries (Reiss *et al*., 2009). Variation within and between populations and stock discrimination within exploited species are important issues for conservation programmes. Identification of non-interbreeding populations is also essential to assess the gene flow between different genetic stocks, and to monitor temporal changes in the gene pools (Carvalho and Hauser, 1994). By characterizing the distribution of genetic variation, population substructuring can be detected and the degree of connectivity among populations estimated (Nesbo *et al*., 2000; Ruzzante *et al*., 2000; Hutchinson *et al*., 2001). Taking into account the influence of present gene flow on the genetic structuring of the species is crucial in order to protect those populations with higher genetic diversity and greater ability to effectively be able to export individuals to other areas (Palumbi, 2004).

Many non-genetic methods of stock discrimination are available to achieve varying degrees of success in distinguishing breeding stocks. With the advent of genetic methods, stock identification based solely upon morphological and meristic differences has become rare. Instead, these data are used in conjunction with genetic data. Molecular markers provide direct assessment of pattern and distribution of genetic variation (Ferguson *et al.,* 1995) thus helping in answering, "If the population is single unit or composed of subunits". The powerful ability of molecular genetic markers to detect genetic variations when combined with new statistical methods having high analytical power, have revolutionized the genetic diversity studies. Various molecular markers now being used in fisheries and aquaculture provide various scientific observations which have importance in species identification, genetic variation

and population structure study in natural populations, comparison between wild and hatchery populations, assessment of demographic bottleneck in natural population when populations experience severe, temporary reduction in size which influence the distribution of genetic variation within and among populations and propagation assisted rehabilitation programmes (Chauhan and Rajiv, 2010)

Molecular tools have become an indispensable part of innumerable systematic and conservation-based studies (Hillis *et al*., 1996), providing information across a large scale of research, ranging from differential heritage of genes within the same individual (Avise, 2004) to population biology and species-level relationships (Rubinoff and Sperling, 2002). For conservation biology specifically, DNA data contribute to research as diverse as fine-scale management of fish stocks through the assignment of individual fish to one of several populations in the same watershed or fishery (Hansen *et al*., 2001; Ruzzante *et al*., 2004), to cryptic and invasive species recognition, identification of appropriate source populations for local reintroduction (Ludwig *et al*., 2003), and even tracking the post harvest use of sensitive species through forensic identification of animal parts (Shivji *et al*., 2002).

Molecular genetic data have become a standard tool for understanding the evolutionary history and relationships among species (Avise, 1994). A critical assumption for phylogenetic analyses is that gene flow among lineages has been rare (Shaklee and Currens, 2003). The inter-specific genetic divergence established through species specific diagnostic molecular markers provides precise knowledge on phylogenetic relationships and also resolve taxonomic ambiguities. Phylogenetic classification specifically attempts to show relationships based on reconstructing the evolutionary history of groups or unique genomic lineages.

2B.2. Molecular markers in use for population and phylogenetic studies

A *molecular marker* is a gene with a known location or clear phenotypic expression that is detected by analytical methods or an identifiable DNA

sequence that facilitates the study of inheritance of a trait or a gene (Okumus and Çiftci, 2003). In the early 1980s, the first population genetic studies based on analysis of mitochondrial DNA emerged (Avise *et al*., 1979). Later, with the advent of the PCR, a number of different techniques emerged, ranging from sequencing of the DNA of interest to methods analysing length polymorphisms, such as microsatellites.

Molecular markers can be classified into type I and type II markers. Type I markers (e.g. Allozymes) are associated with genes of known function, while type II markers are associated with anonymous genomic regions (O'Brien, 1991). The significance of type I markers is becoming extremely important for aquaculture genetics. Sequence conservation within genes are high, allowing type I markers to serve as anchor points for genomic segments to be compared among species. Expressed Sequence Tags (ESTs), are type I markers that are considered as new generation markers. RAPD markers are type II markers because RAPD bands are amplified from anonymous genomic regions via the polymerase chain reaction (PCR). Microsatellite markers are also type II markers unless they are associated with genes of known function. In general, type II markers such as RAPDs, microsatellites, and AFLPs are considered non-coding and therefore selectively neutral. Such markers have found widespread use in population genetic studies to characterize genetic divergence within and among the populations or species (Brown and Epifanio, 2003). Usefulness of molecular markers can be measured based on their polymorphic information content (PIC, Botstein *et al*., 1980). PIC refers to the value of a marker for detecting polymorphism in a population.

Several marker types are highly popular in aquaculture genetics which have been subjected to a number of reviews (Liu and Cordes, 2004; Chauhan and Rajiv, 2010 etc.). The most recent approaches to gathering data relevant to fisheries and aquaculture come from direct assessments of nuclear DNA (nDNA) sequence variation (Brown and Epifanio, 2003) which exhibits the greatest variability of all genetic markers. The nuclear DNA markers include random amplified polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Variable number tandem repeats (VNTRs), Single

Nucleotide Polymorphism (SNP) and nuclear ribosomal DNA markers. RAPD and RFLP are multiple Arbitrary Primer Markers or "anonymous nucDNA markers" used to detect anonymous, or arbitrary, sequences by "multiple arbitrary amplicon profiling". Main applications in fisheries and aquaculture are phylogenetics and phylogeography, population genetic structure, conservation of biodiversity and effective population size, hybridization and stocking impacts, inbreeding, domestication, quantitative traits, and studies of kinship and behavioural patterns.

2B.2.1. Random Amplified Polymorphic DNA (RAPD)

RAPD procedures first developed in 1990 (Welsh and McClelland, 1990; Williams *et al*., 1990), utilizes random 10-base oligonucleotides as primers to amplify anonymous segments of nuclear DNA via PCR. RAPD markers are the amplified products of less functional part of the genome that do not strongly respond to selection on the phenotypic level. Such DNA regions may accumulate more nucleotide mutations with potential to assess inter-population genetic differentiation (Mamuris *et al*., 2002). RAPD can detect high levels of DNA polymorphisms (Williams *et al*., 1990; Welsh and McClelland, 1990). Because the primers are short and relatively low annealing temperatures (often 36-40 °C) are used, the likelihood of amplifying multiple products is great, with each product (presumably) representing a different locus. Because most of the nuclear genome in vertebrates is non-coding, it is presumed that most of the amplified loci will be selectively neutral. Genetic variation and divergence within and between the taxa of interest are assessed by the presence or absence of each product, which is dictated by changes in the DNA sequence at each locus. The potential power is relatively high for detection of polymorphism; typically, 5-20 bands can be produced using a given primer pair, and multiple sets of random primers can be used to scan the entire genome for differential RAPD bands. Because each band is considered a bi-allelic locus (presence or absence of an amplified product), PIC values for RAPDs fall below those for microsatellites and SNPs, and RAPDs may not be as informative as AFLPs because fewer loci are generated simultaneously. RAPD markers are inherited as Mendelian markers in a dominant fashion and scored as present/absent.

Analysis follows the assumption that populations under study follow Hardy-Weinberg expectations.

RAPDs have all the advantages of a PCR-based marker, with the added benefit that primers are commercially available and do not require prior knowledge of the target DNA sequence or gene organization and a large number of loci and individuals can be screened. The method is simple, rapid and cheap, it has high polymorphism, only a small amount of DNA (~20ng) is required no need for molecular hybridization and most importantly, no prior knowledge of the genetic make-up of the organism in question is required (Hadrys *et al*., 1992). RAPDs have gained considerable attention particularly in population genetics, species and subspecies identification (Bardakci and Skibinski, 1994), phylogenetics, linkage group identification, chromosome and genome mapping, analysis of interspecific gene flow and hybrid speciation, analysis of mixed genome samples (Hadrys *et al*., 1992), breeding analysis and as a potential source for single-locus genetic fingerprints (Brown and Epifanio, 2003).

The main drawback with RAPDs is that the resulting pattern of bands is very sensitive to variations in reaction conditions, DNA quality, and the PCR temperature profile (Liu and Cordes, 2004). RAPD markers are subject to low reproducibility due to the low annealing temperature used in the PCR amplification. Extensive standardization is required to get reproducible results. Even if the researcher is able to control the major parameters, other drawbacks of RAPD will remain: homozygous and heterozygous states cannot be differentiated and the patterns are very sensitive to slight changes in amplification conditions, giving problems of reproducibility (Ferguson *et al*., 1995). Some concerns about the reproducibility of RAPD analysis both within a laboratory and among laboratories remain unresolved. It is suggested that if the overall temperature profiles (especially the annealing temperature) inside the tubes are identical among the laboratories, then RAPD fragments are likely to be reproducible (Penner *et al.*, 1993). Also difficulty is there to determine whether bands represent different loci or alternative alleles of a single locus, so that the number of loci under study can be erroneously assessed. This is

especially true if the RAPD is caused by deletion or insertion within the locus rather than at the primer binding sites. Other shortcomings of this type of marker include the difficulty of demonstrating Mendelian inheritance of the loci. The presence of paralogous PCR product (different DNA regions which have the same lengths and thus appear to be a single locus) limits the use of this marker. These difficulties have limited the application of this marker in fisheries science (Wirgin and Waldman, 1994).

2B.2.1a. RAPD technique in decapod crustacean genetics

RAPD analysis has been used to evaluate genetic diversity for species, subspecies and population/ stock identification, taxonomic identity, and systemic details in a wide variety of decapod crustaceans.

Even though RAPD fingerprinting has been used in population genetics of a wide variety of crustaceans, comparatively few investigations are carried out with this marker in the case of lobsters. Population genetic studies in American lobster *Homarus americanus* (Harding *et al*., 1997) and European lobster, *Homarus gammarus* (Ulrich *et al*., 2001) were carried out using RAPD PCR. The technique was used for tissue discrimination of American lobster and European (Hughes and Beaumont, 2004) and species comparisons of Korean slipper lobster (*Ibacus ciliatus*) with Indian Ocean deep sea lobster *Puerulus sewelli* (Park *et al*., 2005). These are the few published works in lobsters with this genetic marker.

RAPD technique was used in the genetic studies of a variety of crustaceans like amphipod *Gammarus locusta* (Costa *et al*., 2004), *Caprella* spp. (Cabezas *et al*., 2010), *Argulus* sp. (Sahoo *et al*., 2011) etc. RAPD-PCR was employed to detect the DNA polymorphism to obtain molecular markers to enable the identification, to assess the phylogenetic relationship and to explore intra and interspecific variation in *Macrobrachium* spp. (Guerra *et al*., 2010). This technique has been used to estimate genetic diversity in penaeid shrimps like *Penaeus monodon* (Garcia and Benzie,1995; Tassanakajon *et al*., 1998), *Metapenaeus ensis* and *Penaeus japonicus* (Meruane *et al*., 1997; Song *et al*., 1999), *Penaeus chinensis* (Shi *et al*., 1999; Zhuang *et al*., 2001), *Penaeus*

stylirostris (Aubert and Lightner, 2000), *Litopenaeus vannamei* (Freitas *et al.*, 2007), *Metapenaeus dobsoni* (Mishra *et al*., 2009), *Penaeus semisulcatus* (Niamaimandi *et al*., 2010), *M. affinis* (Lakra *et al*., 2010), *Fenneropenaeus indicus* (Rezvani Gilkolaei *et al*., 2011), atyid shrimp *C. cantonensis* (Yam and Dudgeon, 2005), sergestid shrimp *Acetes japonicus* (Aziz *et al*., 2010), *Pandalus borealis* (Martinez *et al.*, 2006) and brine shrimp *Artemia* (Sun *et al.*, 1999).

RAPD was used for analysing populations of blue swimming crab *Portunus pelagicus* (Klinbunga *et al.*, 2010) and mud crab *Scylla* spp. (Klinbunga *et al.*, 2000). RAPD variation was surveyed in the freshwater crayfish *Cherax destructor* (Nguyen *et al*., 2005), *Cherax quadricarinatus* (Macaranas *et al*., 1995) and Spanish white-clawed crayfish *Austropotamobius pallipes* (Gouin *et al*., 2001, 2003; Beroiz *et al*., 2008).

2B.2.2. Allozyme studies in decapod crustaceans

Allozyme electrophoresis denotes the technique for identifying genetic variation at the level of enzymes, which are directly encoded by DNA. Allozymes are co-dominant Mendelian characters that are passed from parent to offspring in a predictable manner. They can be used to quantify genetic variation and distinguish among genetic units at the levels of populations, species, and higher taxonomic designations. Disadvantages associated with this type I marker include occasional heterozygote deficiencies due to null (enzymatically inactive) alleles and sensitive to the amount as well as quality of tissue samples. In addition, some changes in DNA sequence are masked at the protein level, reducing the level of detectable variation (Liu and Cordes, 2004).

The amount and pattern of genetic variation and stock structure was assessed by alozyme loci analysis for *Homarus americanus* (Shaklee, 1983; Kornfield and Moran 1990), European lobster *H. gammarus* (Tam and Kornfield, 1996; Jørstad and Farestveit, 1999; Jørstad *et al*., 2005), *Panulirus marginatus* (Shaklee and Samollows, 1984; Seeb *et al*., 1990), and Norway lobster *Nephrops norwegicus* (Maltagliati *et al.*, 1998; Stamatis *et al*., 2006).

Allozyme analysis has been the most commonly used method to determine the levels of variation and genetic structuring for commercially important shrimps species (Garcia-Machado *et al.*, 2001; Barcia *et al.,* 2005; Zitari-Chatti *et al.,* 2008) and crab species (Gomez-Uchida *et al.,* 2003).

2B.2.3. Mitochondrial DNA (mtDNA)

Properties and phylogenetic utilities of the mtDNA

A small portion of (<%1) of the DNA of eukaryotic cells is non-nuclear; it is located within organelles in the cytoplasm called mitochondria. The mtDNA molecule exists in a high copy-number in the mitochondria of cells and has a circular structure (Fig. 2). In decapod crustaceans, the usual size ranges from 14 to 18 kb. Metazoan mtDNAs ordinarily contain 36 or 37 genes as shown in Fig. 2; two for ribosomal RNAs (16SrRNA and 12SrRNA), 22 for tRNAs and 12 or 13 subunits of multimeric proteins of the inner mitochondrial membrane (cytochrome oxidase I-III [COI-III], ATP synthase 6 and 8, NADH dehydrogenase 1-6 and 4L [ND1-6, ND4L], and cytochrome b apoenzyme [Cyt *b*]). In addition, there is usually at least one sequence of variable length that does not encode any gene (e.g. control region or A+T rich region).

The major features of mtDNA: a) It is in general maternally inherited haploid single molecule; b) the entire genome is transcribed as a unit except for the approximately 1-kb control region (D-loop), where replication and transcription of the molecule is initiated; c) not subject to recombination and provides homologous markers; d) mainly selectively neutral and occurs in multiple copies in each cell; e) replication is continuous, unidirectional and symmetrical without any apparent editing or repair mechanism; and f) optimal size, with no introns present (Billington, 2003).

Studies of vertebrate species generally have shown that sequence divergence accumulates more rapidly in mitochondrial than in nuclear DNA (Brown, 1985). This has been attributed to a faster mutation rate in mtDNA that may result from a lack of recombination during replication, hence low efficiency
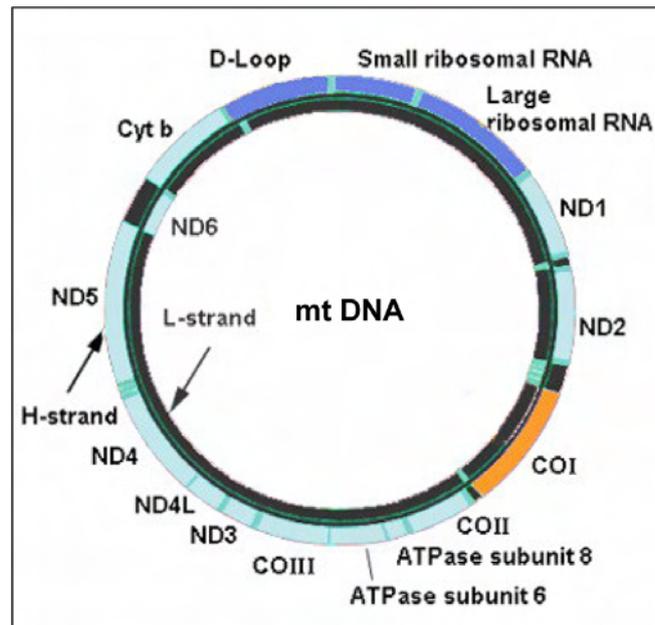
Fig. 2: Mt DNA structure in Eukaryotes

of DNA repair mechanisms (Wilson *et al*., 1985). The mtDNA polymorphism has been used for genetic stock structure analysis because of its rapid evolutionary rate (Avise, 1994). In most species the highly variable mitochondrial DNA (mtDNA) is a good marker for detecting possible genetic differentiation. They are haploid and maternally inherited and consequently are one quarter the effective population size of nuclear genes (Moritz *et al*., 1987, Birky et al., 1989), thus allowing population level studies and systematic studies among recently diverged taxa. MtDNA evolves at a rate faster than single-copy genes in nuclear DNA which makes this molecule extremely useful for phylogenetic analyses (Brown *et al*., 1979). Possibly the most important reason to use mitochondrial genes is the availability of universal mtDNA primer sets that have minimized laboratory time in the initial setting up of a project. The high copy number of mitochondria in tissues makes them relatively easy to isolate. Also, there are already extensive sets of nucleotide sequences from these genes in GenBank.

MtDNA has proven useful for identifying major evolutionary lineages (Bernatchez *et al*., 1992). MtDNA genes have been found to vary considerably

among closely related species,making phylogenetic estimates of recent species radiations possible (Shaw, 2002). Neutral mtDNA markers can provide information about past events, while giving a picture of the overall gene flow between populations (Grant and Waples, 2000). The high mutation rate of mtDNA makes it a useful tool for differentiating between closely related species (Brown *et al*., 1979), a tool that is especially important when significant variations occur between species, but not within species (Blair *et al*., 2006; Chow *et al*., 2006a). MtDNA provided interesting insights into the demographic history of marine populations ('phylogeography'- Avise, 1992; Grant and Waples 2000; Grant and Bowen, 2006).

Different parts of the mitochondrial genome are known to evolve at different rates (Meyer, 1993). Due to the high rate of substitution occurring in the third codon positions (wobble positions) of protein coding genes, the DNA sequences of protein coding genes have frequently been used for species level or population level phylogeny (Navajas *et al*., 1996). Like nuclear DNA, the genome includes coding and non-coding regions and later evolves much faster than coding regions of DNA (Avise, 1994). The D-loop region of the mtDNA is practically the only noncoding region in the entire mtDNA of vertebrates. The control region of mtDNA, the unassigned region, is hypervariable and there exist variations even between individuals. Thus, this region has been mainly used for phylogenetic studies among species, subspecies, or populations (Zhang and Hewitt, 1997). In general, non-coding segments like the D-loop exhibit elevated levels of variation relative to coding sequences such as the cytochrome b gene, presumably due to reduced functional constraints and relaxed selection pressure. 12SrDNA, however, is highly conserved like the nuclear SSU rDNA, which has been employed to illustrate phylogeny of higher categorical levels such as in phyla or subphyla (Ballard *et al*., 1992). 16SrDNA is usually used for phylogenetic studies at mid-categorical levels such as in families or rare genera since it is more variable than 12SrDNA (Hwang and Kim, 1999). Compared to the nuclear rDNA, it is more difficult to design universal primers for amplifying specific regions in mtDNA due to a high variability. That is why only a few mitochondrial genes such as 12SrDNA, 16S rDNA, Cytb, ND1 and COI have been employed in phylogenetic studies. In

general, 12S and 16SrDNAs are the most conserved regions among the mitochondrial genes ((Hwang and Kim, 1999). COI is the most conserved among 3 cytochrome oxidase coding genes, and also ND1 among the seven NADH dehydrogenase coding genes. Cyt *b* is more conserved than ND1 but less than COI (Hwang and Kim, 1999). It implies that the frequency used as gene regions in phylogenetic studies is closely related with the degree of the gene conservation.

Mitochondrial DNA analysis has proven a powerful tool for assessing intraspecific phylogenetic patterns in many animal species (Avise, 1994). Smaller fragments of the mitochondrial genome (D-loop region) have also been targeted by probing or PCR and findings have indicated that it may be best to concentrate on the 'slow evolving' coding sequences for species comparisons, and to use the 'fast evolving' non-coding regions for population investigations. MtDNA variation can resolve relationships of species that have diverged as long as 8-10 million years before present. Afterwards, sequence divergence is too slow to allow sufficient resolution of divergence times.

Application of mtDNA in animals, including fishes has some major problems as well. The drawbacks of mtDNA in population genetics have been thoroughly discussed by Zhang and Hewitt (2003). The effectiveness of using mtDNA in population-genetic studies has been greatly weakened by the fact that mitochondrial *pseudogenes* are present in the nuclear genome of a wide range of organisms. In addition mtDNA data on their own have some important limitations. Since mtDNA represents only a single locus (Avise, 1994), we can look only through a single window of evolution. This window reflects at best only the maternal lineal history (Skibinski, 1994). The phylogenies and population structures derived from mtDNA data may not reflect those of the nuclear genome due to gender-biased migration (Birky *et al*., 1989) or introgression and hence could well differ from that overall of populations or species. Therefore, the inference we make on species/population history is likely to be highly biased and the need for independent, genomic molecular markers to support mtDNA analysis is clear. Second, the effective population size of mtDNA is only a fourth of that of nuclear autosomal sequences; that

means mtDNA lineages have a much faster lineage sorting rate and higher allele extinction rate (Zhang and Hewitt, 2003). In addition, mtDNA markers are subject to the same problems that exist for other DNA-based markers, such as back mutation, parallel substitution, and rate heterogeneity or mutational hot spots (Liu and Cordes, 2004). Although mtDNA loci can exhibit large numbers of alleles per loci and its PIC values higher than those for allozymes it is lower than highly variable nuclear markers such as RAPDs, microsatellites, AFLPs, and SNPs. Use of mtDNA probes and PCR amplification of selected regions have made examination of mtDNA much faster.

2B.2.4. MtDNA in crustacean population genetics

2B.2.4a. MtDNA Cytochrome Oxidase I (COI) gene

Mitochondrial markers have been favored in population genetic studies for several reasons (Wan *et al*., 2004, Galtier *et al*., 2009). Mitochondrial DNA is highly variable in natural populations because of its elevated mutation rate, which can generate some signal about population history over short time frames. Variable regions (e.g. the control region) are typically flanked by highly conserved ones (e.g. ribosomal DNA), in which PCR primers can be designed. Clearly, mtDNA is the most convenient and cheapest solution when a new species has to be genetically explored in the wild. Mitochondrial ribosomal genes 12S and 16S, mitochondrial control region (CR) and coding genes such as Cytochrome Oxidase (COI) and Cyt *b* have been extremely useful in population genetic and systematic studies.

Genetic diversity and population structure of marine species with long-lived larval phase like the lobsters was analyzed using partial sequences of mtDNA COI gene. Nucleotide sequence analysis of mitochondrial COI gene was used to infer population structure of spiny lobsters *Panulirus japonicus* (Inoue *et al*., 2007; Sekiguchi and Inoue, 2010), *Palinurus elephas* (Cannas *et al*., 2006; Palero *et al*., 2008a), *Panulirus regius* (Froufe *et al*., 2011), *P. argus* (Naro-Maciel *et al*., 2011), *P. mauritanicus* (Palero *et al*., 2008a) etc. Genetic connectivity of lobster *Jasus tristani* was studied using the cytochrome oxidase II (COII) gene (Von der Heyden *et al*., 2007). Partial nucleotide sequences of

mitochondrial DNA COI and 16SrDNA regions were used for population genetics in *P. penicillatus* (Chow *et al.*, 2011).

The population genetics and historical demography were assessed using mitochondrial DNA sequences from portions of the cytochrome c oxidase subunit I (COI) and cytochrome b (Cyt *b*) genes of the swimming crab *Callinectes bellicosus* (Pfeiler *et al.*, 2005). COI gene was used to examine the population structure in a number of crab species like *Scylla serrata* (Fratini and Vannini, 2002), *Carcinus maenas* (Roman and Palumbi, 2004), *Erimacrus isenbeckii* (Azuma *et al.*, 2008), *Pachygrapsus crassipes* (Cassone and Boulding, 2006), *Epilobocera sinuatifrons* (Cook *et al.*, 2008), *Portunus trituberculatus* (Liu *et al.*, 2009), *Eriocheir* spp. (Zhao *et al.*, 2002) and *Uca annulipes* (Silva *et al.*, 2010).

The population genetics was examined by nucleotide sequence variation in mtDNA COI gene of in the Antarctic krill species *Euphausia crystallorophias* (Jarman *et al.*, 2002), freshwater shrimp *Caridina zebra* (Hurwood and Hughes., 2001) and in many penaied shrimp species like *Penaeus kerathurus* (Zitari-Chatti *et al.*, 2009), *Fenneropenaeus chinensis* (Quan *et al.*, 2001; Li *et al.*, 2009), *Fenneropenaeus indicus* (De Croos and Palsson, 2010), *Metabetaeus lohena* (Russ *et al.*, 2010) and *Penaeus monodon* (Klinbunga *et al.*, 2001; Khamnamtong *et al.*, 2009).

Levels and patterns of distribution of genetic diversity in crayfish populations were analyzed using mitochondrial COI gene sequences in a number of species like *Austropotamobius pallipes* (Die´Guez-Uribeondo *et al.*, 2008, Stefani *et al.*, 2011), *Cherax quadricarinatus* (Baker *et al.*, 2008), *Austropotamobius italicus* (Zaccara *et al.*, 2005; Matallanas *et al.*, 2011) and *Euastacus* spp. (Ponniah and Hughes, 2006).

Partial sequences of mtDNA COI gene has been used for population structure investigation in a variety of invertebrate organisms like sponge (Whalan *et al.*, 2008), planktonic copepods (Nuwer *et al.*, 2008), may fly (Hughes *et al.*, 2003), giant clam (Kochzius and Nuryanto, 2008), Ark Shell (Cho *et al.*, 2007), octopus (Keskin and Atar, 2011), asteroids (Flowers and

Foltz, 2001; Waters *et al*., 2004), brittle star (Christensen *et al*., 2008) and mysids (Remerie *et al.*, 2009)   in addition to use in decapod crustaceans.

2B.2.4b. Taxonomic and phylogenetic utility of MtDNA genes

The reasons for the adoption of mtDNA as marker of choice are well-known. Experimentally, mtDNA is relatively easy to amplify because it appears in multiple copies in the cell. Mitochondrial gene content is strongly conserved across animals, with very few   duplications, no intron, and very short intergenic regions (Gissi *et al*., 2008). Because of its relatively high substitution rate, mtDNA has been extensively used as a phylogenetic marker at recent time scales, both for tree building and molecular dating. Mitochondrial ribosomal genes such as 12SrRNA, 16SrRNA and coding genes such as COI have been extremely useful in population genetic and systematic studies in Crustaceans (Tudge and Cunningham, 2002).

2B.2.5. Nuclear DNA genes in crustacean phylogenetics

Tsang *et al.* (2008) utilized the nuclear protein-coding genes, phosphoenolpyruvate carboxykinase (PEPCK) and sodium–potassium ATPase a-subunit (NaK) for decapod phylogenetics. The evolutionary relationships and divergence ages were estimated for 37 penaeoid genera using nuclear protein-coding genes (Ma *et al*., 2009). Studies have been undertaken to reconstruct the phylogeny of Palinuridae and its allies using sequences from three nuclear protein-coding genes-phosphoenolpyruvate carboxykinase, sodium– potassium ATPase a-subunit and histone 3 (Tsang *et al.,* 2009). RE digestion of 28S ribosomal DNA was used to differentiates adults or larvae of *Panulirus argus*, *Panulirus guttatus*, and *P. laevicauda* (Silberman and Walsh, 1992). The contribution of the small-subunit l8S ribosomal (r) DNA nuclear gene to crustacean phylogeny is well known and has been useful in investigating relationships across a wide variety of groups (Spears and Abele, 1998; Kim and Abele 1990; Crandall *et al.*, 2000; Morrison *et al.*, 2002; Perez-Losada *et al.*, 2002; Ahyong *et al.*, 2007). The 18s rDNA gene polymorphism of *P. homarus* has been studied using standard markers in five major fish landing centres of Indian peninsula (Mon *et al.*, 2011). Partial 28S rRNA gene has been

used to construct phylogeny of four spiny lobster species in India (Suresh *et al.*, 2012).

2B.3. DNA barcoding

Species are the principal currency of biodiversity and usually the focal taxonomic unit of conservation biology. The majority of conservation programs and legislation are focused on saving species. Dayrat (2005) clearly expressed, 'delineating species boundaries correctly - and also identifying species - are crucial to the discovery of life's diversity because it determines whether different individual organisms are members of the same entity or not'. The DNA barcode itself consists of a 648 bp region 58-705 from the 5'-end of the cytochrome *c* oxidase 1 (COI) gene using the mouse mitochondrial genome as a reference. It is based on the postulate that every species will most likely have a unique DNA barcode and that genetic variation between species exceeds variation within species (Hebert *et al.*, 2003a, b; Hebert *et al.*, 2004).

To make reliable and consistent conservation and fisheries management decisions, accurate, unambiguous, and robust species identifications are needed. DNA barcodes are supposed to increase our ability and efficiency in identifying new species. Specifically, COI as a barcoding tool helps to identify an organism based on DNA sequence variability and assignment to a certain species previously described (Lefe´bure *et al.*, 2006). DNA barcode sequences can be used as a DNA taxonomy tool to perform prediction and classification of potentially new species. However other genes and phylogenetic methods are required to evaluate the evolution information contained in the barcode region of COI (da Silva *et al.*, 2011).

Mitochondrial DNA can be a powerful tool in the effort to identify species, their relationships to each other, and threatened or endangered populations with divergent haplotypes worthy of conservation attention (Moritz 1994, 2002; Avise 2004). DNA barcodes have been proposed as a fast, efficient, and inexpensive technique to catalogue all biodiversity (Hebert *et al.*, 2003b; Stoeckle *et al.*, 2003; Mortiz and Cicero, 2004; Hebert *et al.*, 2004). Sequence variation within species for COI is generally much lower than

sequence variation among species, permitting most unknown samples to be sequenced and allocated correctly to species (Ward *et al*., 2009). DNA barcoding offers taxonomists the opportunity to greatly expand, and eventually complete, a global inventory of life's diversity. It will make the Linnaean taxonomic system more accessible assigning specimens to known species. DNA barcoding will play an increasingly important role as a taxonomic screening tool because of its ability to rapidly reveal the genetic discontinuities that ordinarily separate distinct species (Janzen *et al*., 2005). Based on past results for varied animal groups, DNA barcoding will deliver species-level resolution in 95% to 97% of cases (Hebert *et al*., 2004; Janzen *et al*., 2005; Ward *et al*., 2005).

Despite the potential benefits of DNA barcoding to both the practitioners and users of taxonomy, it has been controversial in some scientific circles (Will and Rubinoff, 2004; Ebach and Holdredge, 2005). Barcode differences appear to accumulate quickly, making it possible to distinguish all but the youngest of sister species. It has also been suggested that, it is undesirable to rely on a single sequence for taxonomic identification (Sites and Crandall, 1997; Mallet and Willmortt, 2003; Matz and Nielsen, 2005). Thus the feasibility of using additional genes, particularly ribosomal RNA genes, as DNA barcodes has also been explored (Blaxter *et al*., 2004; Chu *et al*., 2006).

The use of mitochondrial genes encoding ribosomal (12S and 16S) DNA in broad taxonomic analyses is constrained by the prevalence of base insertions and deletions (indels) that complicate sequence alignments (Doyle and Gaut, 2000; Hebert *et al*., 2003a). Sequence alignment is a major obstacle that limits the effective use of rRNAs for barcode purposes. More recent evidence from the better-studied taxa such as birds and fishes suggests that in most cases barcoding will in fact permit accurate identifications (Kerr *et al*., 2007; Tavares and Baker, 2008; Ward *et al*., 2008). This identification tool can clearly give support to improve classifications and to critically examine the precision of morphological traits commonly used in taxonomy (Frézal and Leblois, 2008).

2B.3a. MtDNA genes in decapod barcoding and crustacean phylogenetics

Cytochrome Oxidase subunit I (COI or COX), was recently elected as the standardized tool for molecular taxonomy and identification (Ratnasingham and Hebert, 2007). It was used in distinguishing lobsters *Panulirus femoristriga*, *P. longipes bispinosus* and *P. longipes longipes* (Ravago and Juinio-Meñez, 2003). The genus *Thenus* was barcoded with COI and 16SrRNA genes to identify five distinct species in the previously monotypic subfamily Theninae (Burton and Davie, 2007). COI gene was amplified and sequenced to identify *Thenus* species in Thailand (Iamsuwansuk *et al*., 2012). Nucleotide sequence analysis of the mtDNA COI gene was performed to identify phyllosoma larvae of spiny lobsters of the genus *Panulirus* (Chow *et al*., 2006b). Nucleotide sequence analysis of mitochondrial 16SrDNA identified *Panulirus echinatus* phyllosoma larva in the central Atlantic which was undescribed (Konishi *et al.*, 2006).

Partial sequences of mitochondrial DNA genes especially 16SrRNA and COI have proved suitable than other gene sequences to resolve the phylogenetic relationships within the family in several group of eukaryotes. Most of the molecular phylogenetic studies using mtDNA genes on spiny lobsters have focused on species-level relationships within a genus. Partial sequences of mitochondrial 12S and 16S genes were used to infer phylogenetic relationships of the recent clawed lobster genera (Chu *et al*., 2006; Tshudy *et al*., 2009). Using 16S mtDNA, Tam and Kornfield (1998) produced a tree including five nephropid lobster genera. Chu *et al.* (2006) produced a 12S mtDNA-based tree for ten clawed lobster genera using *Neoglyphea* as outgroup. The phylogenetic relationships within the family Palinuridae (among the two species of *Palinurus- Palinurus elephas* and *P. mauritanicus*, most of the species of *Panulirus* and all the species of *Jasus*) were examined using mtDNA COI gene (Cannas *et al*., 2006). The phylogenetic relationships of the extant *Linuparus* species, including the colour forms, were investigated using mitochondrial 12SrRNA and COI gene sequence analysis (Tsoi *et al.*, 2011). Sequence data derived from the

mitochondrial DNA 16SrRNA and COI genes were used to determine the phylogenetic relationships among six *Palinurus* spiny lobster species (Groeneveld *et al*., 2007) and in *Jasus* (Ovenden *et al.,* 1997). Phylogenetic relationships among all described species and four subspecies (total of 21 taxa) of the spiny lobster genus *Panulirus* White, 1847 were examined with nucleotide sequence data from portions of two mitochondrial genes, large-subunit ribosomal RNA (16S) and COI gene by Ptacek *et al.* (2001). Phylogeny of Iranian coastal lobsters was inferred from mitochondrial DNA-COI restriction fragment length polymorphism (Ardalan *et al.*, 2010).

Mitochondrial DNA structure of cray fish *Austropotamobius italicus italicus* was assessed using 16S and COI (Pedraza-Lara *et al.*, 2010). Nucleotide variation and phylogenetic relations within and between four species of freshwater crayfish of the genus *Cherax* was investigated using four fragments amplified from the 16SrRNA, 12SrRNA, Cytochrome Oxidase I (COI), and Cytochrome *b* (Cyt *b*) gene regions (Munasinghe *et al*., 2003). Phylogenetic and phylogeographic relationships in crustaceans like copepods (Dippenar *et al.,* 2009) and crayfish genus *Austropotamobius* inferred from mitochondrial COI gene sequences (Trontelj *et al.*, 2005). The sequence analysis of mtDNA has been considered a useful tool for phylogeny and systematics among closely related crab species (Geller *et al*., 1997; Schubart *et al.,* 2001; Tang *et al*., 2003; Imai *et al*., 2004; Sotelo *et al.*, 2008). The mitochondrial DNA markers including 16SrRNA and cytochrome oxidase I (COI) genes were employed to reconstruct the phylogenetic relationships among *Penaeus* spp. (Palumbi and Benzie 1991; Gusmão *et al*., 2000; Quan *et al*., 2004; Lavery *et al*., 2004; Voloch *et al*., 2005), squat lobsters (Machordom and Macpherson, 2004) and in achelate lobsters. Similarly, the mitochondrial 16SrDNA gene has been regularly used to investigate decapod relationships and phylogenetic studies in decapods (Ovenden *et al.*, 1997; Kitaura *et al.*, 1998; Crandall *et al*., 2000; Stillman and Reeb, 2001; Parhi *et al.*, 2008).

2B.4. Use of concatenated sequence data in phylogeny reconstruction in decapod crustaceans

A solid taxonomy is fundamental to all biology, and phylogenies provide a sound foundation for establishing taxonomy. The choice of marker will have a significant effect on divergence estimates obtained (Carvalho and Hauser, 1999). The highly conserved molecular markers and/or gene regions are useful for investigating phylogenetic relationships at higher categorical levels while the hypervariable molecular markers are useful for elucidating phylogenetic relationships at lower categorical levels or recently diverged branches (Hwang and Kim, 1999). Thus Because different genes may reflect different evolutionary histories (Avise 2004), use of multiple genetic markers is often necessary even for many intra- and interspecific studies to provide an accurate perspective on an organism's evolutionary history and systematic/ taxonomic relationships (Funk and Omland 2003). Use of nuclear genes in addition to mitochondrial genes adds to the number of independent markers in a dataset, thus increasing the chances of reconstructing the true species phylogeny. In addition, a larger effective population size, and on average, a lower substitution rate (Moriyama and Powell, 1997), results in nuclear genes evolving slower than mitochondrial genes. Consequently, they may be better at resolving deeper phylogenetic nodes (Chu *et al*., 2009). Hence an integrated approach that uses mtDNA and nuclear DNA, usually in conjunction with morphology and ecology, is better able to access different avenues of inheritance, producing more accurate results that are essential when assessing and managing biodiversity (Rubinoff, 2006).

Most molecular phylogenetic studies of Decapoda have relied heavily on mitochondrial DNA and nuclear ribosomal DNA markers. The former, however, exhibit rapid substitution saturation that limits their utility in resolving deep nodes, whereas the latter suffer from alignment ambiguities. These disadvantages can complicate analysis and hamper accurate recovery of phylogenetic signal (Tsang *et al.*, 2008).

Many studies have shown an increase in resolution when multiple genes are combined in phylogenetic analyses (Ahyong and O'meally 2004; Porter *et al.*, 2005). The nuclear and mtDNA gene sequences have been concatenated to construct phylogeny in a number of animals and also in decapods crustaceans. However, Fisher-Reid and Weins (2011) opined that lower homoplasy of nucDNA characters may outweigh the influence of the larger numbers of variable mtDNA characters and combined-data analyses need not necessarily be dominated by the more variable mtDNA data sets that may lead to widespread discordance between trees from mtDNA and nucDNA. Their results from 14 vertebrate clades showed that combined mtDNA-nucDNA data analyses are not necessarily dominated by the more variable mtDNA data sets.

The concatenated data set of 3139 bp including one mitochondrial gene (16S) and three nuclear genes (18S, 28S, H3) was used to investigate phylogeny in decapoda (Bracken *et al.*, 2009a). The concatenated alignment containing 3398 bp, including the partial 18S, 28S and COI fragments from all four horseshoe crab species yielded an overall stable phylogeny in the combined analysis (Obst *et al.*, 2012). A detailed phylogeny of all *Euastacus* species was constructed using nucleotide sequence data from the 16SrRNA, 12SrRNA, and cytochrome *c* oxidase subunit I (COI) mitochondrial gene regions, and from the 28S rRNA gene region of the nuclear genome. The different gene regions were then concatenated for a single data file for subsequent analyses (Shull *et al.*, 2005).

Few workers have conducted DNA-based cladistic analyses on the clawed lobsters. Ahyong and O'Meally (2004) used 16S mtDNA along with 18S and 28S nuclear DNA data (2,500 bp total) to evaluate reptant decapod phylogeny, including six lobster genera. Porter *et al.* (2005) used 16S mtDNA along with 18S and 28S nuclear DNA data and the histone H3 gene (3,601 bp total) to evaluate decapod phylogeny (43 genera), including four lobster genera.

Patek and Oakley (2003) presented the first attempt to reconstruct the molecular phylogeny of palinurid genera based on morphological characters

and ribosomal DNA evidence (16S, 18S and 28S nuclear and mitochondrial ribosomal RNA gene regions). Different nuclear (18S, 28S, and H3) and mitochondrial (16S and COI) gene regions were sequenced and the concatenated sequence was used to to test conflicting hypotheses of evolutionary relationships within the Achelata infraorder and solve the taxonomic disagreements in the group (Palero *et al*., 2009a). Partial sequences of three mitochondrial genes, the small subunit ribosomal RNA (12SrRNA), the large subunit ribosomal RNA (16SrRNA) and cytochrome c oxidase subunit I (COI) genes, and the nuclear gene histone H3 from all the 17 extant species to reconstruct the phylogeny of the species of *Metanephrops* (Chan *et al*., 2009). To ascertain phylogenetic relationships and monophyly patterns in species of the genus *Palinurus* mitochondrial DNA sequence data and microsatellite markers were used (Palero *et al*., 2009b). Yang *et al.* (2012) collected nucleotide sequence data from regions of five different genes (16S, 18S, COI, 28S, H3) to estimate phylogenetic relationships among 54 species of Scyllaridae.

2B.5. Present study

Commercially, lobsters are generally the most highly prized crustaceans in all parts of the world. Among them, spiny lobsters are one of the most commercially important groups of decapod crustaceans (Palero and Abelló, 2007; Follesa *et al*., 2007) and have received great attention during recent years, including numerous studies on their ecology, phylogeography, and molecular phylogeny (Díaz *et al*., 2001; Patek and Oakley, 2003; Palero *et al*., 2008a). However, the slipper and coral lobsters have been the subject of much less research, probably because they do not include many species of commercial interest (Holthuis, 1991; Lavalli and Spanier, 2007).

Majority of the scientific works on lobsters from the Indian seas have concentrated on fishery and stock assessment, growth and culture and breeding. Except a few works like PCR-RFLP of mtDNA COI gene for larval identification of *P. homarus* (Dharani *et al*., 2009), study using 18S rDNA gene polymorphism in *P. homarus* (Mon *et al*., 2011) and phylogeny construction of

four spiny lobster species using 28SrRNA gene (Suresh *et al.*, 2012), no comprehensive molecular genetic works have been reported on lobsters from India.

The identification of stock structure has been recognized widely as a prerequisite for sustainable management of marine fisheries (Reiss *et al*., 2009). Variation within and between populations and stock discrimination within exploited species are important issues for conservation programmes. The main aim is to recognize genetic stocks within a species which are largely reproductively isolated from each other. Patterns of genetic diversity or variation among populations can provide clues to the populations' life histories and degree of evolutionary isolation. The genetic variation can be observed using molecular markers. Population gnetic structure investgated using RAPD and hypervariable region of COI gene in *P. homarus homarus* and *T. unimaculatus* which are the most dominant lobster species from the Indian Seas (CMFRI, 2011). The stock structure analysis is especially important in the present context of alarming decline in lobster landings in the country to formulate suitable conservation strategies.

Crustaceans are an interesting target for DNA barcoding because they represent one of the most diverse metazoan groups from a morphological and ecological point of view. Morphological identification of crustaceans can be difficult, time-consuming and very often requires highly trained taxonomists. Previous work on crustaceans found DNA barcoding to be a useful tool for specimen identification in both marine and freshwater species (Bucklin *et al*., 2007; Costa *et al*., 2007). Of the 17635 morphologically described freshwater and marine extant species of decapod crustaceans, only 5.4% are represented by COI barcode region sequences (da Silva *et al*., 2011). DNA barcoding of fish and marine life was initiated in India during 2006 and 115 species of marine fish from the Indian Ocean have been barcoded (Lakra *et al*., 2011) .In this work, the barcodes using mtDNA COI were generated for all lobster species of commercial importance from the Indian EEZ. But closely related species may have identical or nearly identical COI sequences (Scott Harrison, 2004; Lorenz *et al*., 2005; Brower, 2006). It is also suggested that it is undesirable to rely on

a single sequence for taxonomic identification (Sites and Crandall, 1997; Mallet and Willmortt, 2003; Matz and Nielsen, 2005). Thus in the present study, the feasibility of using partial sequences of additional genes like 16SrRNA, 12SrRNA and nuclear 18SrRNA has also been explored. These barcodes will be helpful in accurate species identification of lobster larvae too which is usually difficult by visual examination.

Recent advances in morphological and molecular phylogeny studies have created great impacts on the concepts of the evolutionary relationships of marine lobsters and other Decapoda. Some analyses of the last decades suggest that marine lobsters do not comprise a monophyletic group (Schram and Dixon, 2004; Ahyong and O'Meally, 2004; Porter *et al*., 2005). These results also showed that the relationships of the superfamilies and families of marine lobsters are mostly different from the previously well-established scheme of Holthuis (1991). The phylogenetic studies also have yielded significantly contrasting results (Tsang *et al*., 2008; Bracken *et al*., 2009a; Toon *et al*., 2009). The latest and by far the most robust phylogenetic analysis (Tsang *et al*., 2008) utilised newly developed molecular markers, concluding that lobsters are indeed a monophyletic group (Chan, 2010). The present work also aims to reconstruct the phylogeny of eleven species of commercially important lobsters from the Indian EEZ using molecular markers to understand evolutionary relationships.

Chapter 3

MATERIALS AND METHODS

3.1. Collection of samples

3.1.1. Population genetic study of lobsters

A total of 180 specimens of *Panulirus homarus* (Linnaeus, 1758) and 240 numbers of *Thenus unimaculatus* Burton and Davie, 2007 were collected for the population genetic study from sites of fishery along the Indian coast details of which are presented in Table 1 and depicted in Fig. 3. The two species were collected randomly from the landing centres and from exporters of live lobsters.

The lobsters were examined for morphology and tissue samples for DNA extraction were taken from tip of antennae and pleopods, using minimal invasive techniques. The collected tissues were preserved in 1.5 ml microvials containing 1.25 ml of 95% ethyl alcohol and stored in refrigerated condition at 4°C until further analysis.

Table 1. Details of sampling of *P. homarus homarus* and *T. unimaculatus* for population genetic studies.

Species	Indian Coast	Sampling Site	Date of collection	n_1	n_t
P. homarus	WEST	Kollam	14/09/2006-21/09/2006	25	
			17/09/2007-25/09/2007	35	60
	EAST	Chennai	17/8/2006 -23/8/2006	30	
			20/10/2007-27/10/2007	30	60
		Visakha-patnam	6/10/2006-12/10/2006	33	60
			16/10/2007-23/10/2007	27	
T. unimaculatus	WEST	Veraval	17/10/2006-23/10/2006	28	
			10/11/2007-17/11/2007	32	60
		Kollam	18/09/2006-26/09/2006	35	60
			14/10/2007-20/10/2007	25	
	EAST	Chennai	17/8/2006 -23/8/2006	30	
			20/10/2007-25/10/2007	30	60
		Visakha-patnam	6/10/2006-12/10/2006	24	
			16/10/2007-23/10/2007	36	60

n_1 – no. of specimens collected in each sampling, n_t – total no. of specimens.

3.1.2. Species-specific DNA signatures

The samples of eleven commercially important lobster species, eight of which (*P. homarus homarus*, *P. versicolor*, *P. ornatus*, *P. longipes longipes, P. polyphagus, P. penicillatus, Peurulus sewelli* and *Linuparus somniosus*) belong to Palinuridae and three of family Scyllaridae (*Thenus unimaculatus, T. indicus* and *Petrarctus rugosus*) were collected from their places of abundance along the Indian coast for generating species-specific markers with a view to accurately document the lobster diversity in Indian seas. Specimen information and location data along Indian coast are presented in Table 2 and depicted in Fig. 3.

3.2. Genomic DNA isolation

Total DNA was extracted from tissue (10–20 mg) of all the collected individuals following the standard phenol-chloroform method (Sambrook and Russell, 2001) with heat shock modification, steps involved in which are detailed below.

Modified phenol-chloroform protocol for DNA Isolation

Day1

➢ 10-20 mg of lobster muscle was taken for DNA isolation. It was finely minced with scissors and transferred into a 2 ml centrifuge tube. (Ethanol preserved tissues were washed twice in high TE (0.1M Tris and 0.04 M EDTA) at 10,000 rpm for 10 minutes, for the removal of ethanol)

➢ 500 µl Lysis buffer was added to it [10mM Tris-HCl (pH 8.3), 1 mM EDTA, 0.4M NaCl] and suspended properly.

➢ The samples in lysis buffer were incubated at 4°C for overnight in the refrigerator.

Day 2

➢ The samples were taken out from the refrigerator, added 100 µl of 10% SDS (sodium dodecyl sulphate) per sample.
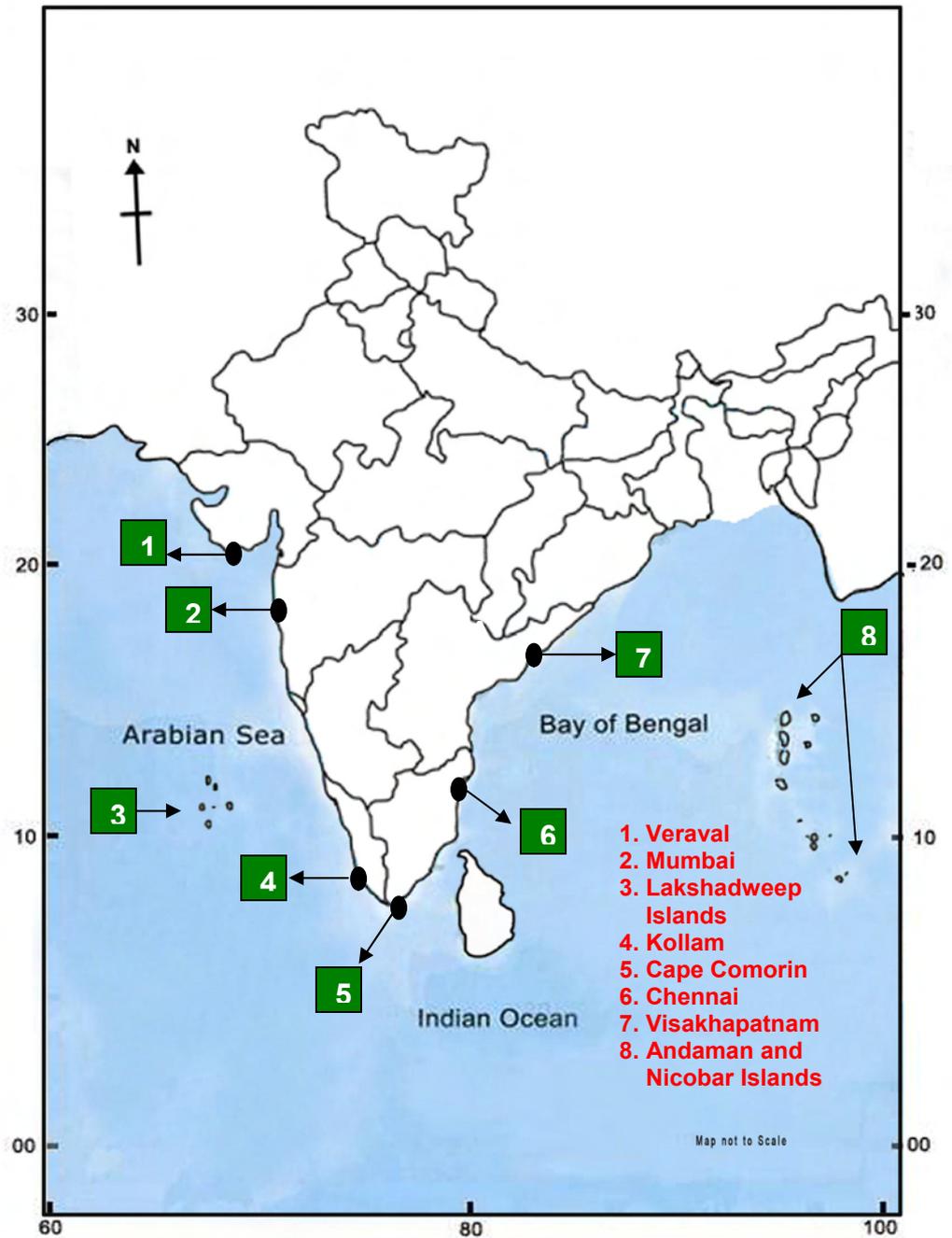
**Fig. 3. Map of India showing the distribution of the sampling
sites of lobster species along the Indian coast for
species- specific marker studies.**

Table 2. Sampling location of commercially important lobsters along the Indian Coast.

LOCATION and CODE (as in map)		Collection locality along the Indian Coast								Number of individuals collected
		WEST COAST				EAST COAST				
		1	2	3	4	5	6	7	8	
		Veraval (VRL)	Mumbai	Lakshadweep Island	Kollam (QLN)	Cape Comorin	Chennai (CHE)	Visakha-patnam (VSK)	Andaman and Nicobar Islands	
STATE IN INDIA		Gujarat	Maha-rashtra	UT	Kerala	Tamil Nadu	Tamil Nadu	Andhra Pradesh	UT	
Geographic Location of sampling site	Latitude	20° 54' N	18° 56' N	8°and12° 13' N	8° 94' N	8°17' N	13°06' N	17 °14' N	6° and14° N	
	Longitude	70° 22' E	72° 45' E	71° and74° E	76° 55' E	77°43' E	80°18 E	83 °17' E	92°and 94° E	
SL NO.	SPECIES									
Family PALINURIDAE										
1	*P. homarus homarus*				✓		✓	✓		180
2	*P. versicolor*				✓		✓			6
3	*P. ornatus*						✓			5
4	*P. longipes longipes*					✓				5
5	*P. polyphagus*	✓	✓							10
6	*P. penicillatus*			✓						5
7	*Peurulus sewelli*				✓					5
8	*Linuparus somniosus*							✓		2
Family SCYLLARIDAE										
9	*T. unimaculatus*	✓		✓			✓	✓		240
10	*T. indicus*							✓		3
11	*Petrarctus rugosus*						✓			5

(N.B .Large sample sizes for P. homarus and T. unimaculatus for population structure analysis)

Day 2 *(continued)*

➢ Kept the samples at -80^0 C for 2 hours.

➢ Immediately after removing from the freezer, the samples were kept in the water bath at $+60^0$ C for 5 minutes to lyse the cells.

➢ The samples were taken out and kept outside for 5 minutes.

➢ Added 7 µl Proteinase K (20 mg/ ml stock) to each sample and kept in water bath at 56^0 C till the tissue is fully digested.

➢ 500µl Tris saturated phenol was added to the samples and mixed gently by slowly inverting the tubes for 10 minutes. Slow shaking avoids the denaturation of DNA.

➢ The samples were centrifuged at 12,000 rpm for 15 min at 4^0 C. The aqueous phase was transferred to a fresh autoclaved tube by using wide bore tips. The organic phase containing the denatured proteins and other debris was discarded.

➢ Equal volume of chloroform: isoamyl alcohol (24:1v/ v) mixture was added to the sample and mixed gently for 10 minutes. Centrifuged at 12,000 rpm for 15 min at 4^0 C.

➢ The aqueous phase of the mixture was transferred to a fresh autoclaved tube and discarded the organic phase containing the lipids and carbohydrates.

➢ 1/10th volume of 3 M sodium acetate (pH - 5.2) was added to the separated aqueous phase and the DNA was precipitated with 2.5 volume of ice-cold ethanol. The tubes were kept overnight at 4^0 C in a refrigerator to get the maximum DNA pellet.

Day 3

➢ The samples were taken out from refrigerator, centrifuge at 12,000 rpm for 10 minutes at 4° C for precipitating the DNA. Decanted the ethanol and the marked the DNA pellet in tube.

- ➢ Washed the DNA pellet with 200 µl of 70% ethanol and mixed gently. Centrifuged the solution at 12,000 rpm for 10 minutes at 4^0 C.

- ➢ The ethanol was discarded carefully without losing the DNA pellet and kept the tubes inverted to drain off remaining ethanol. Allowed the pellet to air-dry and dissolved it in TE buffer (pH - 8) depending upon the quantity of DNA precipitated.

- ➢ 1µl of DNAase free RNAase (10 mg/ ml- Bangalore Genei) was added to each tube and incubated at 37^0 C for two hours for degradation of RNA.

- ➢ The DNA samples were at stored at $^-20^0$ C until further use.

- ➢ 5 µl of extracted DNA was run on on 0.7% agarose gel with ethidium bromide incorporated in 1X TBE buffer to check the purity and quantity of the same.

The reagents required for DNA isolation using phenol-chloroform method along with protocol for neutral phenol preparation (pH - 8) are given in Appendix 1.

DNA Quantification

The quality and quantity of the extracted DNA was checked in UV spectrophotometer (Beckman, USA) by taking the optical density (OD) at 260 nm and 280 nm. The quality was checked by measuring the ratio of absorbance at 260 nm and 280 nm (260/ 280). The value between 1.7 - 1.8 indicates good quality DNA without protein contamination. DNA quantification was done according to the following calculation: sample showing 1 OD at 260 nm is equivalent to 50 µg of DNA/ml. The OD of each DNA sample at 260 nm was measured and quantified accordingly.

3.3. Methods employed in population genetic study of lobsters

The population genetic structure analysis of *P. homarus homarus* and *T. unimaculatus* was carried out using Random Amplification of Polymorphic DNA (RAPD) and partial sequence comparison of hypervariable region of

mtDNA Cytochrome Oxidase I (COI) gene. Sixty individuals each were selected from both the species from all sampling sites for carrying out the RAPD technique. Twenty individuals each per sampling site were selected for mtDNA analysis in *P. homarus homarus* while 18 per sampling site were analyzed in *T. unimaculatus.*

3.3.1. Random Amplification of Polymorphic DNA (RAPD)

3.3.1.1. Screening of RAPD primers

Hundred decamer primers (20 from each series OPA, OPAA, OPAC and OPAH and OPB) (Operon Technologies, Alameda, USA) were used for screening of samples. Eight and nine primers each were selected for population genetic analysis of *P. homarus* and *T. unimaculatus* respectively taking into consideration the repeatability, sharpness and intensity of bands. The primers selected for population genetic analysis of *P. homarus* were OPA-17, OPA-19, OPAC- 01, 0PAC-15, OPAH-04, OPAH-19, OPB-01 and OPB-20 and those selected for the same in *T. unimaculatus* were OPA-13, OPA-18, OPAC-05, OPAC-09, OPAC-11, OPAC-13, OPAC-17, OPAH-06 and OPAH-09. The list of primers used in population genetic analysis using RAPD in *P. homarus homarus* and *T. unimaculatus* are given in Tables 3 and 4 below.

Table 3. Selected primers for RAPD analysis in *P. homarus homarus.*

Sl. No:	Primer	Sequences (5'-3')
1	OPA 17	GACCGCTTGT
2	OPA 19	CAAACGTCGG
3	OPAC 01	TCCCAGCAGA
4	OPAC 15	TGCCGTGAGA
5	OPAH 04	CTCCCCAGAC
6	OPAH 19	GGCAGTTCTC
7	OPB 01	GTTTCGCTCC
8	OPB 20	GGACCCTTAC

Table 4. Selected primers for RAPD analysis in *T. unimaculatus.*

Sl. No:	Primer	Sequences (5'-3')
1	OPA 13	CAGCACCCAC
2	OPA 18	AGGTGACCGT
3	OPAC 05	GTTAGTGCGG
4	OPAC 09	AGAGCGTACC
5	OPAC 11	CCTGGGTCAG
6	OPAC 13	GACCCGATTG
7	OPAC 17	CCTGGAGCTT
8	OPAH 06	GTAAGCCCCT
9	OPAH 09	GGCAGTTCTC

3.3.1.2. PCR amplification

RAPD-PCR reactions were carried out in a PTC 200 gradient thermal cycler (M.J. research, Inc., Watertown, Massachusetts, USA) using the RAPD primers described above. PCR amplifications were performed in 25µl reactions containing 1x reaction buffer (100 mM Tris, 500 mM KCl, 0.1% gelatin, pH 9.0) with 1.5 mM $MgCl_2$ (Genei, Bangalore, India), 7.5 pmoles of primer (random primers), 200 mM dNTPs, 2 U *Taq* DNA polymerase (Genei, Bangalore, India) and 25 ng of template DNA. To check DNA contamination, a negative control was set up omitting template DNA from the reaction mixture. The reaction mixture was pre-heated at 95^0 C for 3 minutes followed by 40 cycles (94^0 C for 1 minute, 40^0 C for 1 minute and 72^0 C for 1.30 minutes). The reaction was then subjected to a final extension at 72^0 C for 10 minutes. The composition of PCR reaction mixture is given in box below.

PCR reaction Mixture	Vol. per reaction
Double distilled water	17.3µl
Assay buffer (10x)	2.5 µl
dNTPs	2.0 µl
Primer (Operon Technologies)	1.5µl
Taq polymerase (Genei, Bangalore)	0.7µl
Template DNA	1.0 µl
TOTAL	25 µl

PCR mix for RAPD

Agarose electrophoresis and visualization of bands

PCR products were electrophoretically analyzed through 1.5% agarose gels stained with ethidium bromide (5µg/ml) in 1x TBE buffer (pH

8.0). The gels were visualized under UV transilluminator and documented using Image Master VDS (Pharmacia Biotech, USA). RAPD-PCR technique can often produce non-reproducible amplification product (Callejas and Ochando, 2001). Reactions were therefore performed following a strict protocol with standardized conditions to make sure consistency and repeatability of fingerprints generated using selected RAPD primers.

3.3.1.3. Analysis of data

a. Scoring of bands

Scoring of bands was done as described below and data was analyzed using POPGENE version 1.31 (Yeh *et al.,* 1999). Gels images were used to analyze banding patterns. A binary matrix was produced whereby the presence or absence of each DNA fragment for each sample was recorded 1 or 0, respectively. Faint or poorly amplified fragments were excluded from the analysis as were fragments with very high (above 2300 bp) or low (below 400 bp) molecular weight. The analysis was based on a few assumptions. First, all RAPD fragments scored represented 2-allele system, *i.e.,* presence (dominant) and absence (recessive) of bands. Second, fragments that migrated at the same position, had the same molecular weight, and stained with the same intensity were homologous bands from the same allele, and the alleles from different loci did not co-migrate. A third assumption was that the populations conformed to Hardy-Weinberg equilibrium, $p^2 + 2pq + q^2 = 1$, with frequencies p (dominant, band present) and q (recessive, band absent) (Clark and Lanigan, 1993; Lynch and Milligan, 1994). From the binary matrix, the total number of RAPD fragments and polymorphic fragments were calculated for all the selected primers. The molecular weights of the bands were calculated by using Image Master 1D Elite software (Pharmacia Biotech, USA) in relation to the molecular marker λDNA with *Eco*R I/ *Hind* III double digest applied along with the samples (Fig. 4 A).

b. Genetic variability analysis-
The genetic variability analysis in RAPD were carried out in individual and overall populations by assessing number of

polymorphic loci, percentage polymorphism, average heterozygosity or Nei`s gene diversity (Nei, 1987) 'h', and Shannon's diversity Index (Lewontin, 1972) using POPGENE version 1.31(Yeh *et al.,* 1999). The percentage of polymorphic loci (%P) values were calculated using the criterion for polymorphism. Average gene diversity index (Nei, 1987) is a measurement of genetic variation for randomly mating populations. It is defined as the mean of heterozygosity (h) for all loci. Shannon diversity Index was calculated to provide a relative estimate of the degree of variation within each population.

c. Genetic differentiation and gene flow- The value of coefficient of genetic differentiation (G_{ST}) and effective migration rate or gene flow (*Nm*) were also calculated using POPGENE version 1.31 (Yeh *et al.,* 1999).

d. Genetic similarity and distance- Genetic similarity/identity and distance between pairs of populations of *P. homarus homarus* and *T. unimaculatus* were estimated using POPGENE Version 1.31 (Yeh *et al.,* 1999). Nei and Li's (1979) pair-wise genetic similarity (SI) among populations were computed and converted by POPGENE into genetic distance (GD) according to Hillis and Moritz's (1990) formula, GD = 1- SI. The SI reflects the proportion of bands shared between the individuals and values range from 0 when no bands are shared between RAPD profiles of two populations to 1, when no difference observed, *i.e.,* all bands are identical. The opposite holds true for 'GD' values.

e. Dendrogram- Cluster analysis was performed and dendrogram plotted based on RAPD data among three populations of *P. homarus* and four populations of *T. unimaculatus*. The method followed was unweighted pair group method using arithmetic averages (UPGMA) based on Nei's (1978) genetic distance which was modified from NEIGHBOR procedure of Phylip version 3.5 in POPGENE. To test the confidence level of each branch of UPGMA based dendrogram, the binary data matrix was bootstrapped 1000 times, using WinBoot (Yap and Nelson, 1996). Bootstrap vlues between 75 and 95 were considered significant (Lehmann *et al.*, 2000).

3.3.2. Hypervariable COI region of Mitochondrial DNA

3.3.2.1. PCR amplification and sequencing

Partal sequences of the hypervariable region of mtDNA COI gene were used for population structure analysis of both the species of lobsters under study. The region was amplified with the primer pair Jerry/ Pat (800 bps, Simon *et al*., 1994) and generated sequences were analysed to determine the population structure. The primer details are given in Table 5. PCR reactions were carried out in PTC 200 gradient thermal cycler (M.J. research, Inc., Watertown, Massachusetts, USA). The amplifications were performed in 25 µl reactions containing 10x assay buffer (100 mM Tris, 500 mM KCl, 0.1% gelatin, pH 9.0) with 15 mM $MgCl_2$ (Genei, Bangalore, India), 5 pmoles of each primer, 200 μM of each dNTP (Genei, Bangalore, India), 1.5 U *Taq* DNA polymerase, 0.5 µl of 25 mM $MgCl_2$ and 25 ng of template DNA. To check DNA contamination, a negative control was set up omitting template DNA from the reaction mixture. The composition of the PCR reaction mixture for mtDNA is given below.

PCR reaction mixture	Vol. per reaction
Double distilled water	18.05 μl
Assay buffer (10x)	2.5 μl
dNTPs	2.0 μl
Primer (forward and reverse)	0.45μl
Taq polymerase (Genei, Bangalore)	0.5μl
Template DNA	1.0 μl
$MgCl_2$	0.5 μl
Total volume	25.0μl

PCR mix for Mt DNA amplifications

The PCR cycling profiles were as follows: 5 min at 95^0 C for initial denaturation, then 30 cycles of denaturation for 45 S at 94^0 C, 30 S annealing at 47^0 C, 45 S extension at 72^0 C, and final extension for 10 min at $72°$ C.

Test Run

3 μl of the PCR product along with a marker (100bp DNA ladder, Genei, Bangalore, India, Fig. 4 B) was loaded in 1.5% agarose gel and

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electrophoresis was performed in 1X TBE buffer for 90 minutes at constant voltage (80 V) and stained with ethidium bromide and the gel was visualized under UV transilluminator and documented using Image Master VDS (Pharmacia Biotech, USA).

Purification of PCR product

      The remaining PCR product was purified using GeNei™ Quick PCR purification kit (Genei, Bangalore, India) following the instructions given by the manufacturer.

Sequencing Reaction

      The cleaned up PCR products were used as the template for sequencing PCR. Sequences were obtained from both directions using the same primer pairs for PCR by cycle sequencing using ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The composition of reaction mixture for Sequencing PCR is given in below.

      Cycle sequencing conditions were 95^0 C for 30 s, 50^0 C for 5 s and 60^0 C for 4 min repeated for 25 cycles. The sequencing PCR products were stored at 4^0 C.

Components	Vol. per reaction
BDT (Big Dye Terminator - kit)	1.0 μl
Buffer (Supplied with Cycle Sequencing kit.)	1.5 μl
DNA (10 - 25 ng /μl)	1.0 μl
Primer (forward or reverse; 10 pmol/μl)	0.5 μl
De-ionized water	6.0 μl.
Total volume	10.0 μl

Composition of reaction mix

Clean up for Sequencing:

The resulting cycle sequencing fragments were cleaned up following the procedure below.

- Add 2.5 μl of 125 mM EDTA to each tube (PCR tube containing the PCR product) and mix.

- Make up the final volume to 100 with double-distilled H_2O.

- Transfer to a 1.5 ml tube and add 10 μl 3 M sodium acetate (pH - 4.6).

- Add 250 μl of 100% ethanol and mix gently.

- Incubate the tubes at room temperature for 15 min.

- Spin tubes at 12,000 rpm, at room temperature, for 20 min.

- Decant the supernatant and add 250 μl of 70% ethanol.

- Spin tubes again for 10 min and the decant ethanol.

- Repeat the above step (ethanol wash).

- Decant the supernatant and the air-dry the pellet for 25 – 30 min.

The cleaned up products were sequenced at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram and SciGenom Labs Pvt. Ltd, Kakkanad, Cochin. The chromatograms were visually inspected with the aid of ABI sequence editor 3.3 (Applied Biosystems).

3.3.2.2. MtDNA analysis

a. Sequence alignment

The raw DNA sequences were edited manually using the software, BioEdit sequence alignment editor version 7.0.9.0 (Hall, 1999). Multiple alignments of sequences were performed using CLUSTAL W (Thompson *et al*., 1994) algorithm in BioEdit and the alignments were manually checked and corrected. Partial sequences of COI region- 666 bp of *P. homarus* and 681 bp of *T. unimaculatus* respectively were used for population study. The accuracy of COI sequences was confirmed by translating the nucleotide data to amino acid sequences.

b. Population genetic analysis

Haplotype number, haplotype frequency among different populations and nucleotide sequence characteristics after alignment were analysed using the program DnaSP v 5.10.01 (Librado and Rozas, 2009). All other

population analyses were performed using Arlequin version 3.0 (Excoffier *et al.*, 2005). Intrapopulation diversity was analysed by estimating gene diversity (*h*), nucleotide diversity (π). Analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) was performed for populations of each species separately to partition the total genetic variation into its variance components within and among populations and to produce F_{ST} statistics. All individuals collected from the same site were treated as a single population. Fixation indices (F_{ST}) were calculated on the basis of the information on haplotypes and their frequencies for populations together as well as for pairs of populations. The statistical significance of F_{ST} values was tested by permutation tests (10,000 replicates). Pair-wise Φ_{ST} values as well as gene flow (*Nm*) (Nei, 1987; Tajima, 1983) among populations were also calculated. The evolutionary analysis was conducted using *MEGA* version 5 (Tamura *et al.,* 2011). Pair-wise sequence divergence among populations was calculated according to Kimura 2-parameter model (Kimura, 1980) as well as the number and rate of transitions/ transversions were calculated with *MEGA* 5. MtDNA parsimony cladograms of haplotypes were constructed for both species (at 95% level connectivity) using the statistical parsimony of Templeton *et al.*, 1992 implemented in the software TCS v1.21 (Clement *et al.*, 2000). Demographic history was investigated using neutrality tests. Evidence of genetic bottleneck as well as population expansion was also tested using Tajima's (1989) '*D* ' and Fu's '*Fs*' test (Fu, 1997) using DnaSP v 5.0 (Librado and Rozas, 2009) and Arlequin version 3.0 (Excoffier *et al.*, 2005) respectively.

3.4. Species-specific DNA signatures and phylogenetic study of 11 species of lobsters

3.4.1. PCR amplification

Amplification of partial sequences of three mitochondrial (COI, 16SrRNA, 12SrRNA) and one nuclear (18SrRNA) genes was accomplished using either universal or designed primers (Table 5). PCR reactions for gene amplifications were carried out in PTC 200 gradient thermal cycler (M.J.

research, Inc., Watertown, Massachusetts). The amplifications were performed in 25 µl reactions containing 10x assay buffer (100 mM Tris, 500 mM KCl, 0.1% gelatin, pH 9.0) with 15 mM $MgCl_2$ (Genei, Bangalore, India), 5 pmoles of each primer, 200 μM of each dNTP (Genei, Bangalore, India), 1.5 U *Taq* DNA polymerase, 0.5 µl of 25 mM $MgCl_2$ and 25 ng of template DNA to a total volume of 25 μl. The PCR cycling profiles were as follows: 5 min at 95^0 C for initial denaturation, 30 cycles of denaturation for 45 S at 94^0 C, 30 S annealing at $42-57^0$ C (depending on genes), 45 S extension at 72^0 C, and final extension for 10 min at 72^0 C. The number of cycles increased to 40 for the Cytochrome oxidase 1 (CO1) gene amplification of scyllarid lobsters. Annealing temperatures for various primers are indicated Table 5. Prior to sequencing, PCR products were purified using GeNei^{TM} Quick PCR purification kit (Genei, Bangalore, India) following the instructions given by the manufacturer. The cleaned up PCR products were used as the template for sequencing PCR. Sequences were obtained from both directions using the same primer pairs for PCR by cycle sequencing using ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The products were sequenced at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram and SciGenom Labs Pvt. Ltd, Kakkanad, Cochin in PRISM ABI/3730 DNA sequencer. The chromatograms were visually inspected with the aid of ABI sequence editor 3.3 (Applied Biosystems).

3.4.2. Phylogenetic analysis using species-specific DNA markers

The raw DNA sequences were edited manually using BioEdit sequence alignment editor version 7.0.9.0. (Hall, 1999). Multiple alignments of sequences were performed using CLUSTAL W (Thompson *et al*., 1994) algorithm in BioEdit and the alignments were manually checked and corrected. Partial sequences of 18SrRNA were compiled using BioEdit. The COI sequences were translated into the corresponding amino acids for all species to check for stop codons.

Nucleotide sequence characteristics after alignment were analysed using the program DnaSP version: 5. v 5.10.01 (Librado and Rozas, 2009).

Number of mtDNA and nuclear DNA haplotypes were calculated for each species using the above programme.

In order to avoid an outgroup selection effect on phylogenetic reconstruction, both closely and distantly related outgroups were included in the analysis. The nephropid lobster *Homarus americanus* (NC_015607.1) and cray fish *Cherax destructor* (NC_011243.1) were included as outgroups for mtDNA sequence analysis. The complete mitochondrial genome sequences of the outgroup species were taken from GenBank and respective regions were aligned and compared with the ingroup taxa. *Homarus americanus* (AF235971) and *Cherax quadricarinatus* (AF235966) from GenBank were included as outgroup species for the nuclear 18SrRNA gene analysis.

Individual data sets for each gene and the combined mtDNA data set were taken for phylogenetic analysis using the software *MEGA* version 5 (Tamura *et al*., 2011). Individual gene sequence data was analysed and gene trees were constructed using distance (Neighbour-Joining) and Maximum Parsimony methods. Relative support for tree topology was obtained by bootstrapping (Felsenstein, 1985) using 1,000 iterations of the data matrix. Pair-wise evolutionary distance among species as well as genera were determined according to Kimura two-parameter model (Kimura, 1980) in *MEGA* 5. The base composition and rate of transitions/ transversions were also calculated using the program. All characters were equally weighted and alignment gaps were treated as missing data in phylogeny reconstruction. Phylogenetic trees were constructed for the global mtDNA data set (1723 bp long) in *MEGA* version 5 by maximum parsimony (MP), Neighbour joining (NJ), and Maximum Likelihood method (ML) which was based on Bayesian Information Content (BIC) criterion. Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. Non-uniformity of evolutionary rates among sites may be modelled by using a discrete Gamma distribution (+*G*) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+*I*). The best-fit model of nucleotide substitution selected for evolutionary

analysis in *MEGA* 5 for the combined mtDNA dataset was Tamura-Nei model (Tamura and Nei, 1993) with a gamma distribution and a proportion of invariable sites (TN93 + G + I). The evolutionary history was inferred by using the Maximum Likelihood method based on this model.

Fig. 4 A	Fig. 4 B
Lambda DNA/ *Eco*R I/ *Hin*d III Double Digest	100 bp DNA Ladder

0.7% Agarose   2% Agarose
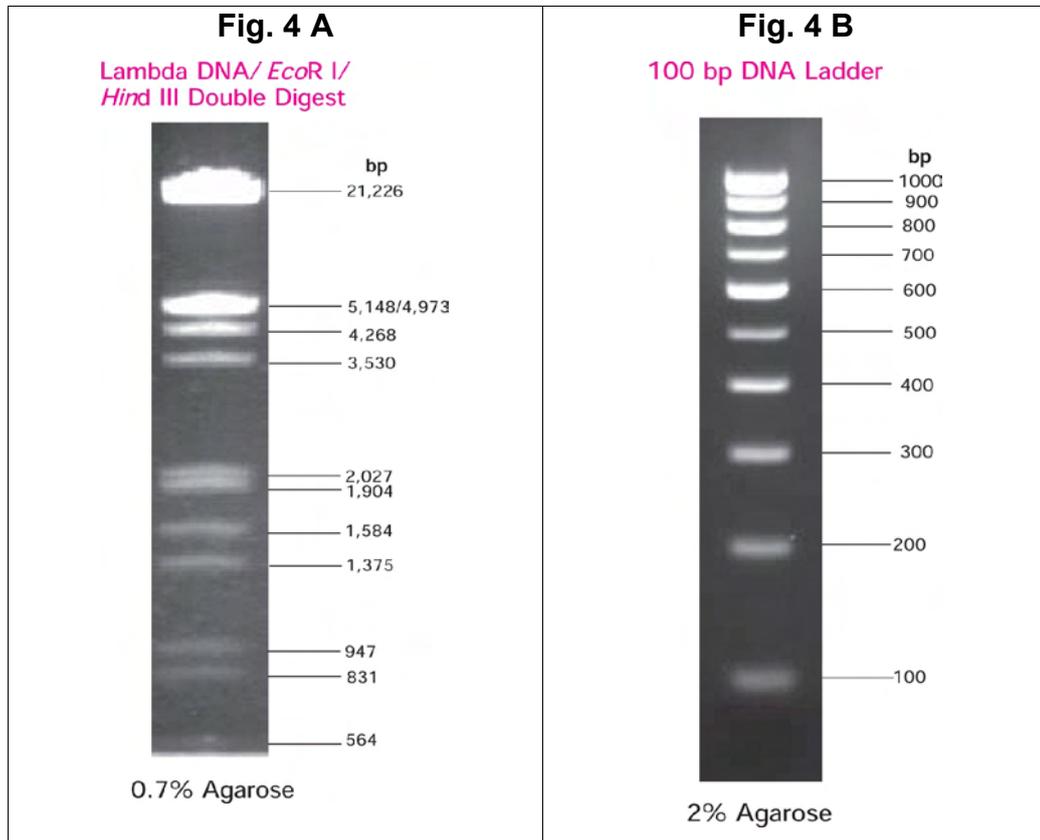
Table 5. Loci and Primers used in this study to amplify the mtDNA and nuclear DNA genes

Locus	Primer Name	Sequence	Source	Annealing Temp (in ^{0}C)	Fragment Size (bp)
GENES USED					
A) Population genetic study in lobsters *P. homarus* and *T. unimaculatus*					
1 hypervariable region of COI	Jerry	5' CAA CAY TTA TTT TGA TTT TTT GG 3'	Simon *et al.*, 1994	47	800 bp
	Pat	5' ATC CAT TAC ATA TAA TCT GCC ATA 3'			
B) 1. Species-specific signatures and genetic divergence study in 11 species of lobsters					
1) Cytochrome Oxidase 1 (CO1)	LCO1490	5`-GGT CAA CAA ATC ATA AAG ATA TTG G-3`	Folmer *et al.* (1994)	42 - Palinuridae 48- Scyllaridae	700
	HCO2198	5`-TAA ACT TCA GGG TGA CCA AAA AAT CA-3`			
2) 16SrRNA	16S-L-2510	5`-CGCCTGTTTATCAAAAACAT- 3`	Palumbi *et al.*, 1991	50	550
	16S- H-3080	5`- CCGGTCTGAACTCAGATCACGT- 3`			
3) 12SrRNA	L13337-12S	5`-YCTACTWTGYTACGACTTATCTC- 3`	Machida *et al.*, 2002	57	600
	H13845-12S	5`-GTGCCAGCAGCTGCGGTTA- 3`			
2. Nuclear gene used in genetic divergence study between lobsters					
1) 18SrRNA	18S 1f	TAC CTG GTT GAT CCT GCC AGT AG	Whiting (2002)	48	900
	18S b2.9	TAT CTG ATC GCC TTC GAA CCT CT			
	18S 5FrRNA	GCG AAA GCA TTT GCC AAG AA	Carranza *et al.* (1996)	50	900
	18S_9RrRNA	GAT CCT TCC GCA GGT TCA CCT AC			

N. B. Y= equimolar concentration of C or T; W= equimolar concentration of A or T

Chapter 4

RESULTS

4A. Population genetic structure analysis of *Panulirus homarus homarus* and *Thenus unimaculatus*

4A.1. Populaton structure analysis using RAPD PCR
4A.1.1. *P. homarus homarus*
a. Genetic characterization of populations

RAPD profiles were generated from all the 180 spiny lobsters using eight Operon random primers *viz.* OPA-17, OPA-19, OPAC-01, 0PAC-15, OPAH-04, OPAH-19, OPB-01 and OPB-20. Figs. 5-12 represent RAPD fingerprints generated by the above primers from Kollam, Chennai and Visakhapatnam respectively. These data were used for further analysis and the interpretations based on the quality of bands-robustness and reproducibility.

b. Genetic variability analysis

Lobster DNA analysed with the above eight primers showed 44 reliable amplified fragments ranging in size from 426 bp to 1860 bp (Table 6). Of these, 24 bands were polymorphic. The total number of RAPD fragments, number of polymorphic loci, average gene diversity for each and overall populations and performance of the Operon random primers on *P. homarus homarus* from the three sampling sites are given in Table 7.

Table 6. **Amplified fragments using selected Operon primers in *P. homarus homarus* populations.**

No. of fragments generated by primers	Size of fragments (in base pairs) generated using primers							
	OPA-17	OPA-19	OPAC-01	OPAC-15	OPAH-04	OPAH-19	OPB-01	OPB-20
1	1860	1720	1228	925	1724	1349	1389	1844
2	1579	1413	872	749	953	1185	1185	1280
3	1365	1181	715	623	690	888	1042	915
4	1255	1058	578	457	611	661	879	731
5	1144	840	-	-	535	590	764	565
6	509	689	-	-	426	-	647	488
7	-	592	-	-	-	-	-	-
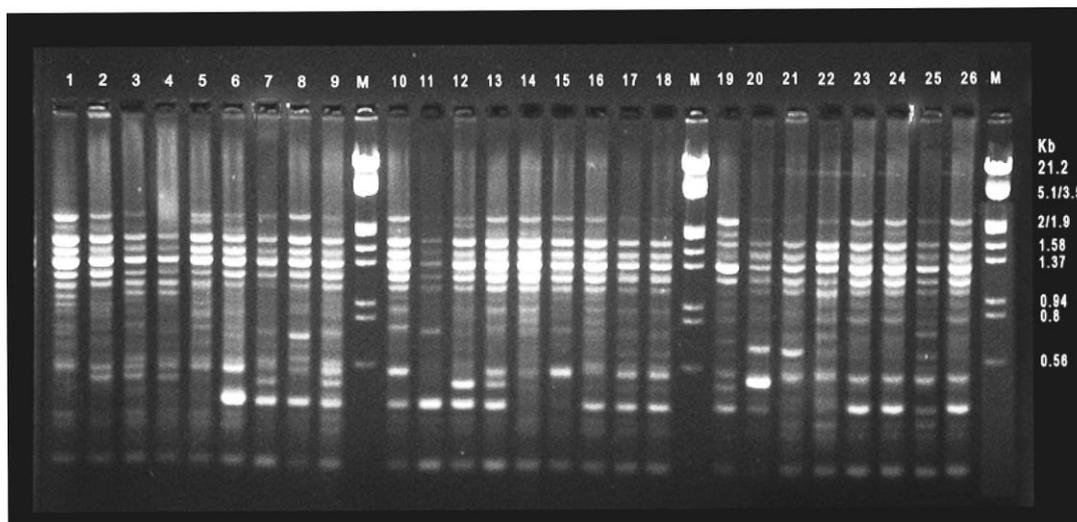
Fig. 5. RAPD profile of *P. homarus homarus* from Kollam (*lanes 1-9*), Chennai (lanes *10-18*) and Visakhapatnam (*lanes 19-26*) showing the amplified fragments by OPA-17. The lanes marked 'M' represent molecular weight marker (λDNA with *Eco*R I / *Hind* III double digest) fragments.
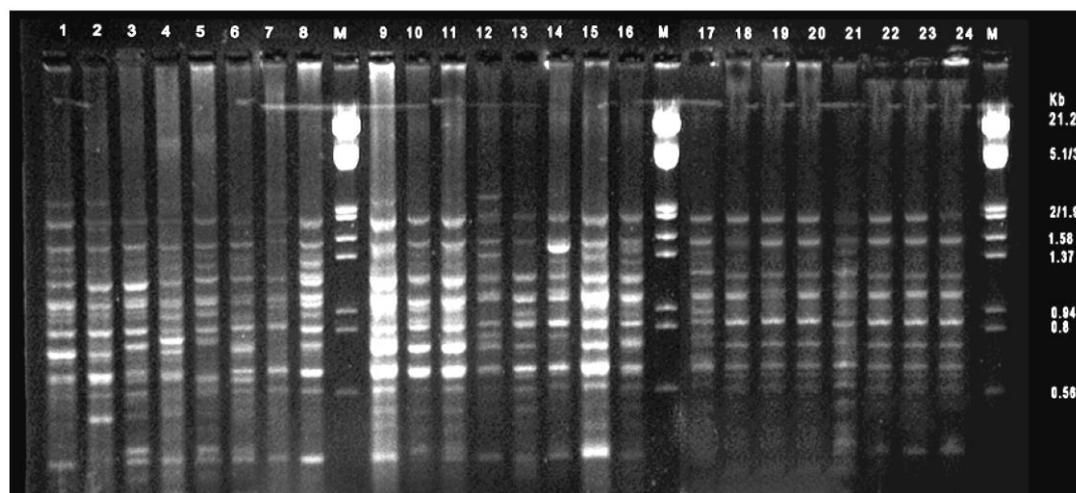
Fig. 6. RAPD profile of *P. homarus homarus* from Kollam (*lanes 1-8*), Chennai (lanes *9-16*) and Visakhapatnam (*lanes 17-24*) generated by OPA-19.
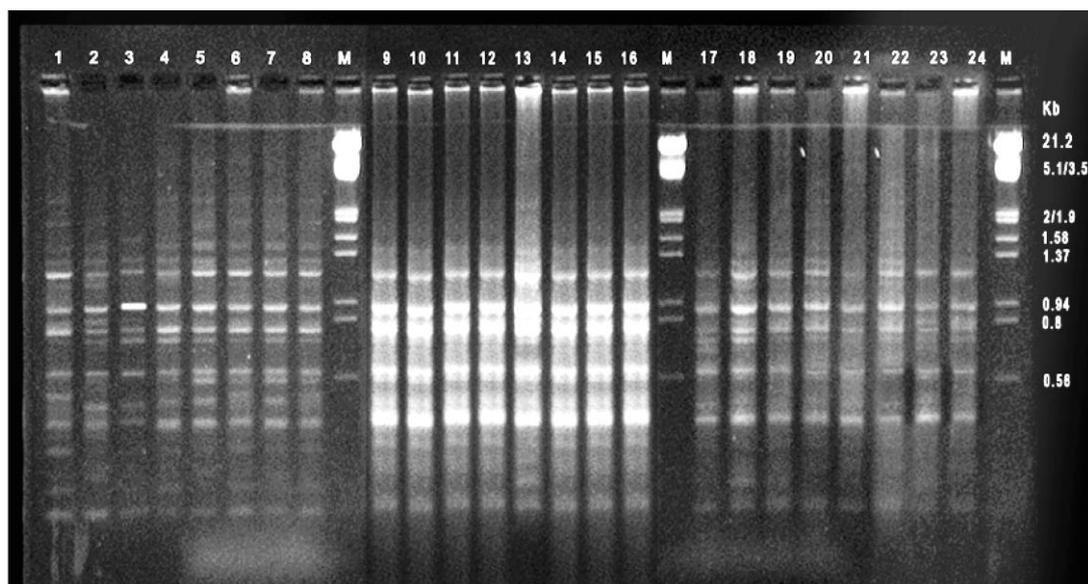
Fig. 7. RAPD profile of *P. homarus homarus* **from Kollam (***lanes 1-8***), Chennai (lanes** *9-16***) and Visakhapatnam (***lanes 17-24***) generated by OPAC-01.**
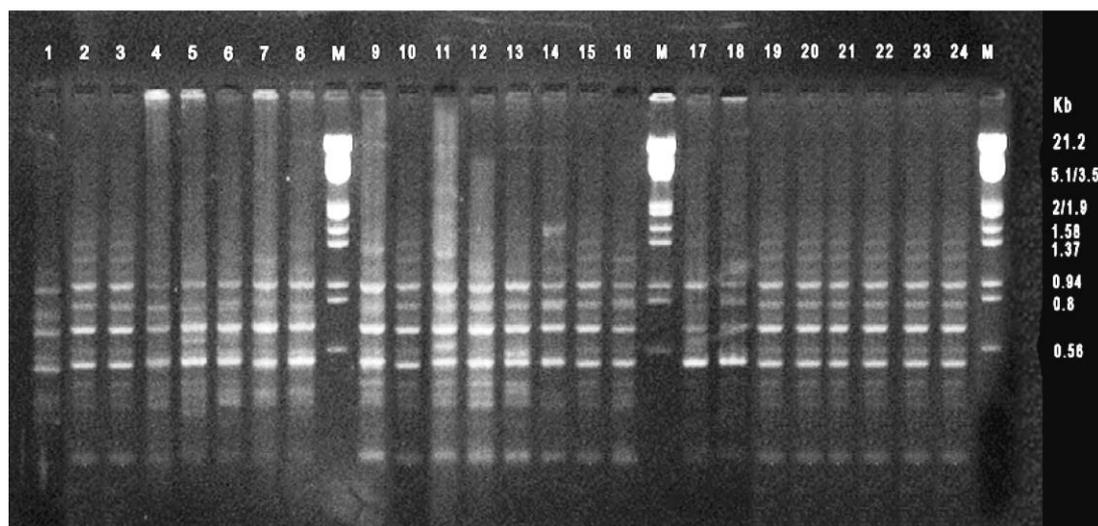
Fig. 8. RAPD profile of *P. homarus homarus* **from Kollam (***lanes 1-8***), Chennai (lanes** *9-16***) and Visakhapatnam (***lanes 17-24***) generated by OPAC-15.**
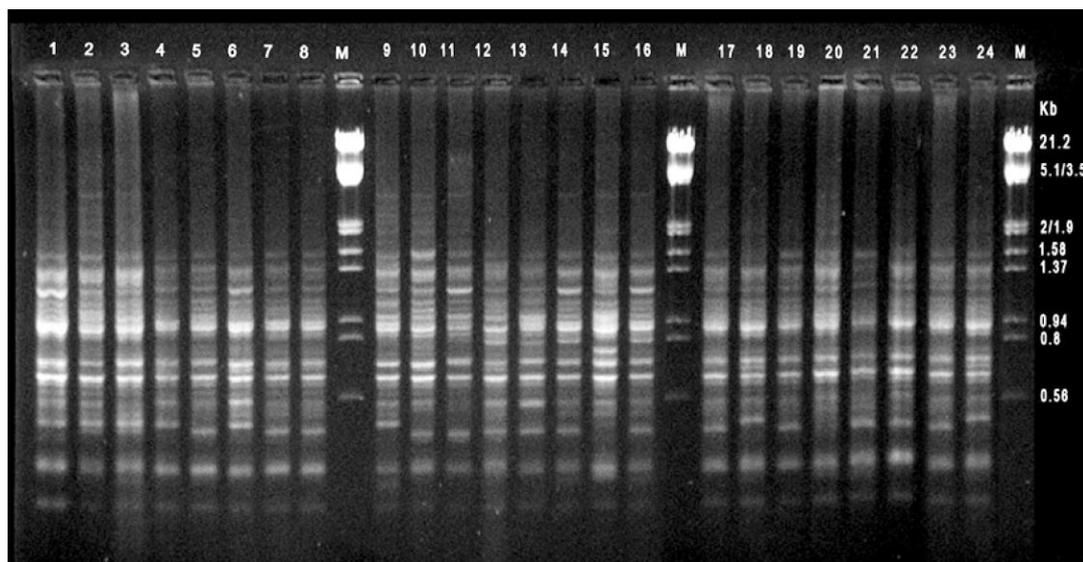
Fig. 9. RAPD profile of *P. homarus homarus* from Kollam (*lanes 1-9*), Chennai (lanes *10-18*) and Visakhapatnam (*lanes 19-27*) generated by OPAH-04.
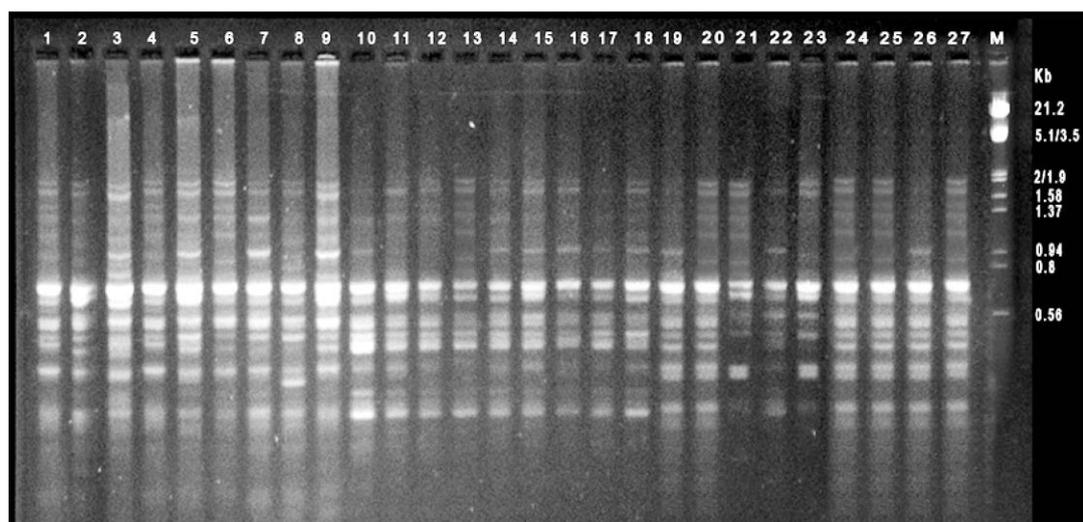
Fig. 10. RAPD profile of *P. homarus homarus* from Kollam (*lanes 1-9*), Chennai (lanes *10-18*) and Visakhapatnam (*lanes 19-27*) generated by OPAH-19.
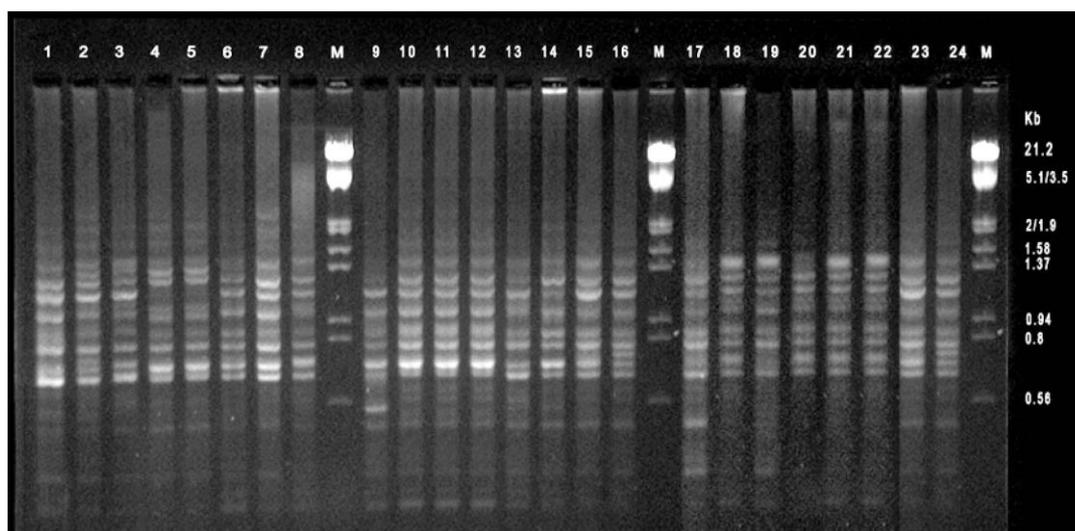
Fig. 11. RAPD profile of *P. homarus homarus* **from Kollam (***lanes 1-8***),
Chennai (lanes** *9-16***) and Visakhapatnam (***lanes 17-24***) generated by
OPB-01.**
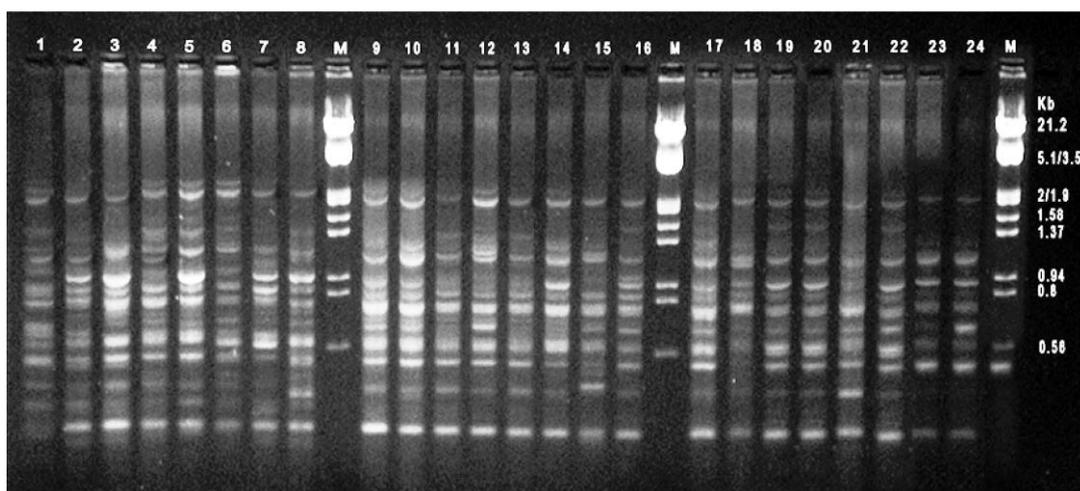
Fig. 12. RAPD profile of *P. homarus homarus* **from Kollam (***lanes 1-8***),
Chennai (lanes** *9-16***), Visakhapatnam (***lanes 17-24***) generated by
OPB-20.**

Table 7. Performance of the Operon random primers on *P. homarus homarus* collected from three locations: Kollam (QLN), Chennai (CHE) and Visakhapatnam (VSK).

Primer Code	Total No.of bands	KOLLAM (QLN)			CHENNAI (CHE)			VISAKHAPATNAM (VSK)			OVERALL POPULATIONS			Range (bp)	Species specific bands, if any (in bp)
		No. P	% P	(h)	No. P	% P	(h)	No. P	% P	(h)	No. P	% P	(h)		
OPA-17	6	3	50	0.20	2	33.3	0.16	3	50	0.23	3	50	0.23	509-1860	1579, 1365, 1144
OPA-19	7	5	71.43	0.33	3	42.86	0.19	2	28.57	0.14	5	71.3	0.26	592-1720	1058,689
OPAC-01	4	0	0	0	3	75	0.34	2	50	0.22	4	100	0.25	578-1228	NIL
OPAC-15	4	2	50	0.23	0	0	0	2	50	0.21	2	50	0.18	451-925	925,457
OPAH-04	6	0	0	0	2	33.3	0.17	3	50	0.22	3	50	0.17	426-1724	611, 426
OPAH-19	5	0	0	0	0	0	0	0	0	5	0	0	0	590-1349	590,661,888, 1185,1349
OPB-01	6	3	50	0.23	1	16.67	0.08	3	50	0.19	4	66.7	0.20	647-1389	764, 647
OPB-20	6	1	16.67	0.07	3	50	0.2	1	16.67	0.07	3	50	0.15	488-1844	1844,1280

The abbreviations 'h' stand for Average Gene Diversity, 'No. P' represents the number of polymorphic bands and '% P' represents percentage polymorphism in populations.

Six fragments were amplified with primer OPA-17, out of which three were polymorphic and it formed 50% polymorphism in populations (Fig. 5). The amplified fragments of 1579 bp, 1365 bp and 1144 bp were common to all individuals in all the three populations. For primer OPA-19, seven fragments were amplified, five were polymorphic and it formed 71.3% polymorphism (Fig. 6). The amplified fragments of 1058 bp and 689 bp were shared by all individuals. Four fragments were amplified with the primer OPAC-01 (Fig. 7). All were polymorphic and it accounted 100% polymorphism in the lobster populations studied. Out of the four fragments amplified by OPAC-15, two were polymorphic and it formed 60 % polymorphism. The amplified fragments of 925 bp and 457 bp were found to be specific (Fig. 8). In the case of primer OPAH-04, six fragments were amplified; of which only three were polymorphic forming 50 % polymorphism (Fig. 9). With this primer, all individuals in all the four populations shared the amplified fragments of 611 bp and 426 bp. All five amplified bands were monomorphic for the primer OPAH- 19 (Fig. 10). For primer OPB- 01, out of the six amplified fragments, four were polymorphic and it formed 66.7% polymorphism (Fig. 11). Six fragments were amplified with the primer OPB-20 (Fig. 12) out of which three were polymorphic and it formed 50% polymorphism in populations. The amplified fragments of 1844bp and 1280 bp were common to all individuals in all the three populations. No polymorphic bands were observed with primers OPAC-01 and OPAH-04 in Kollam population while these were polymorphic in Chennai and Visakhapatnam populations. The primer OPAC-15 didn`t yield polymorphism in the Chennai population while found to be polymorphic in the other two populations.

The estimates of percentage of polymorphism, Nei's (1987) gene diversity (h) and Shannon Information Index (Lewontin, 1972) in the three populations studied are given in Table 8. Same level of polymorphism was observed for Kollam and Chennai populations (31.82%) while a slightly lower value (29.55%) was observed in Visakhapatnam samples. In the present study, almost similar genetic diversity values for 'h' were found

within the populations. For the overall population, Nei's gene diversity value ('h') of 0.1719, percentage polymorphism of 54.55 and Shannon Information Index value of 0.2647 was observed.

Table 8. Genetic variability estimates in each and overall population of *P. homarus homarus*.

Parameter	Kollam	Chennai	Visakhapatnam	Overall populations
No.of polymorphic loci	14	14	13	24
% polymorphism (P)	31.82	31.82	29.55	54.55%
Shannon Information index (I)	0.1992	0.2073	0.1854	0.2647
Gene diversity (h)	0.1388	0.1462	0.1292	0.1719

c. Genetic Differentiation

For overall population, the Coefficient of genetic differentiation (G_{ST}) value observed was 0.0136 (Table 9). Average pair-wise similarity index (SI) and the genetic distance (GD) based on Nei's unbiased measures of genetic identity and genetic distances (Nei, 1978) were calculated for all eight primers together and are given in Table 10. Higher genetic identity values were obtained between Chennai and Visakhapatnam populations (0.9506). The values of 'GD' among populations had an average value of 0.0513 and it ranged from 0.0482 (Chennai-Visakhapatnam) to 0.0551 (Kollam-Visakhapatnam). No significant difference was observed between the genetic distance values of of *P. homarus homarus* populations from the three sampling sites. An unweighted Pair Group Method with arithmetic mean (UPGMA) dendrogram was constructed using the genetic distance values to show the genetic relationships among the *P. homarus homarus* collected from the three locations using POPGEN Software (Fig.13). The dendrogram showed two clusters, the Chennai and Visakhapatnam populations (East Coast of India) of *P. homarus homarus* formed one cluster while the Kollam population (West Coast) formed another cluster with low bootstrap support.

Table 10. Data showing pair wise comparison of similarity index (above diagonal) and genetic distance (below diagonal) of *P. homarus homarus* based on Nei (1978), calculated for eight primers.

Sites	Kollam	Chennai	Visakhapatnam
Kollam	*******	0.9634	0.9570
Chennai	0.0507	*******	0.9506
Visakhapatnam	0.0551	0.0482	*******

Table 9. Coefficient of genetic differentiation (G_{ST}) for overall population with eight RAPD Operon primers in *P. homarus homarus.*

Primers	G_{ST}
OPA-17	0.0112
OPA-19	0.0228
OPAC-01	0.0187
OPAC-15	0.0065
OPAH-04	0.0076
OPAH-19	0.0165
OPB-01	0.0143
OPB-20	0.0112
Mean overall population	0.0136
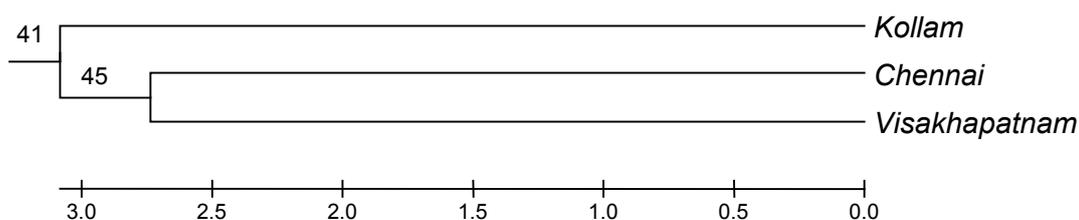
Fig. 13. Dendrogram Based Nei's (1978) Genetic distance for *P. homarus homarus* populations: Method-UPGMA-Modified from NEIGHBOR procedure of PHYLIP Version 3.5.

4A.1.2. *Thenus unimaculatus*

a. Genetic characterization of populations

RAPD profiles were generated from all the 240 slipper lobsters using nine Operon random primers *viz.* OPA-13, OPA-18, OPAC-05, OPAC-09,

OPAC-11, OPAC-13, OPAC-17, OPAH-06 and OPAH-09. Figs.14-22 represent RAPD fingerprints generated by the above primers from Kollam, Veraval, Visakhapatnam and Chennai respectively. These data were used for further analysis and the interpretations based on the quality of bands-robustness and reproducibility.

b. Genetic variability analysis

Lobster DNA analysed with nine primers amplified 46 reliable fragments ranging in size from 409 bp to 2200 bp (Table 11). Twenty seven bands were polymorphic. Performance of the Operon random primers on *T. unimaculatus* collected from four locations is given in Table 12.

Table 11. Amplified fragments using each primer selected for population study in *Thenus unimaculatus*.

No. of Fragments Generated by primers	Size of fragments (in base pairs) generated using primers								
	OPA-13	OPA-18	OPAC-5	OPAC-09	OPAC-11	OPAC-13	OPAC-17	OPAH-06	OPAH-09
1	1125	1229	1196	1824	1192	2200	1253	1297	1200
2	776	1012	979	1752	809	1552	1078	1010	1032
3	618	697	800	1069	682	1360	790	828	744
4	520	568	639	880	573	1007	717	628	604
5	-	409	526	768	510	611	582	574	583
6	-	-	-	662	-	-	-	-	-
7	-	-	-	575	-	-	-	-	-

Four fragments were amplified with the primer OPA-13 (Fig. 14). Two were polymorphic which showed 50% polymorphism in the slipper lobster populations studied. The amplified fragments of 618 and 520 bp were common to all individuals in all the four populations. For primer OPA-18, five fragments were amplified, three were polymorphic and it formed 60% polymorphism (Fig. 15). Out of the five fragments amplified by OPAC-05 (Fig. 16), two were polymorphic and it formed 40 % polymorphism. Seven fragments were amplified with the primer OPAC-09 ((Fig. 17). Five were

polymorphic and it showed 71% polymorphism in the slipper lobster populations studied. In the case of primer OPAC-11, five fragments were amplified (Fig. 18), of which only four were polymorphic forming 80% polymorphism. Using this primer, all individuals in all the four populations shared the amplified fragment of 809 bp. For primer OPAC-13, out of the five amplified fragments (Fig. 19), three were polymorphic and it formed 60% polymorphism. The amplified fragment of 1007 bp and 1360 bp were shared by all individuals. Five fragments were amplified with the primer OPAC-17 out of which four were polymorphic and it formed 80% polymorphism in populations (Fig. 20). The amplified fragment of 1078 bp was common to all individuals in all the populations. OPAH-06 primer produced five amplicons three of which were polymorphic and the fragments 1297 and 628 can be considered specific (Fig. 21). The primer OPAH-09 yielded five bands out of which there was single polymorphic band (Fig. 22). The amplified fragments 1200bp, 1032bp, 744 bp and 604 bp were shared by all four populations and canbe considered specific. No polymorphic bands were observed with primers OPA-13 in Kollam and Visakhapatnam populations; OPA-18, OPAC-05 and OPAC-13 in Veraval and Visakhapatnam populations; OPAH-06 in Kollam and Veraval populations; OPAH-09 in Kollam, Chennai and Visakhapatnam, while these were polymorphic in other populations. The overall estimate of number of polymorphic loci, Nei`s gene diversity (h), Shannon Information Index in *T. unimaculatus* populations studied are given in Table 13. For the over all population, percentage of polymorphism value of 58.7, Nei's gene diversity value 'h' of 0.1446 and Shannon Information Index value of 0.2379 was observed. The level of polymorphism was highest for Kollam populations (30.43%) while lower values (15.22%) were observed in Veraval and Visakhapatnam samples. In the present study, genetic diversity values for 'h' were found to be the highest (0.1375) in Kollam populations and lowest (~0.073) for Visakhapatnam and Veraval samples.
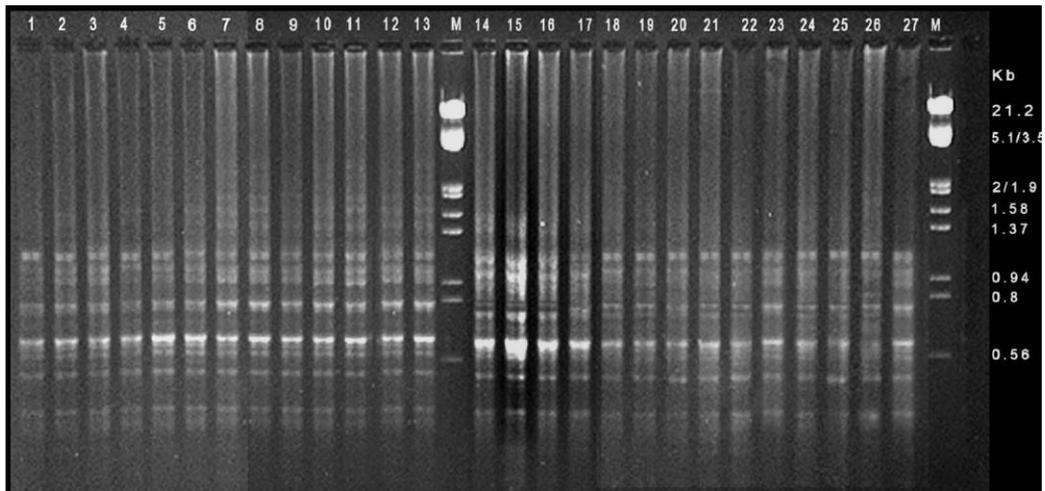
Fig. 14. RAPD profile of *T. unimaculatus* from Kollam (*lanes 1-7*), Veraval (lanes 8-*14*) Visakhapatnam (*lanes 15-21*) and Chennai (*lanes 22-28*) showing the amplified fragments by OPA- 13. The lanes marked 'M' represent molecular weight marker (λDNA with *Eco*R I / *Hind* III double digest) fragments.
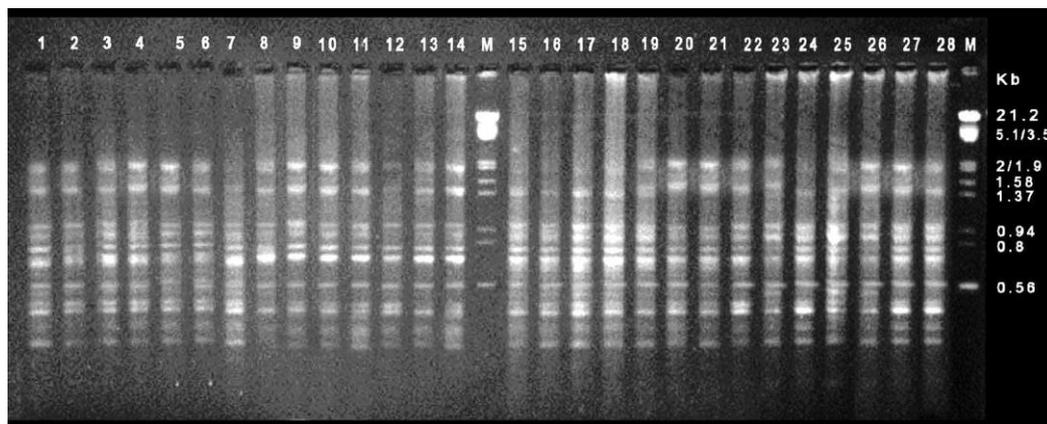
Fig. 15. RAPD profile of *T. unimaculatus* from Kollam (*lanes 1-7*), Veraval (lanes 8-*14*), Visakhapatnam (*lanes 15-21*) and Chennai (*lanes 22-28*) generated by OPA-18.
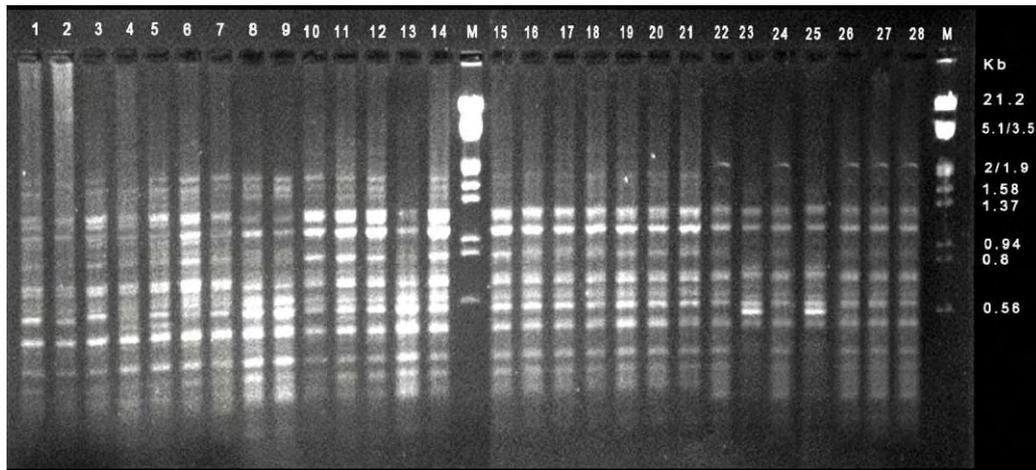
Fig. 16. RAPD profile of *T. unimaculatus* **from Kollam (***lanes 1-7***), Veraval (lanes 8-***14***), Visakhapatnam (***lanes 15-21***) and Chennai (***lanes 22-28***) generated by OPAC-05.**
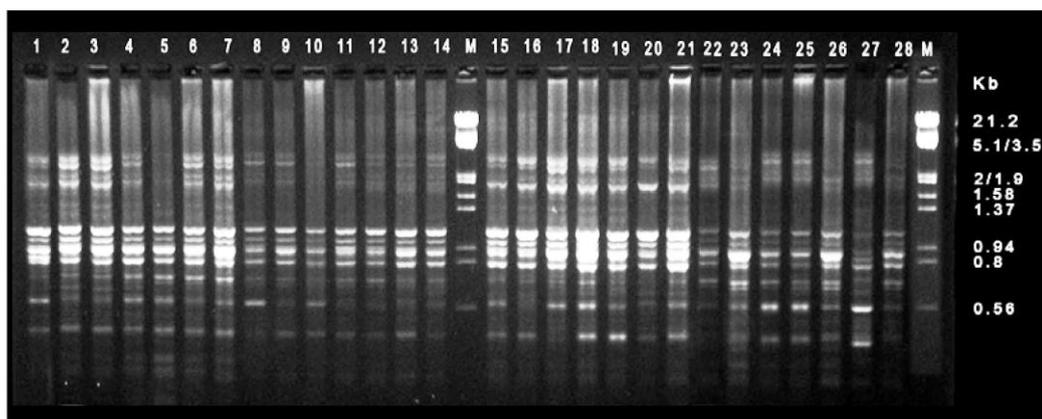
Fig. 17. RAPD profile of *T. unimaculatus* **originating from Kollam (***lanes 1-7***), Veraval (lanes 8-***14***), Visakhapatnam (***lanes 15-21***), Chennai (***lanes 22-28***) generated by OPAC-09**
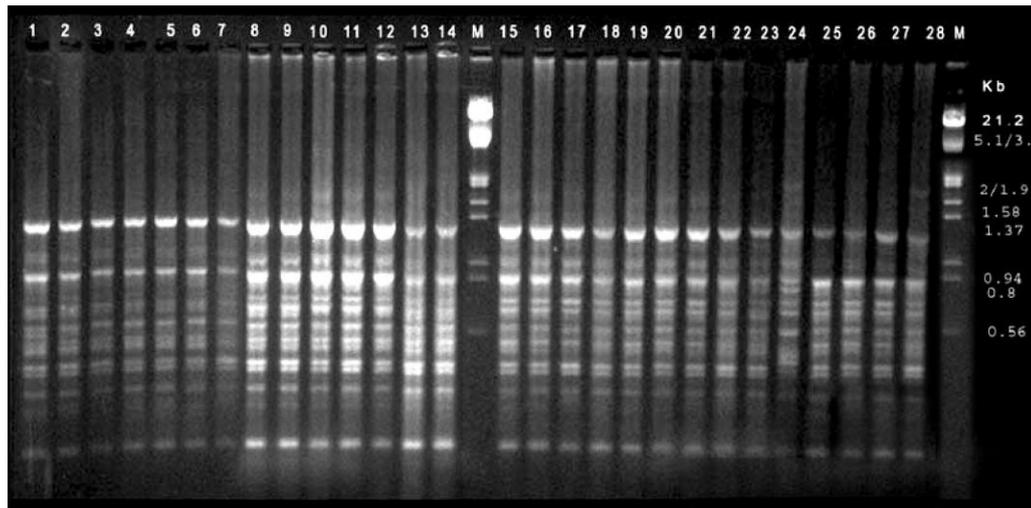
Fig. 18. RAPD profile of *T. unimaculatus* **from Kollam (***lanes 1-7***), Veraval (lanes 8-***14***), Visakhapatnam (***lanes 15-21***) and Chennai (***lanes 22-28***) generated by OPAC-11.**

Fig. 19. R.APD profile of *T. unimaculatus* **from Kollam (***lanes 1-7***), Veraval (lanes 8-***14***), Visakhapatnam (***lanes 15-21***) and Chennai (***lanes 22-28***) generated by OPAC-13.**
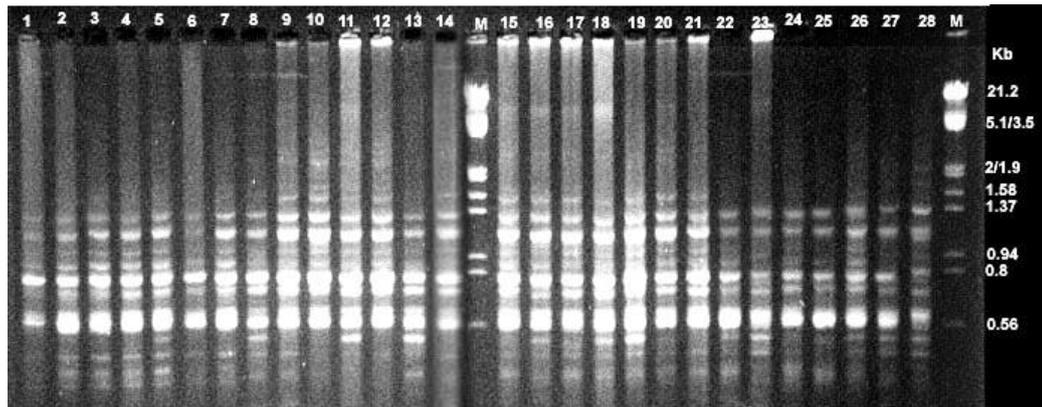
Fig. 20. RAPD profile of *T. unimaculatus* from Kollam (*lanes 1-7*), Veraval (lanes 8-*14*), Visakhapatnam (*lanes 15-21*) and Chennai (*lanes 22-28*) generated by OPAC-17.
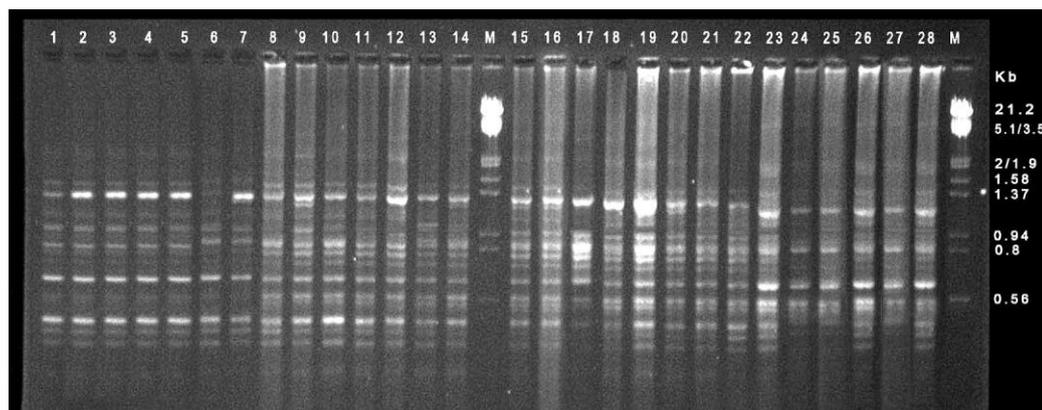
Fig. 21. RAPD profile of *T. unimaculatus* from Kollam (*lanes 1-7*), Veraval (lanes 8-*14*), Visakhapatnam (*lanes 15-21*) and Chennai (*lanes 22-28*) generated by OPAH-06.
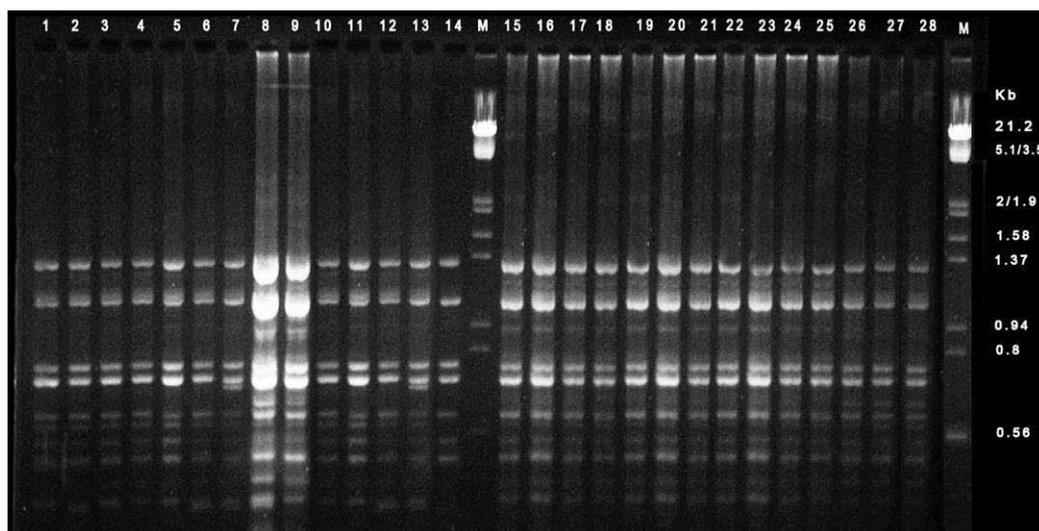
Fig. 22. RAPD profile of *T. unimaculatus* from Kollam (*lanes 1-7*), Veraval (lanes 8-*14*), Visakhapatnam (*lanes 15-21*), Chennai (*lanes 22-28*) generated by OPAH-09.

Table 13. Genetic variability estimates in each and overall population of *T. unimaculatus*.

Parameter	Veraval	Kollam	Chennai	Visakha patnam	Overall population
No.of polymorphic loci	7	14	13	7	27
Percentage polymorphism	15.22	30.43	28.26	15.22	58.70
Shannon Information index (I)	0.1022	0.1957	0.1794	0.1011	0.2379
Gene diversity (h)	0.0728	0.1375	0.1261	0.0718	0.1446

c. Genetic differentiation

The Coefficient of genetic differentiation (G_{ST}) for overall populations with nine RAPD primers was 0.0442 (Table 14). Average pair wise similarity index (SI) and the genetic distance based on Nei's unbiased measures of genetic identity and genetic distances (Nei, 1978) were calculated for all nine primers together and are given in Table 15. The values of Nei's unbiased genetic distance 'GD' among populations had an average value of 0.0768 and it ranged from 0.0720 (Chennai- Visakhapatnam) to 0.0815

(Veraval- Visakhapatnam). An unweighted Pair Group Method with arithmetic mean (UPGMA) dendogram was constructed usingthe genetic distance values to show the genetic relationships among the*T. unimaculatus* collected from the four locations (Fig. 23). No significant difference was observed between the genetic distance values of of populations from the four sampling sites. The dendrogram showed two clusters, the Veraval and Kollam populations (West Coast of India) of *T. unimaculatus* formed one cluster while the Chennai and Visakhapatnam populations (East Coast) formed another cluster.

Table 14. Coefficient of genetic differentiation (G_{ST}) for overall population with nine primers in *T. unimaculatus*.

Primers	G_{ST}
OPA-13	0.0527
OPA-18	0.0326
OPAC-5	0.0499
OPAC-09	0.0464
OPAC-11	0.0299
OPAC-13	0.0263
OPAC-17	0.0419
OPAH-06	0.0681
OPAH-09	0.0500
Mean overall population	0.0442

Table 15. Data showing pair-wise comparison of similarity index (above diagonal) and genetic distance (below diagonal) of *T. unimaculatus* based on Nei (1978), calculated for nine primers.

Sites	Veraval	Kollam	Chennai	Visakhapatnam
Veraval	*******	0.9264	0.9228	0.9185
Kollam	0.0736	*******	0.9239	0.9203
Chennai	0.0772	0.0761	*******	0.9280
Visakhapatnam	0.0815	0.0797	0.0720	*******

Table. 12. Performance of the Operon random primers on *T. unimaculatus* collected from 4 locations: Veraval (VRL), Kollam (QLN), Chennai (CHE) and Visakhapatnam (VSK).

Primer Code	Total No.of band	VERAVAL (VRL)			KOLLAM (QLN)			CHENNAI (CHE)			VISAKHAPATNAM (VSK)			OVERALL POPULATIONS			Range (bp)	Species specific bands, if any (in bp)
		No. P	% P	(h)	No. P	% P	(h)	No.P	% P	(h)	No. P	% P	(h)	No. P	% P	(h)		
OPA-13	4	1	25	0.12	0	0	0	2	50	0.19	0	0	0	2	50	0.14	520-1125	618, 520
OPA-18	5	0	0	0	2	40	0.19	2	40	0.19	0	0	0	3	60	0.13	409-1129	1229, 697
OPAC-5	5	0	0	0	1	20	0.09	1	20	0.09	0	0	0	2	40	0.08	526-1196	979, 639,526
OPAC-09	7	2	29	0.13	3	43	0.21	2	29	0.13	2	29	0.13	5	71	0.20	431-1824	768,1069
OPAC-11	5	2	40	0.19	2	40	0.19	2	40	0.19	1	20	0.09	4	80	0.21	510-1192	809
OPAC-13	5	0	0	0	2	40	0.19	1	20	0.09	0	0	0	3	60	0.11	611-2200	1007,1360
OPAC-17	5	1	20	0.09	3	60	0.21	2	40	0.19	1	20	0.09	4	80	0.19	582-1253	1078
OPAH-06	5	0	0	0	0	0	0	2	40	0.19	3	60	0.29	3	60	0.19	574-1297	1297,628
OPAH-09	5	1	20	0.09	0	0	0	0	0	0	0	0	0	1	20	0.05	604-1200	1200,1032,744,604

The abbreviations 'h' stand for Average Gene Diversity, 'No. P' represents the number of polymorphic bands and '% P' represents percentage polymorphism in population
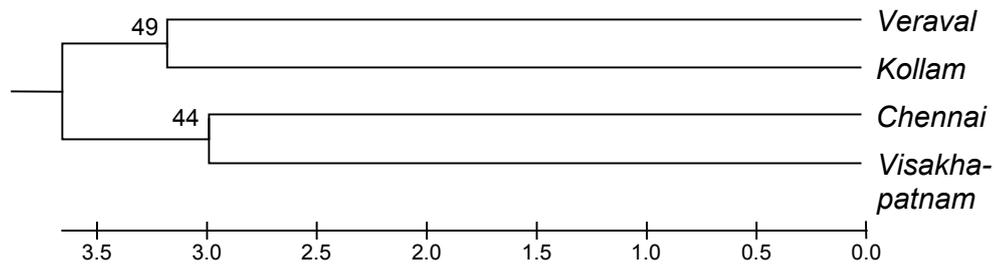
Fig. 23. Dendrogram Based Nei's (1978) Genetic distance for *Thenus unimaculatus* populations: Method UPGMA -- Modified from NEIGHBOR procedure of PHYLIP Version.

4A.2. Population genetic structure analysis of lobster species using hypervariable COI region of mtDNA

4A.2.1. *P. homarus homarus*

The variable/ fast-evolving region of COI gene was used to infer intra-specific/population level phylogenies of *P. homarus homarus*. For this, Jerry-Pat primers, C1-J-2183 and TL2-N-3014 (Simon *et al*., 1994) were employed which generated ~ 750bp product. The partial sequences of COI gene was generated from 60 samples of *P. homarus homarus* from three different geographic locations. A 666 bp region was finally obtained for analysis after sequence editing. The accuracy of COI sequences was confirmed by translating the nucleotide data to amino acid sequences. DNA sequence of a representative haplotype and the translated protein are given in Fig. 24. The sequences generated from this study were deposited in the NCBI GenBank (Table .18).

a. COI sequence variations

From the 666-bp fragment of COI region of 60 *P. homarus homarus* samples, 23 different haplotypes which contained 45 (6.76%) divergent nucleotide sites without indels were obtained. There were 621 invariable /monomorphic characters (93.2%) and 45 (6.69%) variable sites out of which 37 were informative for parsimony. According to codon position, the informative ones were in the third position. All the substitutions were

synonymous or 'silent', not resulting in an amino acid change. The empirical percentages of the different nucleotides were A = 26%, C = 20.8%, G = 20.2% and T = 33 %. The transition-to- transversion ratio (Ts/Tv) estimate for the ingroup was 8.96. The variable nucleotide positions in the mitochondrial DNA sequences of the COI region for locations Kollam (QLN), Chennai (CHE), Visakhapatanam (VSK) are given in Table 16. The molecular diversity indices are given in Table 17.

b. Amino Acid Translations

Nucleotide base pairs of the different haplotypes from all populations were translated into amino acid residues. Out of 222 total residues, no characters were variable and parsimony informative.

Table 17. Molecular diversity indices of 666 bp fragment of the variable COI gene across each population of *P. homarus homarus*.

Statistics	*P. homarus homarus*		
	Kollam	Chennai	Visakhapatnam
No. of transitions	30	20	16
No. of transversions	1	3	3
No. of substitutions	31	23	19
No. of indels	0	0	0

c. Genetic variability

Comparison of the sequences revealed 23 different haplotypes of *P. homarus homarus* out of 60 individuals from three different geographic locations, defined by 123 divergent nucleotide sites. Haplotypes and their relative frequencies among populations were presented in Table 18. The haplotypes Hap2 and Hap11 were found to be shared among the three populations. The haplotypes Hap9 and Hap19 were found to be shared between Kollam-Chennai and Chennai-Visakhapatnam populations respectively. Unique haplotypes were observed in all the three populations.

The genetic diversity analysis for each and overall population done using DnaSP version 5 and results are presented in Table 19. The nucleotide

diversity at three sampling sites was in the range of 0.0071-0.0102 with an overall estimate of 0.0089 and haplotype diversity in the range of 0.8947–0.9368 with an overall value of 0.9226.

d. Genetic Differentiation and gene flow

Mean pairwise distances (%) between the haplotype of each population calculated by the Kimura 2-parameter method in *MEGA* 5 and the values which ranged from 0.008 (Chennai and Visakhapatnam populations) to 0.01 (Kollam and Visakhapatnam populations) indicated similar genetic structuring (Table 20).

Table 18. Distribution of 23 haplotypes, with 666 bp fragment size of COI gene among the populations of *P. homarus homarus*.

Haplotypes	GenBank accession number	No.of individuals possessing the haplotype from sampling site		
		Kollam	Chennai	Visakha patnam
*Hap1	JQ229885	2	1	1
*Hap2	JQ229886	1	3	2
*Hap3	JQ229916	2	1	0
*Hap4	JQ229917	1	0	1
*Hap5	JQ229918	2	1	1
*Hap6	JQ229919	2	0	1
*Hap7	JQ229920	1	1	0
*Hap8	JQ229921	1	1	1
*Hap9	JQ229883	1	1	0
*Hap10	JQ229887	2	0	1
*Hap11	JQ229888	2	1	2
Hap12	JQ229884	0	2	0
*Hap13	JQ229910	0	1	1
*Hap14	JQ229911	1	1	1
Hap15	JQ229912	0	1	0
Hap16	JQ229913	0	1	0
Hap17	JQ229914	0	1	0
Hap18	JQ229915	0	1	0
*Hap19	JQ229925	1	2	1
Hap20	JQ229922	0	0	2
Hap21	JQ229923	0	0	2
*Hap22	JQ229924	1	0	2
Hap23	JQ229926	0	0	1
TOTAL		20	20	20

* indicates shared haplotypes

Table 16. Variable nucleotide (nucl.) positions in the mitochondrial DNA sequences of the COI region of *P. homarus homarus* for locations Kollam (K), Chennai (C), and Visakhapatnam (V). Numbers in the uppermost column refer to nucleotide position from the beginning of the COI region. Identity with the nucleotide of Hap1 is indicated by dots.

Nucl. position	84	105	114	123	129	141	180	192	201	216	249	255	288	300	312	321	324	348	366	372	381	398	401	418	467	470	488	491	500	507	519	515	528	523	545	554	567	568	586	600	608	614	629	638	648	Loca-tion (n)
Hap No.1	G	G	C	T	T	A	C	G	A	T	C	G	T	T	T	G	G	T	G	A	C	G	C	C	A	G	A	C	C	G	A	A	T	G	T	T	C	A	A	T	G	A	T	G	A	K(2), C(1),V(1)
2	.	.	.	C	.	.	.	A	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	K(1),C(3),V(2)
3	.	.	.	.	C	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	K(2),C(1)
4	.	A	.	.	.	G	.	A	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	K(1), V(1)
5	A	.	.	.	.	.	T	A	.	C	.	.	.	.	.	.	.	C	.	A	.	.	.	.	.	G	A	.	.	.	.	A	.	G	.	.	C	.	.	.	C	.	.	C	.	K(2),C(1), V(1)
6	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	A	G	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	C	.	.	.	.	.	.	.	K(2), V(1)
7	.	.	T	.	.	.	.	T	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K(1),C(1)
8	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	K(1), C(1), V(1)
9	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	K(1),C(1)
10	.	.	.	.	.	.	.	A	G	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	T	.	.	.	A	.	.	.	.	A	.	K(2),V(1)
11	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	K(2),C(1),V(2)
12	.	.	.	C	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C(2)
13	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	C(1), V(1)
14	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K(1), C(1), V(1)
15	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	G	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	C(1)
16	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	G	T	.	.	.	.	.	.	.	.	.	.	.	T	.	G	.	.	C(1)
17	.	.	.	.	.	.	.	A	.	.	T	.	.	.	.	.	.	C	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	C(1)
18	.	.	.	.	.	T	A	.	C	.	.	.	.	.	C	.	A	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	G	.	.	.	C	.	.	.	.	C	C	C(1)
19	.	.	.	C	.	.	.	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K(1),C(2),V(1)
20	.	.	T	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	V(2)
21	.	.	.	.	.	T	.	A	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	G	.	.	V(2)
22	.	.	T	.	.	.	.	A	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	T	.	.	.	.	C	.	.	.	.	.	G	.	A	.	.	K(1),V(2)
23	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V(1)

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Table 19. Genetic diversity analysis for each and for overall population of
P. homarus homarus.

	Kollam	Chennai	Visakha-patnam	Total data estimtes
No.of sequences	20	20	20	60
No. of variable/polymorphic sites	31	23	19	45
No.of haplotypes (n_h)	11	11	8	23
Nucleotide diversity(π)	0.0102	0.0071	0.0087	0.0089
Haplotype (gene) diversity (h)	0.9368	0.9053	0.8947	0.9226

The genetic differentiation among scalloped spiny lobster populations was assessed using pair-wise F_{ST} and Φ_{ST} value comparisons. F_{ST} and Φ_{ST} values(Nei, 1977) based on haplotype frequencies and pair-wise sequence divergence values respectively, were tested using 1000 permutations using Arlequin version 3.0 (Excoffier _et al._, 2005). The significance for F_{ST} and Φ_{ST} values was tested at 5% level with a Bonferroni corrected _P_-value. They were found to be insignificant between populations indicating no population subdivision (Table 21). The AMOVA analysis indicated that 96.06% of the total molecular variance was distributed within populations while only 3.94% attributed to differences among poulations (Table 22). Exact tests for population differentiation based on haplotype frequencies (Raymond and Rousset, 1995a) with 10,000 randomizations of Markov chain steps were performed using Arlequin version 3.0 and no significant differences were observed. The gene flow (_Nm_) estimated was high among populations (Table 23).

e. Inference on Demographic history

Inferences on patterns of demographic history were obtained by using Tajima's (1989) _D_ and Fu's (1997) F_S tests (Table 24). Tajima's _D_ statistic values and negative Fu's F_S statistics values were found to be significant (P<0.01) for all populations.

f. Geographical relationships among haplotypes-The statistical parsimony of Templeton *et al*., 1992 implemented in the TCS v1.21 software was used to reconstruct a minimum-spanning haplotype network. Relationships among the above 23 haplotypes detected in the present study is shown in Fig. 25. Many haplotypes were found to be shared between two populations. There was no characteristic geographic distribution pattern for the haplotypes.

Table 23. Matrix of *Nm* (gene flow) values for mtDNA COI for *P. homarus* populations

	Kollam	Chennai	Visakhapattanam
Kollam	****		
Chennai	14.74359	****	
Visakhapattanam	12.71520	9.84236	****

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a. COI - 666 bp partial sequence

```
1    AAAGATATTG GTACCTTATA TTTTATTTTC GGAGCATGAG CTGGGATAGT GGGAACTTCT

61   TTAAGACTTA TTATTCGAGC AGAGCTCGGT CAACCAGGAA GACTGATTGG AGACGACCAA

121  ATTTATAATG TAGTAGTAAC AGCCCACGCT TTTGTGATAA TTTTCTTTAT AGTTATGCCC

181  ATTATAATTG GGGGATTCGG AAACTGGCTC GTTCCTATTA TGTTAGGTGC CCCAGATATG

241  GCATTTCCCC GAATGAATAA CATAAGATTC TGACTTTTAC CTCCCTCTCT AACGCTTCTT

301  CTAGCTAGTG GTATAGTGGA GAGGGGAGTA GGAACTGGCT GAACAGTTTA TCCCCCCCTA

361  GCAGGGGCAG TAGCCCATGC CGGAGCATCA GTAGATTTGG GTATTTTCTC CCTCCATCTT

421  GCCGGTGTGT CATCAATTCT AGGAGCCGTA AATTTTATTA CAACAGTAAT TAATATGCGA

481  TCTTCAGGTA TAACATTCGA CCGAATGCCA CTATTTGTAT GATCTGTGTT TATTACTGCC

541  ATTTTACTTC TACTTTCTCT TCCCGTACTA GCTGGAGCTA TTACTATACT TCTTACTGAT

601  CGTAATTTGA ACACATCATT CTTTGACCCA GTAGGAGGGG GAGATCCAAT TCTCTATCAA

661  CATCTA
```

b. COI - translated protein sequence

```
1    KDIGTLYFIF GAWAGMVGTS LSLIIRAELG QPGSLIGDDQ IYNVVVTAHA FVMIFFMVMP

61   IMIGGFGNWL VPIMLGAPDM AFPRMNNMSF WLLPPSLTLL LASGMVESGV GTGWTVYPPL

121  AGAVAHAGAS VDLGIFSLHL AGVSSILGAV NFITTVINMR SSGMTFDRMP LFVWSVFITA

181  ILLLLSLPVL AGAITMLLTD RNLNTSFFDP VGGGDPILYQ HL
```

Fig. 24. DNA sequence of COI gene and translated protein sequence of a representative haplotype of *P. homarus homarus*.

Table 20. **Mean pairwise K2Pdistances between populations (below diagonal) based on 666 bp region of mtDNA COI gene from haplotype data information of *P. homarus homarus* populations.**

	P. homarus homarus		
	Kollam	**Chennai**	**Visakhapatnam**
Kollam	****		
Chennai	0.009	****	
Visakhapatnam	0.010	0.008	****

Table 21. Pairwise F_{ST} values (above diagonal) and Φ_{ST} values (below diagonal) among three populations of *P. homarus homarus* populations.

	Kollam	**Chennai**	**Visakhapatnam**
Kollam	---------	0.0370 (-)	0.0454(-)
Chennai	0.03280(-)	---------	0.043(-)
Visahkapattanam	0.03784(-)	0.04834(-)	---------

(-) indicates not significant at 5% level

Table 22. **Results of the hierarchical analysis of molecular variance (AMOVA) of populations of *P. homarus homarus* based on variable mitochondrial COI region.**

Source of variation	*Degrees of freedom*	Variance components	% total variation	Fixation Index
Among populations within species	2	0.1183 (Va)	3.94	**Overall F_{ST} value**=0.0394 *Not significant*
Within populations	57	2.8842 (Vb)	96.06	
TOTAL	59	3.0025 (Vt)		

Fig. 25. TCS haplotype networks based on COI region in *P. homarus*: size of circle is proportional to haplotype frequency. The Haplotype numbers are given in circles.

Table 24. Results of non-random distribution tests for _P. homarus_ _homarus_ populations.

population	Tajima's _D_ statistic	Fu's F_S statistic
Kollam	-0.8759	-16.076
Chennai	-1.0513	-20.109
Visakhapatnam	-0.3176	-17.739

Tajima's D values significant at P<0.01; Fu' F_S Significant at P<0.01

4A.2.2. Analysis of _Thenus unimaculatus_ populations

The partial sequence of variable region of COI gene was generated from 80 samples of _Thenus unimaculatus_ from four different geographic locations using Jerry-Pat primers, C1-J-2183 and TL2-N-3014 (Simon _et al.,_ 1994). The size of amplified products was approximately 750 bp. A 681 bp region was finally obtained for analysis after sequence editing. The accuracy of COI sequences was confirmed by translating the nucleotide data to amino acid sequences. DNA sequence of a representative haplotype and the translated protein are given in Fig. 26. The sequences generated from this study were deposited in the NCBI GenBank (Table 27).

a. COI sequence variations

From the 681–bp fragment of COI region of _T. unimaculatus_ samples, 20 different haplotypes were obtained which contained 26 (3.8%) divergent nucleotide sites without indels. There were 655 invariable/ monomorphic characters (96.2%) and 26 variable sites all of which were informative for parsimony. According to codon position, 24 informative sites were in the third position. The single substitutions in first and second positions resulted in amino acid changes which were not parsimony informative. The empirical percentages of the different nucleotides were A = 25.4%, C = 20.5%, G = 19.2% and T = 34.8%. The transition-to-transversion ratio (Ts/Tv) estimate for the ingroup was 5.22. The variable nucleotide positions in the mitochondrial DNA sequences of the COI region for locations Veraval (VRL), Kollam (QLN), Chennai (CHE) and Visakhapatnam (VSK) are

given in Table 25. The molecular diversity indices of the *T. unimaculatus* populations are given in Table 26.

b. Amino Acid Translations

Nucleotide base pairs of the different haplotypes from all populations were translated into amino acid residues. Out of 227 total residues, two characters were variable but not parsimony informative.

c. Genetic variability

Comparison of the sequences revealed 20 different haplotypes out of 72 individuals from three different geographic locations, defined by 123 divergent nucleotide sites. Unique haplotypes were observed within all populations. 'Hap3' was found to be the dominant haplotype shared between all populations. Hap1, 2, 8 and 12 were shared between three populations. Haplotypes and their relative frequencies among populations were presented in Table 27. The genetic diversity analysis for each and overall population done using DnaSP version 5 and results are presented in Table 28. The nucleotide and haplotype diversities at three sampling sites were in the ranges of 0.005–0.008 and 0.758–0.928, respectively.

d. Genetic differentiation and gene flow

Mean pairwise distances between the haplotypes of each population calculated by the Kimura 2-parameter method in *MEGA* version 5 and the values which ranged from 0.005 -0.008 indicated shallow genetic structuring (Table 29). The overall mean pairwise distances within populations too was too low (0.008).

Pair-wise population F_{ST} and Φ_{ST} values (Nei, 1977) were tested using 1000 permutations using Arlequin version 3.0 (Excoffier *et al*., 2005). The significance for F_{ST} and Φ_{ST} values was tested at 5% level with a Bonferroni corrected *P*-value. They were found to be insignificant between populations indicating no population subdivision (Table 30). F_{ST} pairwise values low in general (0.0062- 0.0686). Fixation index over all samples (F_{ST}) was 0.0468, and showed no significant differences.

Table 26. Molecular diversity indiceses of 681 bp fragment of the variable region of COI gene across each population of *T. unimaculatus*.

Statistics	*Thenus unimaculatus*			
	Veraval	Kollam	Chennai	Visakhapattaanam
No. of transitions	7	14	11	14
No. transversions	2	3	1	2
No. of substitutions	9	16	12	15
No. of indels	0	0	0	0

Table 27. Distribution of 20 haplotypes, with 681 bp fragment size of COI gene   among the   populations of *T. unimaculatus*.

Haplotypes	GenBank accession number	No.of individuals possessing the haplotype from sampling site			
		Veraval	Kollam	Chennai	Visakha-patnam
*Hap1	JQ229897	4	2	0	1
*Hap2	JQ229927	2	2	1	0
*Hap3	JQ229936	5	4	7	5
Hap4	JQ229898	2	0	0	0
Hap5	JQ229895	0	1	0	0
*Hap6	JQ229896	1	1	0	0
*Hap7	JQ229933	0	1	0	1
*Hap8	JQ229931	1	2	1	0
*Hap9	JQ229935	1	2	0	0
Hap10	JQ229934	0	2	0	0
Hap11	JQ229937	0	1	0	0
*Hap12	JQ229893	1	0	1	3
Hap13	JQ229894	0	0	2	0
Hap14	JQ229930	0	0	2	0
*Hap15	JQ229929	1	0	1	1
Hap16	JQ229928	0	0	1	0
Hap17	JQ229932	0	0	2	0
Hap18	JQ229900	0	0	0	4
Hap19	JQ229899	0	0	0	2
Hap20	JQ229938	0	0	0	1
TOTAL		18	18	18	18

Table 28. Genetic diversity analysis for each and for overall population of

**   *T. unimaculatus***

	Veraval	Kollam	Chennai	Visakha patnam	Total
No.of individuals analyzed (n_t)	18	18	18	18	72
No. of variable sites	9	16	12	15	26
No.of haplotypes (n_h)	4	10	9	5	20
Nucleotide diversity(π)	0.0053	0.0063	0.0046	0.0081	0.0063
Haplotype (gene) diversity (h)	0.778	0.928	0.843	0.791	0.873
Population specific F_{ST} indices	0.0536	0.0448	0.0599	0.0287	0.0468

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a. COI – 681 bp partial sequence

```
1    GATATTGGTA CTCTATATTT TATTTTCGGA GCTTGGGCTG GTATAGTAGG AACTTCTCTA

61   AGATTGATTA TCCGAGCAGA GTTGGGACAA CCCGGTAGAC TAATTGGAGA TGACCAAATT

121  TATAACGTGG TTGTAACCGC TCATGCATTT ATTATAATTT TTTTTATAGT TATACCCATC

181  ATAATTGGAG GGTTTGGAAA TTGACTGGTC CCTCTTATAT TAGGAGCCCC AGATATAGCT

241  TTCCCACGAA TGAACAATAT AAGATTCTGA CTTCTTCCCC CTTCCTTAAT GCTACTCCTC

301  TCTAGAGGAA TAGTAGAAAG AGGAGTTGGT ACAGGATGAA CTGTGTACCC CCCTCTCTCA

361  GCAGCTGTTG CACATGCAGG AGCCTCGGTA GATCTCGGTA TTTTTTCACT TCATCTAGCA

421  GGTGTTTCAT CAATTTTAGG AGCAATTAAC TTTATAACAA CCGTTATTAA TATGCGATCT

481  AGAGGAATAA GAATGGATCG CATACCTCTT TTCGTATGAT CTGTCTTTAT TACAGCTGTC

541  CTTCTTCTTC TGTCTCTACC AGTATTAGCC GGGGCTATTA CTATACTTTT AACAGATCGA

601  AATCTTAATA CTTCTTTTTT TGACCCTGCT GGAGGAGGGG ATCCTATCCT TTACCAGCAT

661  CTCTTCTGAT TTTTTGGTCA C
```

b. COI – translated protein sequence

```
1    DIGTLYFIFG AWAGMVGTSL SLIIRAELGQ PGSLIGDDQI YNVVVTAHAF IMIFFMVMPI

61   MIGGFGNWLV PLMLGAPDMA FPRMNNMSFW LLPPSLMLLL SSGMVESGVG TGWTVYPPLS

121  AAVAHAGASV DLGIFSLHLA GVSSILGAIN FMTTVINMRS SGMSMDRMPL FVWSVFITAV

181  LLLLSLPVLA GAITMLLTDR NLNTSFFDPA GGGDPILYQH LFWFFGH
```

Fig. 26. DNA sequence of COI gene and translated protein sequence of a representative haplotype in *T. unimaculatus*.

Table 25. Variable nucleotide positions in the mitochondrial DNA sequences of the COI region in *T. unimaculatus* for locations Veraval (Ve), Kollam (QI), Chennai (Ch), and Visakhapatnam (Vsk). Numbers in the uppermost column refer to nucleotide position from the beginning of the COI region. Identity with nucleotides of Hap1 is indicated by dots.

Haplotypes	GenBank Accession Numbers	27	36	39	84	87	99	111	114	123	129	240	252	255	279	327	330	345	348	397	423	482	552	558	570	573	636	Location (n)
Hap1	JQ229897	C	G	T	G	A	A	T	C	T	G	T	G	C	C	T	T	G	C	G	T	G	G	A	C	G	A	Ve(4),QI (2),Vsk (1)
Hap2	JQ229927	.	A	C	.	G	C	.	.	.	.	.	.	.	.	.	A	.	.	G	A	.	.	.	.	.	G	Ve (2),QI (2),Ch(1)
Hap3	JQ229936	.	A	.	.	G	C	.	.	.	.	.	.	.	.	.	A	.	.	G	.	.	.	.	.	.	.	Ve (5),QI (4),Ch (7),Vsk (5)
Hap4	JQ229898	.	A	.	.	G	C	.	.	.	.	.	.	.	.	.	A	T	.	G	.	.	.	.	.	.	.	Ve (2)
Hap5	JQ229895	.	A	C	.	G	C	.	.	.	.	.	.	.	.	C	A	.	.	G	.	.	.	.	.	.	G	QI (1)
Hap6	JQ229896	.	A	.	.	G	C	.	.	.	.	.	.	.	.	.	A	.	.	G	.	.	.	.	.	A	.	Ve(1), QI (1)
Hap7	JQ229933	.	A	C	.	G	C	.	.	.	.	.	.	.	A	.	A	.	.	A	.	.	.	.	T	.	.	QI (1), Vsk(1)
Hap8	JQ229931	.	A	C	.	.	C	.	.	.	.	.	.	.	.	.	A	.	.	G	.	.	.	.	.	.	.	Ve(1),QI (2),Ch (1)
Hap9	JQ229935	T	A	.	.	G	C	.	.	.	.	.	.	.	.	T	T	.	.	A	.	.	.	.	.	.	G	Ve(1),QI (2)
Hap10	JQ229934	.	.	C	.	G	C	.	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.	.	G	QI (2)
Hap11	JQ229937	.	A	.	.	G	C	.	.	.	.	.	.	.	.	.	A	.	.	G	.	.	G	.	.	.	G	QI (1)
Hap12	JQ229893	.	.	C	.	G	C	.	.	.	.	.	.	.	.	.	A	.	A	G	.	.	.	.	.	.	G	Ve(1),Ch (1),Vsk (3)
Hap13	JQ229894	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	Ch (2)
Hap14	JQ229930	.	A	.	.	.	C	.	T	.	.	.	.	.	.	.	T	.	.	G	.	A	.	.	.	.	.	Ch (2)
Hap15	JQ229929	.	.	C	.	.	C	.	.	.	.	.	.	.	.	.	A	.	.	G	.	.	.	.	.	.	.	Ve(1),Ch (1),Vsk(1)
Hap16	JQ229928	T	A	.	.	G	C	.	.	.	.	.	.	.	.	.	A	.	.	G	.	.	.	.	.	.	G	Ch (1)
Hap17	JQ229932	.	.	C	.	G	C	C	.	.	.	.	.	.	.	.	A	.	.	G	.	.	.	.	.	.	G	Ch (2)
Hap18	JQ229900	.	A	.	A	.	.	.	.	.	C	A	.	A	T	T	.	.	.	.	.	.	.	.	.	.	.	Vsk (4)
Hap19	JQ229899	.	.	C	.	G	C	.	.	.	.	C	.	.	.	.	A	.	.	G	.	.	.	.	.	.	.	Vsk (2)
Hap20	JQ229938	.	.	.	.	G	G	.	.	.	.	.	.	.	.	.	A	.	.	G	.	.	.	.	.	.	.	Vsk (1)

AMOVA analysis showed that 95.32% of the total molecular variance was distributed within samples and 4.68% among poulations (Table 31). Exact tests for population differentiation based on haplotype frequencies (Raymond and Rousset 1995a) with 10,000 randomizations of Markov chain steps were performed using Arlequin version 3.0 and no significant differences were observed. High gene flow (*Nm*) values were obsreved among the populations from four locations (Table 32).

Table 29. Mean pairwise K2Pdistances between populations (below diagonal) based on 681 bp region of mtDNA COI gene from haplotype data information of *T. unimaculatus* populations.

	T. unimaculatus			
	Veraval	**Kollam**	**Chennai**	**Visakhapatnam**
Veraval	****			
Kollam	0.005	****		
Chennai	0.006	0.006	****	
Visakhapatnam	0.008	0.007	0.005	****

e. Demographic history

Inferences on patterns of demographic history were obtained by using Tajima's (1989) *D* and Fu's (1997) *F*$_S$ tests (Table 33). Tajima's *D* statistic values and Fu's *F* statistics were negative and were found to be significant (P<0.01) for *T. unimaculatus* populations.

f. Geographical relationships among haplotypes

The statistical parsimony of Templeton *et al.* (1992) implemented in the TCS v1.21 (Clement *et al.*, 2000) software was used to reconstruct a minimum-spanning haplotype network. Relationships among the above 20 haplotypes detected in the present study is shown in Figure 27. One dominant haplotypes (Hap3, *n*= 21) were shared among all populations. Many haplotypes were found to be shared among populations**.** Hap3 was

centered, but there was no characteristic geographic distribution pattern for the haplotypes.

Table 30. Pairwise F_{ST} values (below diagonal) and Φst values above diagonal among four populations of *T. unimaculatus*.

	Veraval	Kollam	Chennai	Visakhapatnam
Veraval	---------	0.0436	0.0598	0.0408
Kollam	0.0491(-)	---------	0.0032	0.0403
Chennai	0.0686(-)	0.0062(-)	---------	0.0627
Visakhapatnam	0.0468(-)	0.0429(-)	0.0629(-)	---------

(-) indicates not significant at 5% level after sequential Bonferroni adjustment.

Table 31. Results of the hierarchical analysis of molecular variance (AMOVA) of populations of *T. unimaculatus* based on mitochondrial COI region.

Source of variation	*Degrees of freedom*	Variance components	% total variation	Overall F_{ST} value
Among populations within species	3	0.1014 (Va)	4.68	0.0468 *Not significant at P<0.01*
Within populations	68	2.0678 (Vb)	95.32	
TOTAL	71	2.1692 (Vt)		

Table 32. Matrix of *Nm* (gene flow) values for mtDNA COI for *T. unimaculatus*.

	Veraval	Kollam	Chennai	Visakhapatnam
Veraval	****			
Kollam	9.6818	****		
Chennai	6.5833	8.8571	****	
Visakhapatnam	10.186	11.1315	7.447	****

Fig. 27. TCS haplotype networks based on COI region in *T. unimaculatus*: size of circle is proportional to haplotype frequency. The Haplotype numbers are given in circles.

Table 33. Results of non-random distribution tests for *T. unimaculatus* populations.

Population	Tajima's D	Fu's F_S statistic
Veraval	-1.33	-19.7813
Kollam	-0.2989	-17.8966
Chennai	-0.3998	-21.4372
Visakhapatnam	-1.0273	-15.3163

Tajima's D values significant P<0.01, Fu's F_S values significant at P<0.01

4B. DNA barcoding and phylogeny

The DNA barcoding and phylogenetic study comprised eleven species of lobsters that belonged to families Palinuridae and Scyllaridae represented by three and two genera respectively. The species of family Palinuridae represented by the genera *Panulirus* (*P. homarus homarus*, *P. versicolor*, *P. ornatus*, *P. longipes longipes, P. polyphagus* and *P. penicillatus*), one representative from *Puerulus* (*Peurulus sewelli*) and one from *Linuparus* (*Linuparus somniosus*). Those from Scyllaridae belonged genera *Petrarctus* (*Petrarctus rugosus*) and two species of *Thenus* (*T. unimaculatus* and *T. indicus*). A total of 81 individuals from the eleven were used for partial sequence analysis of the various mitochondrial (COI, 16SrRNA and 12SrRNA) and nuclear (18S rRNA) genes (Table 34).Sequences of *Homarus americanus* (NC_015607.1) and *Cherax destructor* (NC_011243.1) from GenBank were used as outgroups for all the mitochondrial gene analyses. The complete mitochondrial genome sequences of the outgroup species were taken from GenBank and respective regions were aligned and compared with the ingroup taxa. For the nuclear 18S rRNA gene analysis, *Homarus americanus* (AF235971) and *Cherax quadricarinatus* (AF235966) from GenBank were included as outgroups. All sequences generated from this study were deposited in the NCBI GenBank (Table 35) and details are given in Appendix 6.

Table 34. Number of individuals sequenced for the study and the number of haplotypes

Sl No.	Species	No. of individuals sequenced	No. of haplotypes obtained			
			COI	16S	12S	18S
	Palinuridae					
1	*Panulirus homarus homarus*	15	6	6	6	1
2	*P. ornatus*	5	-	2	2	1
3	*P. versicolor*	6	1	1	2	1
4	*P. polyphagus*	10	-	1	2	1
5	*P. penicillatus*	5	1	1	1	1
6	*Panulirus longipes longipes*	5	1	1	1	1
7	*Linuparus somniosus*	2	1	1	1	1
8	*Puerulus sewelli*	5	1	1	1	1
	Scyllaridae					
1	*Petrarctus rugosus*	5	1	1	1	1
2	*Thenus indicus*	3	2	1	1	1
3	*Thenus unimaculatus*	20	8	5	2	1

4B.1. Mitochondrial DNA analysis of eleven species of lobsters along the Indian coast

4B.1a. COI gene

The COI dataset included 26 sequences from all the species. The unaligned sequences generated using the primer set LCO1490/ HCO2198 (Folmer *et al*., 1994) varied in length from 655 bp in *Linuparus somniosus* to 702 bp in *P. homarus homarus*. The final dataset consisted of an alignment of 655 bp with no indels. Out of the 655 sites 396, 259, 253 and 6 were conserved, variable, parsimony informative and singleton respectively. The A+T base frequency was 58.7%. The average T_S/T_V ratio across pairwise sequence comparisons was 1.24. Sequence divergence between conspecific individuals ranged from 0.3-0.7% in the ingroup taxa. The alignment of partial DNA sequences of the mitochondrial COI gene of 11 lobster species in this study are presented in Appendix 2.

Sequence divergence between the eight species of Palinuridae ranged from 15.3-27.6% with an average evolutionary divergence of 17.7%. It was 16.5-23.3% in Scyllaridae with an average value of 10.7% (Table 36). The divergence in the ingroup taxa ranged from 15.3-28.6% with mean evolutionary diversity of 20.8% in entire dataset. Inter-generic distance ranged from 21.5-26.4% among three genera

Table 35. GenBank depository of species from the present study

Sl No.	Species	No. of individuals sequenced	GENBANK ACCESSION NUMBERS			
			COI	16S	12S	18S
	Palinuridae					
1	*Panulirus homarus homarus*	15	JQ229883-JQ229888 ; JQ229910-JQ229926	JQ229862 ; JQ229866-JQ229871	JQ229841-JQ229848	JQ229940
2	*Panulirus ornatus*	5	*HM446347; *GQ223286	JQ229863-JQ229864	JQ229850-JQ229851	JQ229942
3	*Panulirus versicolor*	6	JQ229882	JQ229877	JQ229858-JQ229859	JQ229948
4	*Panulirus polyphagus*	10	*AF339469; *JN418939	JQ229873	JQ229852-JQ229853	JQ229943
5	*Panulirus penicillatus*	5	JQ229881	JQ229874	JQ229854	JQ229944
6	*Panulirus longipes longipes*	5	JQ229879	JQ229872	JQ229849	JQ229941
7	*Linuparus somniosus*	2	JQ229880	JQ229865	JQ229840	JQ229939
8	*Puerulus sewelli*	5	JQ229890	JQ229876	JQ229857	JQ229947
	Scyllaridae					
1	*Petrarctus rugosus*	5	JQ229889	JQ229875	JQ229855-JQ229856	JQ229945-JQ229946
2	*Thenus indicus*	3	JQ229890-JQ229891	JQ229878	JQ229860-JQ229861	JQ229949
3	*Thenus unimaculatus*	20	JQ229893-JQ229900 JQ229927-JQ229938	JQ229901-JQ229909	KC951871	JQ229950
TOTAL			51	26	22	12

*5 individuals each from KLM, CHE and VSK were sequenced for P. homarus homarus and 5 each from QLN, VRL, CHE and VSK for T. unimaculatus. * indicates*
*indicates sequences retrieved from GenBank for comparison.

of Palinuridae and 21.4 % between two genera of Scyllaridae (Table 37). For the five genera taken together, the value ranged from 21.3% (between *Petractus* and *Thenus*) to 26.9% (*Linuparus* and *Thenus*).

The evolutionary history of the COI data set was inferred using the Neighbor-Joining and Maximum-parsimony analyses in the software *MEGA* version 5. In all analyses, 1000 bootstrap replicates were performed to obtain a measure of the relative robustness of clades. Tree topologies from the Maximum Parsimony (MP) analysis (Fig. 28) and Neighbur-Joining (NJ) (Fig. 29) and indicated four major clades. *P. homarus homarus, P. versicolor, P. ornatus* and *P. polyphagus* formed clade I, *P. longipes longipes* and *P. penicillatus* formed the second clade, *Linuparus somniosus* and *Puerulus sewelli* formed the third and *Petrarctus rugosus*, *Thenus unimaculatus* and *T. indicus* formed the fourth clade. *P. versicolor* and *P. ornatus* were found to be sister taxa in the first clade. *Thenus unimaculatus* and *T. indicus* formed one sub-clade within the fourth clade. *Linuparus somniosus* and *Puerulus sewelli* were grouped together with the Palinuridae with weak to moderate bootstrap support and formed a basal group to the rest of the Palinurid species. Conspecific individuals from different sampling localities were always clustered together and are represented in the tree by only one individual.

Table 36. Average K2P distances of COI gene between species (below diagonal) for lobsters.

		1	2	3	4	5	6	7	8		9	10
1	*P. homarus homarus*	0.000										
2	*P. ornatus*	0.153										
3	*P. versicolor*	0.165	0.180									
4	*P. polyphagus*	0.166	0.174	0.173								
5	*P. longipes longipes*	0.214	0.267	0.249	0.239							
6	*P. penicillatus*	0.255	0.265	0.246	0.265	0.202						
7	*L. somniosus*	0.271	0.276	0.235	0.255	0.256	0.262					
8	*P. sewelli*	0.230	0.245	0.197	0.218	0.266	0.264	0.215				
9	*Thenus unimaculatus*	0.255	0.257	0.268	0.254	0.280	0.278	0.276	0.265			
10	*T. indicus*	0.264	0.268	0.266	0.265	0.266	0.296	0.245	0.241		0.165	
11	*P. rugosus*	0.257	0.262	0.274	0.265	0.251	0.276	0.237	0.236		0.207	0.233

N. B. Comparisons involving averages of haplotypes sequenced

Table 37. Average K2P distances of COI gene between five genera (below diagonal) of lobsters.

	Panulirus	*Linuparus*	*Puerulus*	*Thenus*
Family Palinuridae				
Panulirus	*******			
Linuparus	0.264	*******		
Puerulus	0.234	0.215	*******	
Family Scyllaridae				
Thenus	0.262	0.269	0.260	*******
Petrarctus	0.261	0.237	0.236	0.213
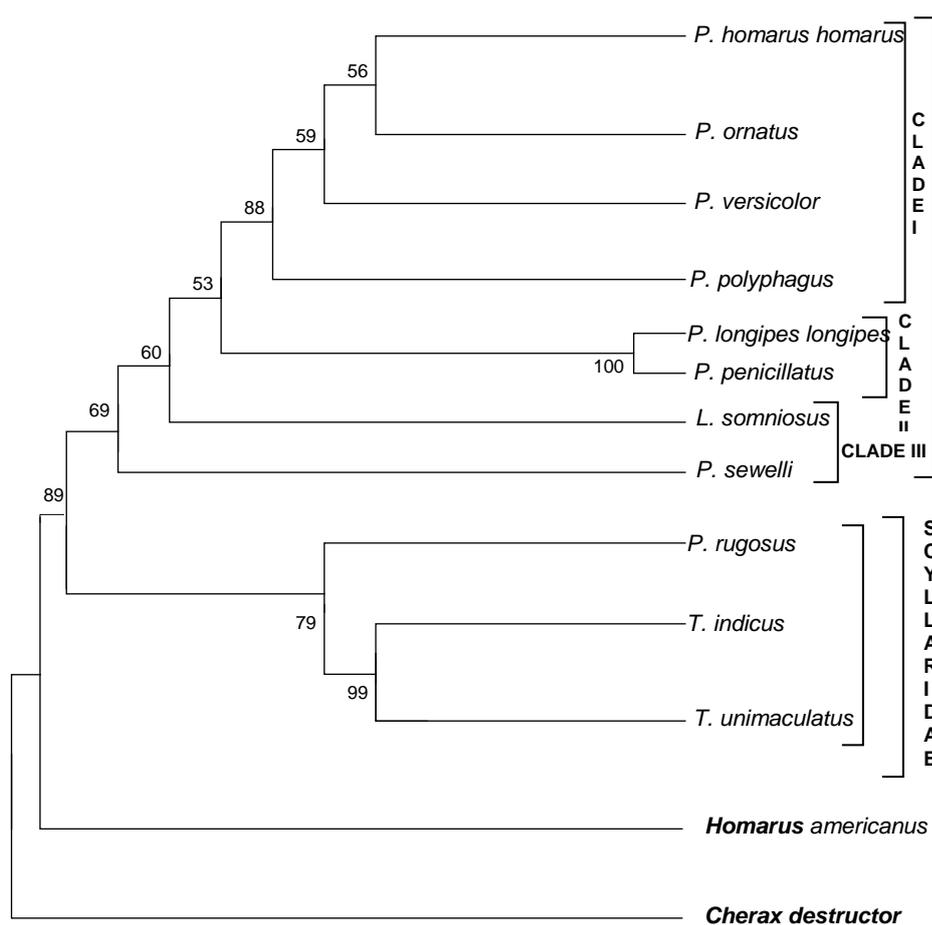
Fig. 28. Maximum parsimony tree of 11 lobster species based on mitochondrial COI gene.
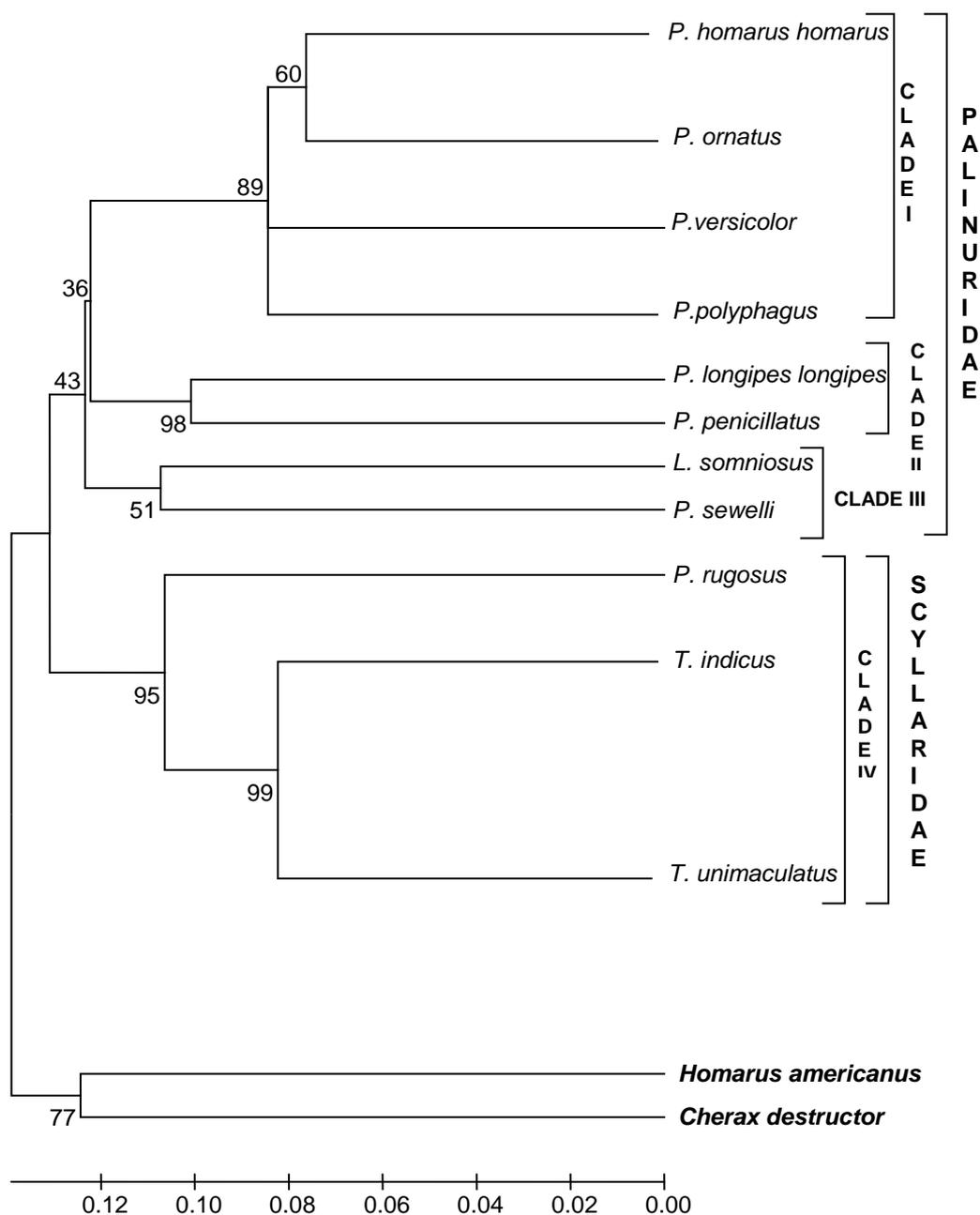
Fig. 29. Neighbour-joining tree of 11 lobster species based on mitochondrial DNA COI gene. *Homarus americanus* (NC_015607.1) *and Cherax destructor* (NC_011243.1) from GenBank and are included as outgroup species. Bootstrap values are shown at nodes.

4B.1b. 16SrRNA

The analysis involved 23 nucleotide sequences including the two outgroups. The unaligned parial sequences generated using the primer set 16S-L 2510, 16S- H 3080 (Palumbi *et al.*, 1991) varied in length from 528 bp in *Linuparus somniosus* to 567 bp in *P. homarus homarus*. The final dataset of the ingroup taxa consisted of an alignment of 541 bp including indels out of which 310, 230, 172 and 58 were conserved, variable, parsimony informative and singleton characters respectively. The A+T bases frequency was 66.3%, indicating moderate AT bias. Sequence divergence between conspecific individuals ranged from 0.2-0.5% in the ingroup taxa. The average T_S/T_V ratio across pairwise sequence comparisons was 1.09. The alignment of partial DNA sequences of the mitochondrial gene 16SrRNA of 11 lobster species in this study are presented in Appendix 3.

The inter-specific sequence divergence observed ranged from 4.6 - 26.4% in family Palinuridae and 4.9-18.1% in Scyllaridae (Table 38). The overall divergence in the ingroup taxa ranged from 4.6-32.2% in the analysis. The average evolutionary divergence over sequence pairs was 10.9% and 6.7% within Palinuridae and Scyllaridae respectively. The mean evolutionary divergence over sequence pairs was 0.173 for the entire dataset. Inter-generic distance ranged from 19.8-22% in Palinuridae and 18% in Scyllaridae. It ranged from 18% (between genus *Petrarctus* and *Thenus*) to 32.1% (*Linuparus* and *Thenus*) among five genera of lobsters (Table 39). The methods yielded trees with the same overall topology with four major clades. Bootstrap analys of the 16SrRNA data resulted in 100% support for the monophyly of all eight Palinurid species and the three Scyllarid species. Tree topologies derived from NJ (Fig. 30) and MP (Fig. 31) was congruent except that the latter recovered a topology similar to the Neighbour-Joining with minor differences in relationships among species within the first major clade.

Table 38. Average K2P distances of 16S gene between species (below diagonal) for lobsters.

		1	2	3	4	5	6	7	8	9	10
1	*P. homarus homarus*										
2	*P. ornatus*	0.046									
3	*P. versicolor*	0.062	0.05								
4	*P. polyphagus*	0.065	0.080	0.071							
5	*P. longipes longipes*	0.146	0.166	0.165	0.142						
6	*P. penicillatus*	0.170	0.167	0.186	0.187	0.110					
7	*Linuparus somniosus*	0.211	0.207	0.216	0.220	0.249	0.264				
8	*Puerulus sewelli*	0.193	0.212	0.214	0.208	0.22	0.243	0.198			
9	*Thenus unimaculatus*	0.243	0.240	0.225	0.244	0.246	0.276	0.312	0.303		
10	*Thenus indicus*	0.240	0.249	0.244	0.251	0.266	0.251	0.298	0.284	0.049	
11	*Petrarctus rugosus*	0.212	0.226	0.221	0.236	0.256	0.253	0.278	0.267	0.181	0.164

N. B. Comparisons involving averages of haplotypes sequenced

Table 39. Average K2P distances of 16S gene between five genera (below diagonal) of lobsters.

	Panulirus	*Linuparus*	*Puerulus*	*Thenus*
Family Palinuridae				
Panulirus				
Linuparus	0.220			
Puerulus	0.207	0.198		
Family Scyllaridae				
Thenus	0.246	0.321	0.307	
Petrarctus	0.222	0.305	0.279	0.180
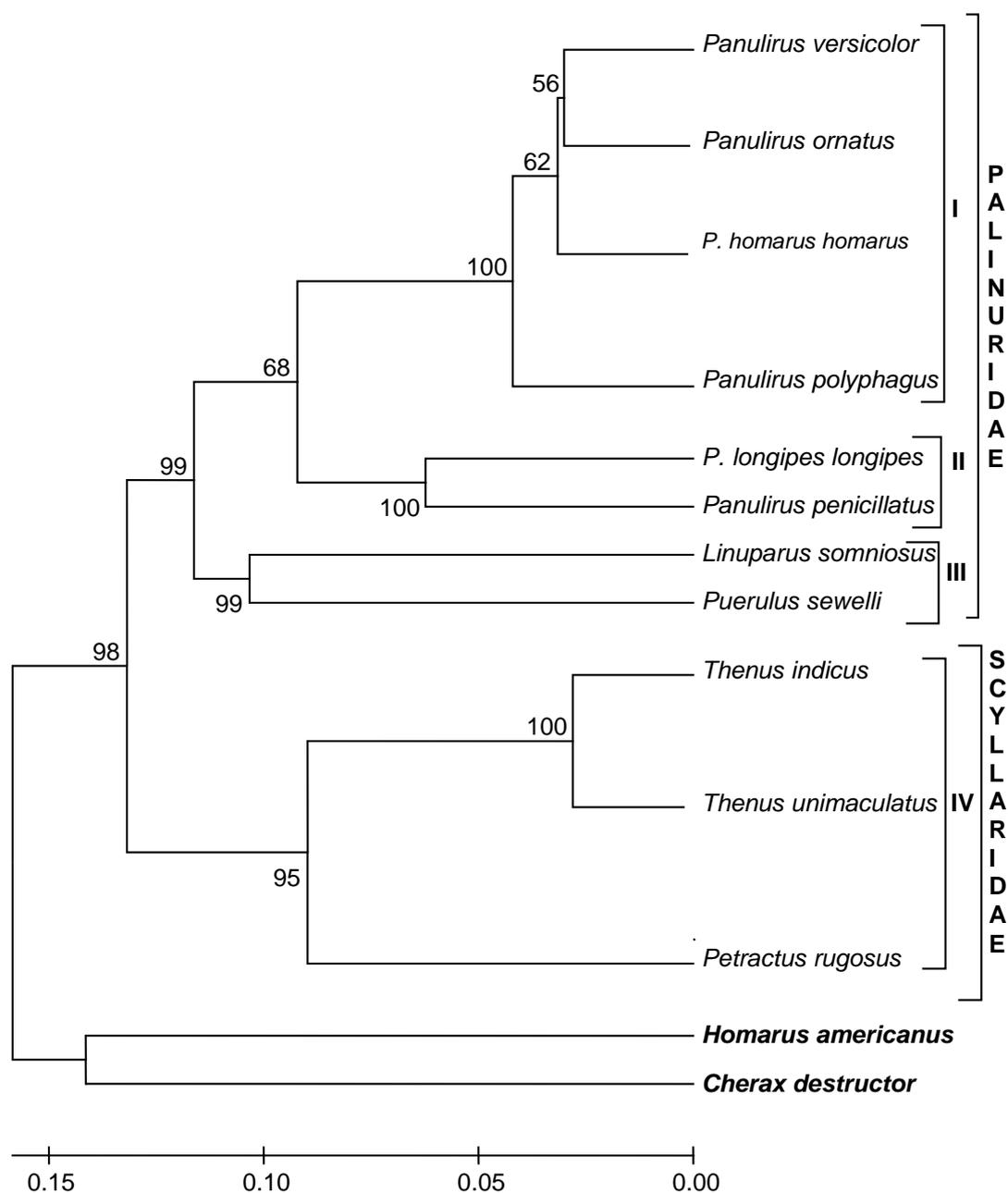
Fig.30. **Neighbour-joining tree of 11 lobster species based on mitochondrial 16SrRNA gene.** *Homarus americanus* **(NC_015607.1) and** *Cherax destructor* **(NC_011243.1) from GenBank and are included as outgroup species.**
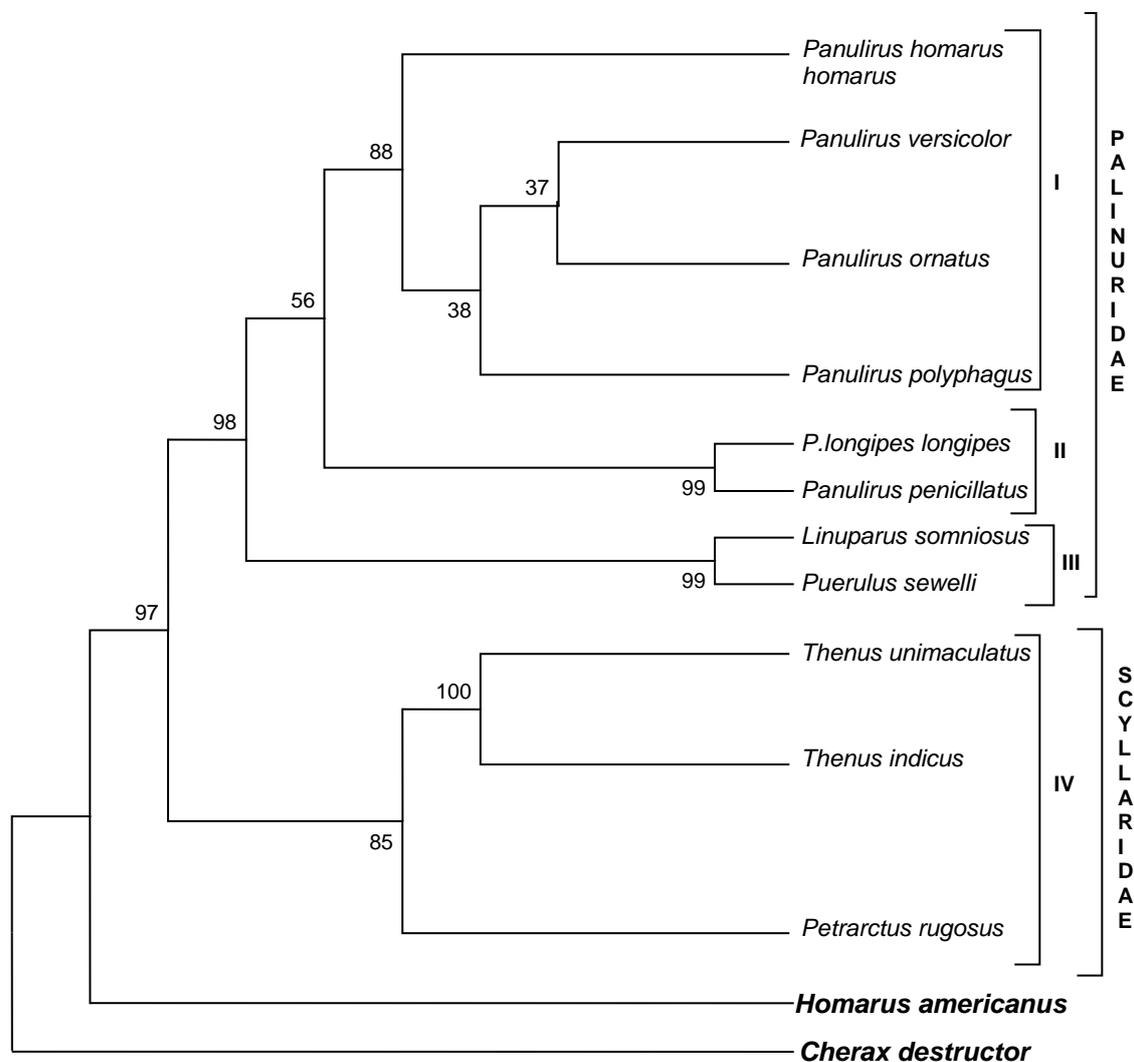
**Fig. 31. Maximum parsimony tree of 11 lobster species based
on mitochondrial 16SrRNA gene.**

4B.1c. 12S rRNA

The analysis involved 22 nucleotide sequences including the two outgroups. The unaligned parial sequences generated using the primer set L13337-12S and H13845-12S (Machida *et al.*, 2002) varied in length from 576 bp in *Linuparus somniosus* to 608 bp in *P. penicillatus*. The final dataset

consisted of an alignment of 592 bp with indels. Among the ingroup taxa, there were 334 variable sites out of which 293 were parsimony informative. There was moderate A+T bias of 68.6%. Sequence divergence between conspecific individuals ranged from 0.4-0.6% in the ingroup taxa. The alignment of partial DNA sequences of the mitochondrial gene 12SrRNA of 11 lobster species in this study are presented in Appendix 4.

The inter-specific sequence divergence for 12SrRNA ranged from 5.8% to 38.6% within Palinuridae and 7.9 to 30% within Scyllaridae (Table 40). The average evolutionary divergence over sequence pairs within Palinuridae was 21.8% and it was 17.5% within Scyllaridae. The mean evolutionary divergence over sequence pairs for the entire dataset was 30%. The average T_S/T_V ratio across pairwise sequence comparisons was 1.02. Inter-generic distance ranged from 26.8-36.6% in three genera of Palinuridae and 28.1% in Scyllaridae. It ranged from 26.8% (between *Puerulus* and *Linuparus*) to 46.1% (*Linuparus* and *Thenus*) among the five genera of lobsters (Table 41). The MP (Fig. 32) and NJ (Fig. 33) and methods yielded trees with the same overall topology similar to the previous analyses. *Linuparus somniosus* and *Puerulus sewelli* clustered together with the other Palinurid lobster species with a weak bootstrap support.

Table 40. Average K2P distances of 12SrRNA gene between species (below diagonal) for lobsters

		1	2	3	4	5	6	7	8		9	10
1	*P. homarus homarus*	0.00										
2	*Panulirus ornatus*	0.058										
3	*Panulirus versicolor*	0.108	0.127									
4	*Panulirus polyphagus*	0.104	0.115	0.136								
5	*P. longipes longipes*	0.245	0.248	0.265	0.259							
6	*Panulirus penicillatus*	0.275	0.304	0.315	0.295	0.188						
7	*Linuparus somniosus*	0.340	0.377	0.364	0.378	0.350	0.386					
8	*Puerulus sewelli*	0.337	0.345	0.319	0.327	0.335	0.361	0.268				
9	*Thenus unimaculatus*	0.430	0.421	0.425	0.401	0.396	0.436	0.447	0.386			
10	*Thenus indicus*	0.450	0.452	0.435	0.436	0.416	0.420	0.466	0.411		0.079	
11	*Petrarctus rugosus*	0.397	0.416	0.419	0.401	0.417	0.412	0.435	0.389		0.284	0.300

N. B. Comparisons involving averages of haplotypes sequenced

Table 41. Average K2P distances of 12SrRNA gene between five genera (below diagonal) of lobsters

	Panulirus	*Linuparus*	*Puerulus*	*Thenus*
Family Palinuridae				
Panulirus				
Linuparus	0.366			
Puerulus	0.334	0.268		
Family Scyllaridae				
Thenus	0.431	0.461	0.398	
Petrarctus	0.410	0.431	0.388	0.281

N. B. Comparisons involving averages of haplotypes sequenced
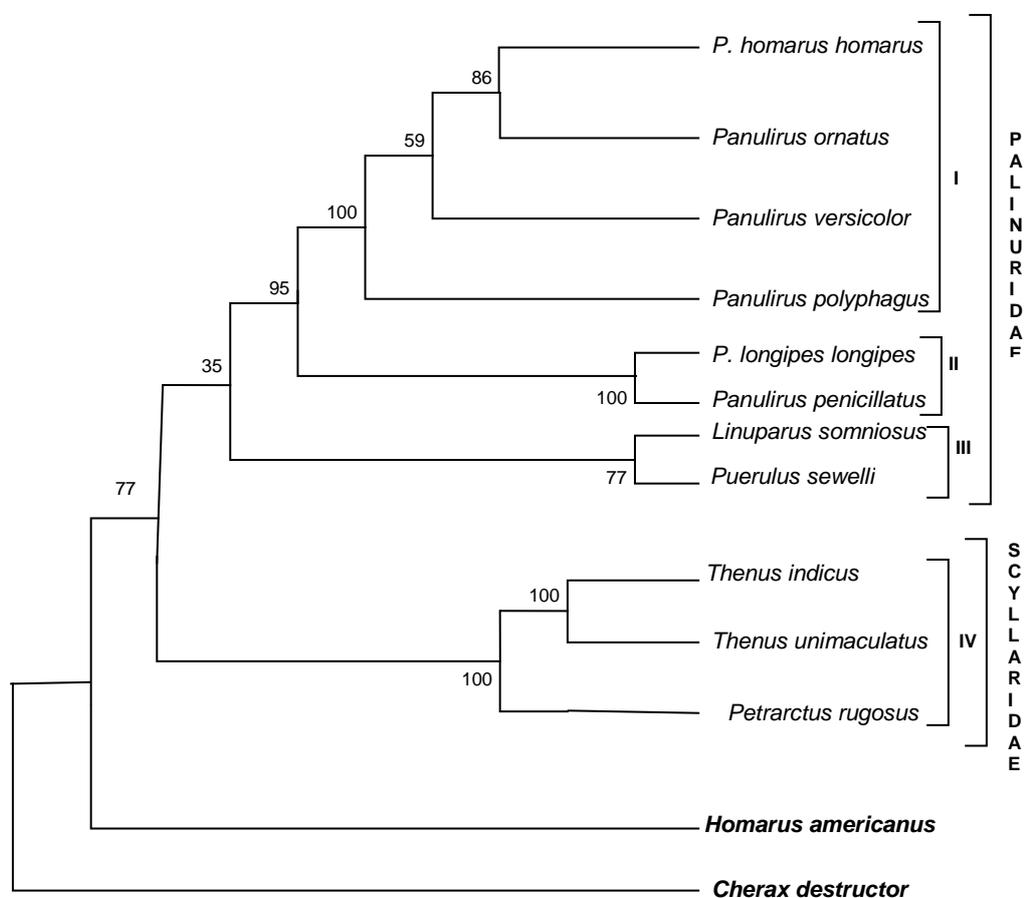
Fig. 32. Maximum parsimony tree of 11 lobster species based on mitochondrial 12SrRNA gene.
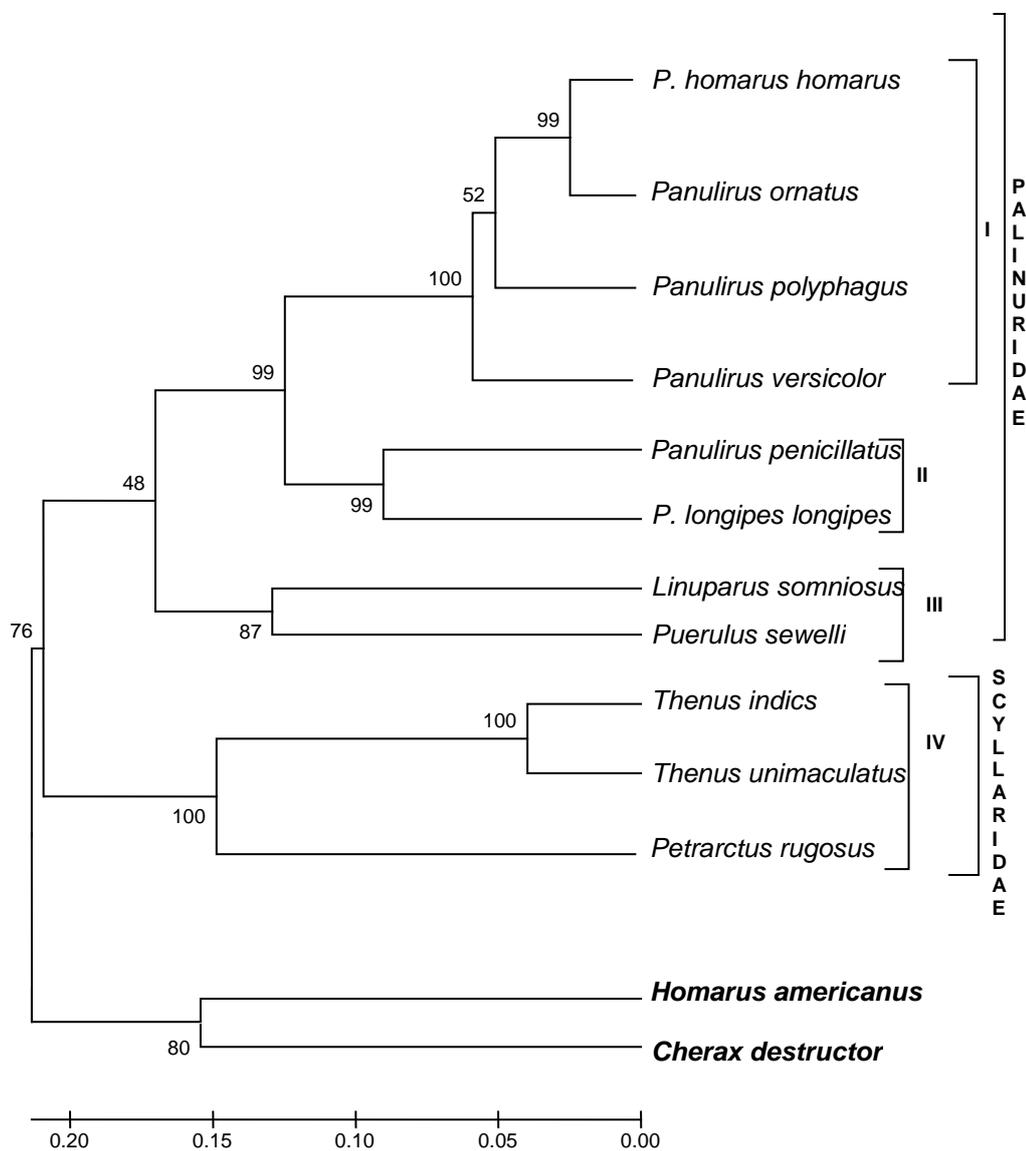
Fig.33. NJ tree of 11 lobster species based on 12SrRNA gene. *Homarus americanus* **(NC_015607.1) and** *Cherax destructor* **(NC_011243.1) from GenBank and are included as outgroup species.**

4B.2. Analysis of nuclear 18SrRNA gene among 11 species of lobsters

The analysis involved 11 nucleotide sequences without outgroups. The unaligned partial sequences generated using the primer set (18S 1f, 18S b2.9: Whiting (2002); 18S 5FrRNA, 18S_9RrRNA: Carranza *et al.*, 1996) varied in length from 1683 bp to 1726 bp. The final dataset of the ingroup taxa consisted of an alignment of 1723 bp with indels out of which there were 1531, 183, 118 and 65 characters that are conserved, variable, parsimony informative and singleton respectively. The overall A+T content of 49.2%. No intraspecific variation was observed in the sequences. The alignment of partial DNA sequences of the nuclear 18SrRNA gene of 11 lobster species in this study are presented in Appendix 5. The inter-specific sequence divergence ranged from 0.3% (*P. penicillatus-P. longipes longipes*) to 7.8% (*L. somniosus-P. homarus homarus*; *L. somniosus-P. ornatus*) within Palinuridae and 0.2% (*T. indicus-T. unimaculatus*) to 1% within Scyllaridae (Table 42). The average evolutionary divergence over sequence pairs was 4.8% and 0.4% within Palinuridae and Scyllaridae respectively .The mean evolutionary divergence over sequence pairs was 3.9%. The average T_S/T_V ratio in the dataset was 1.22 .The evolutionary history was inferred using the Neighbor-Joining and Maximum-parsimony methods (trees not shown) which couldn`t resolve the phylogeny.

Table 42. Average K2P distances of 18S gene between species (below diagonal) for lobsters.

		1	2	3	4	5	6	7	8	9	10
1	*P. homarus homarus*										
2	*Panulirus ornatus*	0.026									
3	*Panulirus versicolor*	0.037	0.038								
4	*P. polyphagus*	0.026	0.026	0.016							
5	*P. longipes longipes*	0.075	0.072	0.070	0.058						
6	*P. penicillatus*	0.074	0.072	0.070	0.058	0.003					
7	*L. somniosus*	0.078	0.078	0.072	0.064	0.018	0.016				
8	*Puerulus sewelli*	0.076	0.073	0.070	0.060	0.009	0.008	0.016			
9	*Thenus unimaculatus*	0.074	0.072	0.070	0.059	0.007	0.005	0.015	0.008		
10	*Thenus indicus*	0.074	0.074	0.070	0.059	0.008	0.006	0.017	0.010	0.002	
11	*Petrarctus rugosus*	0.073	0.070	0.069	0.059	0.010	0.008	0.015	0.010	0.004	0.007

4B.3. Combined mitochondrial DNA dataset analysis

Three mtDNA gene sequences obtained from the same individual were combined based on the rate of evolution and position of each gene in the outgroup species. The pooled sequences of two individuals from each species from varied populations were selected for the analysis. The analysis involved 32 nucleotide sequences of ingroup taxa. The combined mitochondrial data set (COI, 16SrRNA and 12SrRNA) was 1790 bp long including indels and excluding the two outgroup species. In the ingroup taxa, 746 were parsimony informative of 829 variable characters. There were 1674 positions in the final ingroup dataset excluding all indels. The A+T base frequency for the concatenated mtDNA data set was 64%. The summary of molecular characterization and phylogenetic information content of each gene and in the combined data set for the eleven lobster species are presented in Table 45.

The inter-specific sequence divergence ranged from 9-34.7% within Palinuridae and 10.2-25.3% within Scyllaridae. The overall divergence value in the ingroup taxa ranged from 9.0-39.4% (Table 43). The average evolutionary divergence over sequence pairs was 20.9% within Palinuridae and 8.7% within Scyllaridae .It was 25.7% over all sequence pairs. The mean evolutionary divergence over sequence pairs between Palinuridae and Scyllaridae was 37.6%. The mean evolutionary diversity for the entire dataset was 27.5%. The net sequence divergence between Palinuridae and Scyllaridae was 22.7% .The average T_S/T_V ratio across all pairwise sequence comparisons in the dataset was 1.04.

The Inter-generic distance ranged from 22.4% (*Linuparus* and *Puerulus*) to 28.3% (*Linuparus* and *Panulirus*) in family Palinuridae and 22 % (*Thenus* and *Petrarctus*) in family Scyllaridae. It ranged from 22.4% (*Petractus* and *Thenus*) to 33% (*Linuparus* and *Thenus*) among the five genera of lobsters (Table 44).

The evolutionary history among the species of lobsters was inferred using the Neighbor-Joining (Fig. 34), Maximum-parsimony (Fig. 35) and maximum-likelihood analyses (Fig. 36). The tree topologies were identical and supported by >80% bootstrap support at all nodes except for conspecific individuals. The evolutionary trees indicated the independent monophyly of all

eight Palinurid species and the three Scyllarid species. Four major clades were recognized congruent with the individual gene trees with mtDNA genes.

Table 44. Average K2P distances of concatenated mtDNA sequences between five   genera (below diagonal) of lobsters.

	Panulirus	*Linuparus*	*Puerulus*	*Thenus*
Family Palinuridae				
Panulirus				
Linuparus	**0.283**			
Puerulus	**0.259**	**0.224**		
Family Scyllaridae				
Thenus	**0.302**	**0.330**	**0.315**	
Petrarctus	**0.294**	**0.309**	**0.288**	**0.222**

Table 43. Average K2P distances of total mtDNA data set (below diagonal) between haplotypes of different species of lobsters.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
	PALINURIDAE																	
1	*P. homarus homarus*- QLN1																	
2	*P. homarus homarus*- CHE1	0.002																
3	*P. homarus homarus*- VSK1	0.005	0.004															
4	*Panulirus ornatus*	0.093	0.092	0.090														
5	*Panulirus versicolor*	0.121	0.119	0.118	0.127													
6	*Panulirus polyphagus*	0.119	0.119	0.118	0.129	0.140												
7	*P. longipes longipes*	0.226	0.226	0.228	0.256	0.257	0.241											
8	*Panulirus penicillatus*	0.262	0.260	0.260	0.282	0.271	0.283	0.176										
9	*Linuparus somniosus*	0.320	0.321	0.324	0.333	0.311	0.326	0.330	0.347									
10	*Puerulus sewelli*	0.291	0.289	0.288	0.305	0.277	0.285	0.308	0.326	0.250								
	SCYLLARIDAE																	
11	*Thenus unimaculatus*-QLN1	0.365	0.365	0.362	0.363	0.374	0.353	0.365	0.367	0.394	0.369							
12	*T. unimaculatus*-QLN2	0.365	0.365	0.362	0.363	0.372	0.353	0.367	0.367	0.391	0.366	0.003						
13	*T. unimaculatus*-CHE1	0.366	0.366	0.363	0.364	0.375	0.354	0.366	0.370	0.392	0.366	0.003	0.004					
14	*T. unimaculatus*-VSK1	0.369	0.369	0.366	0.365	0.377	0.355	0.367	0.372	0.392	0.370	0.005	0.004	0.003				
15	*T. unimaculatus*-VSK2	0.365	0.365	0.362	0.361	0.373	0.351	0.367	0.370	0.391	0.366	0.004	0.002	0.004	0.003			
16	*T. unimaculatus*-VER1	0.364	0.364	0.360	0.360	0.369	0.351	0.369	0.368	0.392	0.367	0.005	0.005	0.005	0.004	0.003		
17	*Thenus indicus*	0.371	0.371	0.370	0.370	0.368	0.361	0.363	0.364	0.384	0.349	0.104	0.102	0.104	0.107	0.105	0.105	
18	*Petrarctus rugosus*	0.345	0.345	0.346	0.353	0.363	0.355	0.353	0.359	0.368	0.333	0.244	0.243	0.245	0.248	0.244	0.245	0.253

Note: QLN, CHE, VSK and *VRL represent sampling sites Kollam, Chennai, Visakhapattanam* and *Veraval respectively*.

Table 45. Summarized data on the molecular characterization and phylogenetic information content of the nuclear 18SrRNA and mitochondrial DNA regions in the eleven commercially important lobster species along Indian coast.

	18S (n)	Mitochondrial genes			Combined mt DNAdata set (n)
		COI (n)	16S (n)	12S (n)	
No. of bases analysed	1723 (11)	655 (26)	541 (23)	592 (21)	1790 (32)
Nucleotide composition					
% A	24.8	25.4	32.8	33.9	30.2
% T	24.4	33.3	33.5	34.7	33.8
% G	26.9	19.5	21.0	11.0	17.2
% C	23.9	21.8	12.7	20.4	18.7
% A+T	49.2	58.7	66.3	68.6	64
Invariable (monomorphic) sites	1531 *(88.86%)*	396 *(60.46%)*	311 *(57.30%)*	257 *(43.41%)*	958 *(53.52%)*
Variable (polymorphic) sites	183 *(10.68%)*	259 *(39.54%)*	230 *(42.51%)*	334 *(56.42%)*	829 *(46.31%)*
Singleton variable sites	65 *(3.77%)*	06 *(0.92%)*	56 *(10.72%)*	41 *(6.93%)*	83 *(4.64%)*
Parsimony informative sites	118 *(11.52%)*	253 *(38.63%)*	173 *(31.79%)*	293 *(49.49%)*	746 *(41.68%)*
Indel (insertion / deletion) sites/missing data	51 *(2.96)*	2 *(0.31%)*	36 *(6.65%)*	76 *(12.84)*	116 *(6.48%)*
Ts/Tv	1.22	1.24	1.09	1.02	1.04

n = no. samples analyzed, Ts = transitions, T_V = transversions.

Fig. 34. **Neighbour-joining tree of the 11 lobster species inferred from haplotype sequence variation of the 1790 bp mtDNA region. The optimal tree with the sum of branch length = 1.547 is shown .Numbers at nodes indicate the bootstrap values. *Homarus americanus* (NC_015607.1) and *Cherax destructor* (NC_011243.1) from GenBank are included as outgroup species. Abbreviations VRL, QLN, CHE and VSK represent samples from Veraval, Kollam, Chennai and Visakhapatnam respectively.**

Fig. 35. Maximum parsimony tree of the 11 lobster species based on combined mitochondrial DNA dataset. The most parsimonious tree is shown.
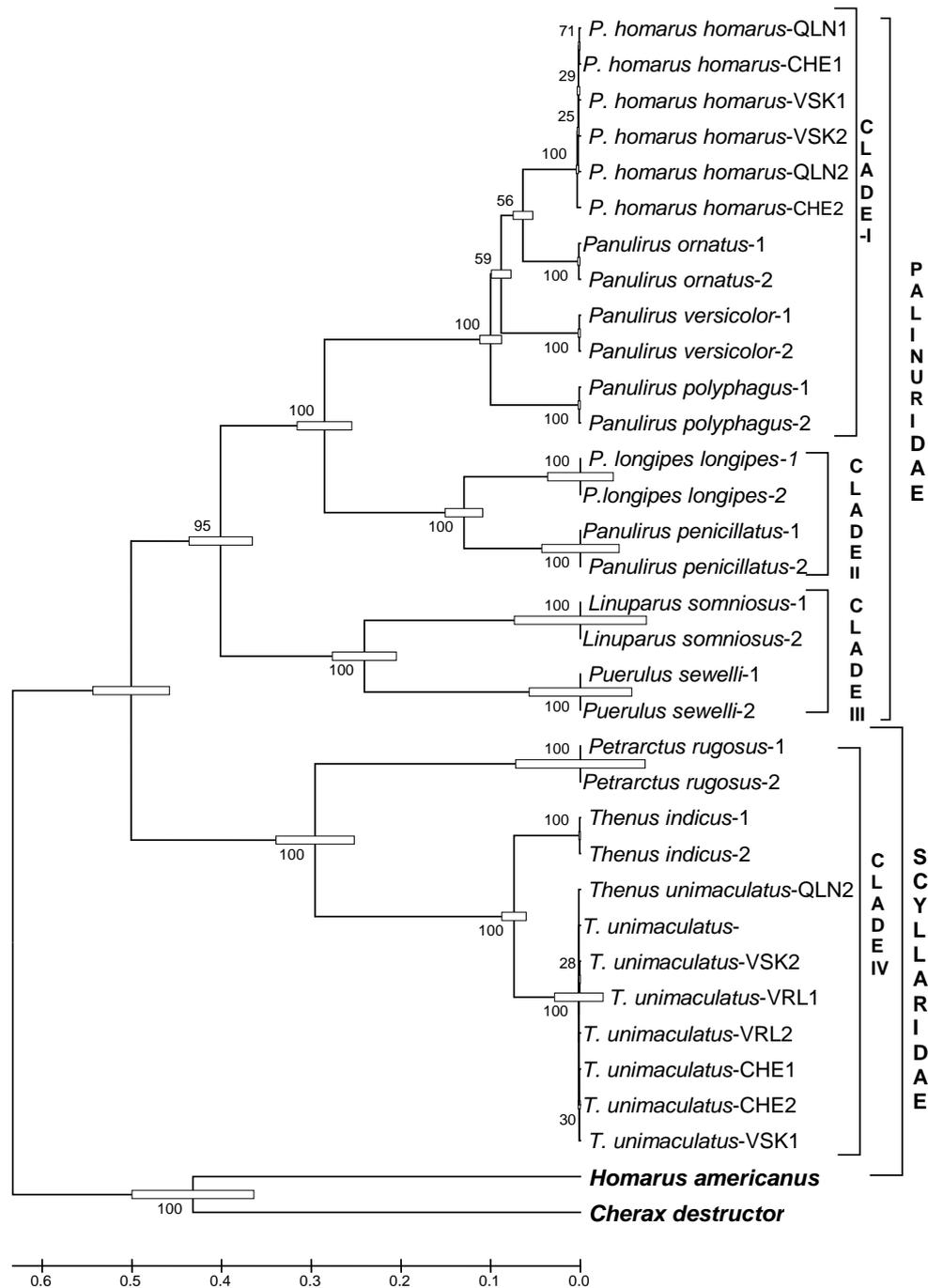
Fig. 36. Maximum likelihood tree of the 11 lobster species based on best-fitting nucleotide substitution model (TN93+G+I) in *MEGA* version 5, inferred from haplotype sequence variation of the 1790 bp mtDNA region. The tree with the highest log likelihood (-11909.7592) is shown. Numbers at nodes indicate the bootstrap values. *H. americanus* (NC_015607.1) and *Cherax destructor* (NC_011243.1) from GenBank are included as outgroup species.

Chapter 5

DISCUSSION

5A.   Genetic structure analysis of *Panulirus homarus homarus* and *Thenus unimaculatus* along the Indian coast

Information concerning the stock structure/intra-specific level of the species is indispensable for rational management and long-term sustainability of any fisheries resource whether directed for exploitation or conservation. With little knowledge of the stock structure, over-fishing can decimate isolated breeding populations to a level where recruitment cannot sustain the harvest, and the isolated populations, which contribute to the total harvest, can collapse (Keenan, 1997). Much of the difficulty in successfully managing marine species arises from the lack of knowledge of population connectivity in organisms with a pelagic larval stage (Carr *et al*., 2003). The lobsters like many marine invertebrates belong to the group with a lengthy pelagic larval life and hence potential for wide dispersal. By characterizing the distribution of genetic variation, population sub-structuring can be detected and the degree of connectivity among populations can be estimated (Nesbo *et al*., 2000; Palof *et al*., 2011).

A number of approaches have been tried to separate the lobster population into stocks or subpopulations for a more meaningful and efficient management of the fishery. These include morphometric (García-Rodríguez *et al*., 2004; Radcliffe, 2011; Øresland and Ulmestrand, 2013) and molecular methods (e.g. Inoue *et al.,* 2007; Palero *et al*., 2008; Pampoulie *et al*., 2011; Kennington *et al*., 2013). Considerable research has been devoted to study the biology and fisheries of both *P. homarus* and *Thenus* spp. throughout their geographic range along the Indian coast especially with regard to reproduction, ethology, stock assessment and growth (e.g. Radhakrishnan and Vijayakumaran, 2003; Vijayakumaran *et al*., 2009; Kabli and Kagwade, 1996 a, b, c; Deshmukh, 2001; Subramanian, 2004; Kizhakudan *et al.,* 2004a). Little information exists, however, about its population structure of both the species. The lobster species of the Indian coast have shown evidence of overexploitation and signs of capture decline from the peak of 4075 MT in 1985

to 1715 MT in 2010 (Radhakrishnan *et al*., 2005; CMFRI, 2002-2012) which could be affecting the species survival. In this framework, we need to assess genetic variability in the wild population through molecular markers to adopt monitoring measures for proper conservation and management decision taking.

Population genetic study with molecular markers can be considered as one of the effective approaches to provide significant information of population structure and genetic diversity. Since different genetic methodologies used in the same species may scan different regions of the genome (Karl and Avise, 1992), it is important to employ several kinds of molecular markers. The combined analysis of mitochondrial and nuclear markers though not frequent, particularly in marine species, and is a step forward in this direction (Babbucci *et al*., 2010). Hence, in the present study, population structure investigations of both the species of lobsters *P. homarus homarus* (family Palinuridae) and *T. unimaculatus* (family Scyllaridae) were carried out using a combination of two markers- the first one was RAPD PCR, a nuclear type II marker and second marker used was partial sequences of the hypervariable region of mtDNA Cytochrome Oxidase I gene. The RAPD profiles of 60 individuals each for both species were generated for analysis and mtDNA partial sequences of 20 and 18 each per sampling locations for *P. homarus* and *T. unimaculatus* respectively were compared. The results from both the classes of markers were concordant and no significant differences in genetic profiles were observed among the populations of either *P. homarus homarus* or *T. unimaculatus* which indicated lack of population heterogeneity within these species of lobsters.

5A.1. RAPD markers

The RAPD (Welsh and McClelland, 1990) technique has been proved a quick and effective method for the detection of intra- and inter-specific genetic polymorphism in Crustacea (Baratti *et al*., 2003). RAPD profiles were used to identify genetic similarity and diversity among populations of *P. homarus homarus* and *T. unimaculatus* lobsters collected from geographically separate regions along the Indian coast. The applications of this nuclear type II marker for population genetic analysis is well known and the marker has extensively been used to evaluate the population structure in a variety of decapod

crustaceans having pelagic larvae like shrimps (Lakra *et al.*, 2010), crab (Klinbunga *et al.*, 2010), cray fishes (Beroiz *et al.*, 2008) and relatively few in lobsters (Harding *et al.*, 1997; Ulrich *et al.*, 2001; Park *et al.*, 2005).

A disadvantage of RAPD technique is reproducibility of the results (Penner *et al.*, 1993). In this study, we used high quality DNA, constant experimental conditions and scoring of only bands with frequencies of at least 10% to avoid non-specific amplifications (Castro and Madi-Ravazzi, 2000; Castiglioni and de Campos Bicudo, 2005) in order to ensure reproducibility of results which is often cited as a major problem with use of RAPD markers (Liu and Cordes, 2004).

5A.1.1. Genetic variability in RAPD analysis

In our study, RAPD profiles were generated from 180 scalloped spiny lobsters (*P. homarus homarus*) using eight Operon primers and from 240 slipper lobsters (*T. unimaculatus*) with nine Operon primers. Liu and Cordes (2004) suggested that six or seven primers were sufficient to assess genetic variability within and among populations of highly polymorphic species. Harding *et al*. (1997) used eight and Uthike *et al*. (2001) used nine polymorphic primers to document the population diversity of *Homarus gammarus* and *H. americanus* lobsters respectively. Five primers could reveal stock structure in the shrimp *Metapenaeus affinis* (Lakra *et al.*, 2010) and crab *Portunus pelagicus* (Klinbunga *et al.*, 2010) from Indian and Thai waters respectively. Presuming that marine lobsters would reveal high genetic polymorphism in RAPD-PCR as observed in *Puerulus sewelli* (Park *et al.*, 2005) the number of primers and the sample size used in this study were expected to be adequate to resolve the genetic polymorphism among the lobster populations.

There were specific bands across populations in RAPD for both species of lobsters. The species identification at larval stages in lobsters is often difficult (Phillips and Mc William, 2009). These specific bands from RAPD can be utilized for developing SCAR (Sequence Characterised Amplified Region) markers for accurate identification of larvae and brood stock of the species. However, the species-specificity of the bands has to be confirmed by using the technique in other lobster species too. Similarly the stock-specific/population

specific RAPD profiles will be of great help in discrimination of genetic stocks of both species in fishery management programmes.

The percentage 'P' value was calculated using the criterion for polymorphism in which the frequency of the most common allele was ≤ 0.95. The primers used in our study were found to generate polymorphic pattern in both the species of lobsters. The percentage polymorphism (P) was almost similar in three populations of *P. homarus homarus* ranging from (29.55-31.82%) but with a high overall value (55%) for all populations taken together. Of the eight primers selected for study, seven revealed clear polymorphisms except the primer OPAH-19 which appeared invariant. The total number of polymorphic loci was 24 (54%). The percentage polymorphism (P) in *T. unimaculatus* ranged from 15.22% (Visakhapatnam and Veraval) to 30.43% (Kollam). Even though, the intra-population percentage polymorphism was moderate, in overall population the percentage was high (58.5%). Polymorphic bands were observed with the same primers in different populations, which suggest that the populations have not been isolated long enough for species-specific DNA fragments to be gained or lost in particular areas.

In a similar study, polymorphism was observed in three populations of *Homarus americanus* (Harding *et al*., 1997) even though there was a high degree of uniformity among individuals. The variation in the RAPD bands using some of the primers was often restricted to a few individuals in the present study. The percentage polymorphism observed within lobster populations in the present study were lower than those detected for a number of crustaceans like shrimps (Tassanakajon *et al.*, 1998; Klinbunga *et al.*, 2001; Mishra *et al*., 2009; Aziz *et al.*, 2010) and crabs (Klinbunga *et al.*, 2010). However, low level of polymorphism within populations has been reported in crustaceans like shrimps (Zhuang *et al*., 2001; Niamaimandi *et al*., 2010).

According to Nei (1987), a more appropriate measure of genetic variation is average heterozygosity or gene diversity (h). Gene diversity values were observed to be variable (0.1292 to 0.1462) in *P. homarus homarus* with a value of 0.1719 in overall population. Highest genetic diversity was found within the Chennai samples (0.1462), while lowest was found in Visakhapatnam

samples (0.1292). Gene diversity values (h) were observed to be variable among the four populations of *T. unimaculatus* (0.0718 in Visakhapatnam population to the highest of 0.1375 in Kollam). The value was 0.1446 for the entire population. The 'h' values observed in this study are comparatively low among crustaceans. Similar values for 'h' has been observed in lobsters like *Homarus gammarus* (0.004-0.091 for allozymes- Jørstad *et al*., 2005), *Nephrops norwegicus* (0.165-0.187 for allozymes- Stamatis *et al*., 2006) and in shrimps like *Penaeus chinensis* (0.2176 for RAPD- Shi *et al*., 1999) and *Penaeus japonicus* (0.2157 for RAPD- Song *et al*., 1999). Higher 'h' values of 0.2565-0.357 was reported in *Metapenaeus affinis* (Lakra *et al*., 2010).

Shannon diversity index estimates, which provides a relative estimate of the degree of variation within each population (Yeh, 1999). In *P. homarus homarus*, the Shannon index ranged from 0.1854 (Visakhapatnam) to 0.2073 (Chennai) with an overall value of 0.2647 for the entire population. For *T. unimaculatus* populations, the value ranged from the lowest of 0.1011 (Visakhapatnam) to the highest of 0.1957 (Kollam) with an overall value 0.2379 for the overall population.

The genetic variability estimates *viz.* level of polymorphism (P), gene diversity (h) and Shannon's Index were moderate within populations of both spiny and slipper lobster species in our study. At least 10-15 primers may be required for species with low level of genetic diversity (Liu and Cordes, 2004) to reveal considerable amount of polymorphism, which may be the case of lobster populations in the present study unlike assumed. The major reasons for the reduced genetic variation in populations may be result of population size contraction due to increased fishing efforts as evident from the catch data over the years of lobsters especially *Thenus* spp. at Veraval (Radhakrishnan *et al*., 2007) and small effective population sizes or through founder effects. Another possibility is similar contiguous environmental conditions though geographically distant, may show homogeneous populations with low genetic diversity value among populations (da Silva Cortinhas *et al*., 2010). *Thenus* species occurs in sedimentary substrates and is adapted to an extensive habitat with less extensive offshore migrations (Webber and Booth, 2007). The species is reported to have minimum movement from its territory (Kizhakudan *et al*.,

2004a). Hence chances are there for the species to have a low genetic diversity level.

5A.1.2. Genetic differentiation and gene flow

The coefficient of genetic differentiation (G_{ST}) values were 0.0136 and 0.0442 respectively for *P. homarus homarus* and *T. unimaculatus*. According to Wright (1978), genetic differentiation could be classified into four categories based on F_{ST} / G_{ST} values.*viz.* 0-0.05- little genetic differentiation, 0.05-0.15- moderate genetic differentiation, and 0.15-0.25- large genetic differentiation and above 0.25- very large genetic differentiation. Based on these guidelines, the G_{ST} values in the present study indicated very weak genetic differentiation among the populations of *P. homarus homarus* and *T. unimaculatus*. G_{ST} values in the present study have been compared with values observed by other researchers for decapod crustacean species having pelagic larvae (Table 46). The low levels of G_{ST} in this study is comparable to the F_{ST} value observed in *Homarus americanus* (F_{ST}-0.000 to 0.073; Harding *et al*., 1997), which indicated the absence of genetic structuring in Gulf of St. Lawrence and the Gulf of Maine. The F_{ST} values of 0.013 for allozymes in *N. norwegicus* populations (Stamatis *et al*., 2006) and 0.001 to 0.016 in *H. gammarus* throughout its distribution range (Jørstad *et al*., 2005) indicated only shallow genetic structuring. Higher G_{ST}/F_{ST} value indicating significant population structuring was recorded in shrimps like in *Metapenaeus affinis* (F_{ST} -0.165-0.187-Lakra *et al*., 2010). The lower G_{ST} values in *P. homarus homarus* (0.0136) and in *T. unimaculatus* (0.0442) could be attributed to the high level of genetic mixing of the populations along the Indian coast.

The gene flow (*Nm*) value (Slatkin, 1993) allows evaluating whether or not each of the considered population of a species evolves as an independent unit. Theoretically value of *Nm*>1 is sufficient to prevent random differentiation by genetic drift (Slatkin, 1993). While it is difficult to quantify precisely the level of exchange required to produce the values of G_{ST} reported in this study, especially as G_{ST} is low (Wright, 1978), it can be estimated that as few as 4-4.5 migrants per generation are needed to account for the little genetic differentiation observed among our sampling locations. The high gene flow

between the populations may be leading to high level of genetic mixing from the sampled regions preventing differentiation among lobster populations by genetic drift. Lack of stock differentiation may have a biological explanation, or may simply be due to the failure to detect variation due to inadequacy in sampling scheme or markers utilized. With an adequate number of samples (60 each from different populations) and polymorphic RAPD profiles in the present study, the reason for the lack of genetic heterogeneity among populations can be attributed to the panmixia. The high *Nm* value in the present study indicates high gene flow between the populations of both species, leading to high level of genetic mixing from the sampled regions. PLD (Planktonic Larval Duration) is conventionally believed to be a good predictor of dispersal of planktonic larvae and species with a longer PLD should have extensive gene flow (panmixia) and, therefore, low or no population structuring (Weersing and Toonen, 2009). This view is recently opposed and hence the population dynamics of many species are probably more 'closed' than previously believed (Shanks *et al.*, 2003; Weersing and Toonen, 2009). It has been observed that the long lived phyllosoma larvae of some lobster species have the potential for long-distance dispersal favoured ocean currents (Caputi *et al.*, 1996; Griffin *et al.*, 2001; Sekiguchi and Inoue, 2010). The hypothesis for larval connectivity among lobster populations along the Indian coast is discussed in detail in 5A.3. (pp. 150-156).

5A.1.3. Genetic distance between populations

Nei's unbiased genetic distances (Nei, 1972) are considered suitable for long evolutionary processes, with population divergences due to genetic drift and mutational events (Weir, 1990). Average pair wise similarity index (SI) and the genetic distance (GD) based on Nei's unbiased measures of genetic identity and genetic distances between the three *P. homarus homarus* populations studied were 0.957 and 0.0513 respectively. For the four *T. unimaculatus* populations, it was 0.928 and 0.077 respectively. The low genetic distance and high genetic identity estimated for the populations of the two species indicated that they act as single interbreeding population, possibly with high levels of gene flow between them. The genetic similarity and distance values from the present study were similar to those reported for different

invertebrate species and populations like shrimps *Acetes japonicus*, *Penaeus chinensis* etc. (Table 46). Similarly low intraspecific 'GD' values of 0.0296-0.0661 were also reported in other crustaceans such as *Penaeus monodon* (Tassanakajon *et al*., 1998), *Penaeus vannamei* (0.077-0.216, Garcia *et al*., 1994) and in mangrove crabs (0.0056-0.0589, Thangaraj *et al*., 2012).

The absence of a clear geographical pattern of genetic differentiation among the populations of both species of lobsters studied is further evident in the UPGMA dendrogram, constructed based on Nei's (1978) genetic distances. Even though populations of both species formed clusters, it was weakly supported by bootstrap analysis (all were lower than 50%, out of 1000 iterations) and does not show strong support for population grouping based on geographical proximity.

Based on the results of genetic distance estimates, G_{ST} analyses, and tests for genetic differentiation in the present study, it can be concluded that a homogenous genetic structure exists within *P. homarus homarus* and *T. unimaculatus* populations. Sofia *et al*. (2008) reported similar results showing low polymorphism value of 28-39%, gene diversity value of 0.12-0.15 and unbiased genetic distances ranged from 0.0253 to 0.0445 indicating moderate genetic differentiation among populations of catfishes and attributed it to the eroding genetic variation to different factors related to its habitat. Low levels of gene diversity and a low level of differentiation as well as genetic structuring among populations has led to the idea of a unique homogenous population in the marine fish *Atherinella brasiliensis* (da Silva Cortinhas *et al*., 2010) too. Little genetic population sub-structuring has been detected with this technique in parrot fishes (Geertjes *et al*., 2004) leading to a conclusion of high migration rate and relatively open sub-populations. However, further in-depth analysis of the planktonic samples of lobsters from the Indian coast using the population-specific molecular markers would improve the direct evidence of larval movement leading to panmixia of lobster species. Another reason as suggested by Chapman *et al*. (1999) is that similar marine environments, even geographically distant, may show homogeneous populations. The fragmentation effects over the structuring of the genetic diversity may still be low and the isolation time or the number of generations may not be enough to

promote a possible differentiation and genetic structuring between the specimens of lobsters from the sampling locations.

Although RAPD PCR is sensitive to several reaction factors, it is quite useful, when used with caution, for several applications in marine organisms. In the present study, we demonstrated the use of RAPD analysis to study the population structure of lobsters along the Indian coast. Polymorphic fragments isolated through these studies could be used and probes be constructed which may be helpful for single species identification. But, while interpreting the RAPD data, absence of amplicons/ bands should be carefully interpreted. Combination of mitochondrial and nuclear markers was helpful in elucidating a more reliable and detailed picture of historical and present-day population structure of the lobsters (Palero *et al.*, 2008; Babbucci *et al.*, 2010). Hence another marker of mitochondrial origin was used in this study.

Table 46. Genetic variability indices reported for different marine crustacean populations. Comments are based on the degree of genetic differentiation as suggested by Wright (1978).

Species	Category	Marker used	Nei's gene diversity 'h'	F_{ST} Or G_{ST} or Φ_{ST}	Genetic Similarity among populations	Genetic distance (GD) among populations	Comments on Genetic differentiation	Reference
Homarus americanus	Lobster	RAPD	....	F_{ST}-<0.000 to 0.073	....	0.002-0.006	No genetic differentiation	Harding *et al*., 1997
H. gammarus	Lobster	RAPD	....	0.1166	0.35-0.58(BSI)	High	High	Ulrich *et al*., 2001
Nephrops norwegicus	Lobster	Allozyme	0.165-0.187	0.013	....	0.0013-0.0186	Shallow, but significant	Stamatis *et al*., 2006
P. stylirostris	Shrimp	RAPD	......	Φ_{ST} -0.1473	85.27% (AMOVA)	14.73%	Significant	Aubert and Lightner, 2000
Metapenaeus affinis	Shrimp	RAPD	0.2565-0.357	....	0.894-0.923	0.134-0.224	High	Lakra *et al*., 2010
Penaeus chinensis	Shrimp	RAPD	....	0.309	0.9049-0.956	0.0561-0.1235	Shallow	Zhimeng *et al*., 2001
Pandalus borealis	Shrimp	RAPD	....	-0.003-0.14	>96% (AMOVA)	0-3.22	Moderate	Martinez *et al*., 2006
Acetes japonicus	Shrimp	RAPD	......	....	0.9049-0.95	0.04-0.099	High	Aziz *et al*., 2010
Portunus pelagicus	Crab	RAPD	......	0.17-0.36	0.7811-0.911	0.09-0.18	High	Klinbunga *et al*., 2010
P. homarus homarus	Lobster	**RAPD**	0.1719	0.0136	0.95-0.96	0.5-0.6	No genetic differentiation	**Present study**
T. unimaculatus	Lobster	Lobster	**RAPD**	0.1446	0.0442	0.92-0.95	0.07-0.08	-do-	**Present study**

5A.2. Hypervariable mitochondrial COI marker

Mitochondrial DNA (mtDNA) has been extensively used to investigate patterns of intra-specific partition of the genetic polymorphism as well as to infer the evolutionary and demographic history of populations and species (Ballard and Whitlock, 2004). Fast evolution rate of COI allows the discrimination of not only closely allied species, but also different populations of the same species (Cox and Hebert, 2001). Greater detail of population history can be inferred by examining the evolutionary relationships between mtDNA haplotypes to determine whether populations have been a stable size or have grown exponentially (Slatkin and Hudson 1991; Rogers, 1995).

Analysis of COI sequence data from *P. homarus homarus* and *T. unimaculatus* indicated lack of population structure along Indian coastline suggesting panmictic populations with high level of gene flow in spite of their patchiness in distribution along the coast. The mitochondrial COI marker is found to be efficient to detect population structure and estimate genetic diversity and divergence times in a variety of marine invertebrate species having pelagic larvae and occupying many geographical areas like lobsters (Palero *et al*., 2008; Froufe *et al*., 2010; Naro-maciel *et al*., 2011; Chow *et al*., 2011), crabs (Cassone and Boulding, 2006; Azuma *et al*., 2008 ; Liu *et al*., 2009; Sotelo *et al*., 2009; Silva *et al*., 2010), shrimps (Li *et al*., 2009; Zitari-Chatti *et al*., 2009; Khamnamtong *et al*., 2009; De Croos and Palsson, 2010; Russ *et al*., 2010; Kelly and Palumbi, 2010), bivalves (Kochzius and Nuryanto, 2008; Mao *et al*., 2011; Krakau *et al*., 2012), krill (Jarman *et al*., 2002), asteroids (Kochzius *et al*., 2009; Pérez-Portela *et al.*, 2010; Hunter and Halanych, 2010) and sea urchins (Lessios *et al*., 2003).

The number of mitochondrial 666 bp long COI sequences selected for the population study in lobsters- *P. homarus homarus* (20 each: 3 sampling sites) and 681 bp of *T. unimaculatus* (18 each: 4 sampling locations) were comparable to the sample sizes selected for the population structure analysis using the same gene in *Panulirus elephas* (Palero *et al*., 2008) and *Panulirus japonicus* (Inoue *et al*., 2007).

Nucleotide composition of *P. homarus homarus* and *T. unimaculatus* indicated an A-T bias of 59% and 60.2% respectively congruent with diagnosis of the same in crab *Callinectes bellicosus* (Pfeiler *et al*., 2005), lobsters like *Palinurus elephas* (Cannas *et al*., 2006), *Panulirus argus (*Naro-Maciel *et al*., 2011), *Jasus* species (Ovenden *et al*., 1997) and *Panulirus* species (Ptacek *et al.,* 2001) suggesting mitochondrial COI gene is under influence of directional selection towards A-T. This A-T rich sequence structure made these sequences useful molecular markers even in populations level (Sanchis *et al*., 2001). Decapod mitochondrial genome is observed to be A+T biased with highest A+T content in the putative control regions (Lin *et al*., 2012). Higher A-T rich regions (>70%) have been reported in the mtDNA control region of various lobsters like *P. elephas* (Babbucci *et al*., 2010). Regions rich in A+T nucleotides are indicators of reduced selective constraints (Nigro *et al*., 1991) and regions which have high A+T content usually are hypervariabe (e.g. non-coding control regions). Findings of this study showed that the mitochondrial COI sequences were sensitive enough to discriminate lobster populations in terms of A-T bias.

The Ts/Tv ratio reported for *P. homarus homarus* and *T. unimaculatus* populations were 8.96 and 5.22 respectively. Pfeiler *et al*. (2005) has reported a similarly high ratio of 10 in the population study of crab *C. bellicosus* using the same marker. Value of 3.8 has been reported in *P. argus* populations along the Carribean Sea (Naro-Maciel *et al*., 2011). The high Ts/Tv ratio indicated a lack of population structure in these studies similar to the observation in the present study.

5A.2.1. Intra-specific variability and population structure

Haplotype diversity is a measure of the uniqueness of a particular haplotype in a given population (Nei and Tajima, 1981). The haplotype diversity '*h*' ranged from 0.8947-0.9368 with an average value of 0.9226 for *P. homarus homarus* and from 0.778-0.928 (0.873 on average) for *T. unimaculatus*. Out of the 23 haplotypes observed in *P. homarus* many were found to be shared among populations. Frequencies of the non-private

haplotypes varied among samples from different locations. For example, Hap9 was found in samples from all locations except Visakhapatnam (Table 18). Many private haplotypes were also detected in all populations, but in low frequencies. Similarly, out of the 20 haplotypes observed in *T. unimaculatus*, the most abundant haplotype was Hap3 found in all the samples. As in the case of *P. homarus homarus*, frequencies of non-private haplotypes varied among samples. Private haplotypes detected were less in number for the species from various locations, which ranged from none in Veraval to three in Kollam (Table 27).

The frequency distribution of the dominant haplotypes was not significantly different among the sample locations. The private haplotypes, which are singletons, detected in all populations of *P. homarus homarus* and *T. unimaculatus* however cannot be used as population markers due to their incidence in low frequencies. Similar results showing occurrence of fewer number of abundant haplotypes and fairly high number of private haplotypes has been observed in many lobster species like *P. elephas* (Palero *et al.*, 2008), *P. japonicus* (Inoue *et al*., 2007), *Panulirus interruptus* (García-Rodríguez and Perez-Enriquez, 2006) and *H. gammarus* (Triantafyllidis *et al.*, 2005). The presence of a higher number of private haplotypes may be due to increased mutation rates, large female effective population sizes, or a combination of these factors.

Nucleotide diversity is a measure of genetic variation, which is used to measure the degree of polymorphism within a population (Nei, 1979). The nucleotide diversity (π) value was found to be low for both species (0.0089 on an average for   *P. homarus homarus* and 0.007 for *T. unimaculatus*). Higher haplotype diversity '*h*' ranged from 0.8947-0.9368 (average value-0.9226) in *P. homarus homarus* and 0.778-0.928 (average value- 0.873) in *T. unimaculatus* populations respectively.

Similar results with high values for '*h*' and very low value for 'π' has been observed in population structure analysis using mtDNA markers in many invertebrate species with pelagic larvae like lobsters (Table. 47), asteroids (Colgan *et al.*, 2005; Kochzius *et al*., 2009), echinoderms (Uthike

and Benzie, 2003), crabs (Cassone and Boulding, 2006) and shrimps (Russ *et al.*, 2010). Unlike this, low values for '*h*' and '*π*' in some marine shrimps like *Penaeus japonicus* (Tsoi *et al.*, 2007), crab *Uca* (Silva *et al.*, 2010) etc., as well as moderate values for the same in crabs (Azuma *et al.*, 2008) and in lobster *P. elephas* (Palero *et al.*, 2008) were also reported.

Grant and Bowen (1998) classified fishes into four categories based on different combinations of haplotypes diversity ('*h*') and nucleotide diversity ('*π*') of mtDNA sequences to interpret different scenarios of population history. Based on the results from mtDNA markers in the present study, *P. homarus homarus* and *T. unimaculatus* populations belong to group II (high '*h*' and low '*π*') which indicates possibility of genetic bottleneck events, with subsequent population expansion and formation of new haplotypes, which are found in low frequencies (Grant and Bowen, 1998). The high haplotype diversities and low nucleotide diversities can be interpreted as evidence of recent population expansion (Rogers, 1995) after a genetic bottleneck seems applicable in the present study too. A demographic or spatial expansion that rapidly increased *Ne* would result in many low frequency haplotypes, but with low overall nucleotide diversity. The rapid population growth enhances the retension of new mutations (Avise *et al.*, 1984). Recent diversification within each population limits the amount of variation among haplotypes, since sufficient evolutionary time has not elapsed for haplotypes to acquire significant nucleotide differences.

The pair-wise F_{ST} and Φ_{ST} population comparisons and AMOVA were not sufficiently different to show a clear biogeographic pattern among the populations of *P. homarus homarus* and *T. unimaculatus* lobsters under our study which remained not significant at 5% significance level after applying sequential Bonferroni correction, indicating a panmictic population. Clades associated with particular geographic regions were not evident. The fixation index (F_{ST}) which is a measure of population differentiation due to genetic structure is having an overall low value for the populations of *P. homarus homarus* (0.0394) and *T. unimaculatus* (0.0468) which were not significant ($P<0.01$) either. Statistical parsimony based haplotype network (TCS) for species did not show clear demographic differentiation patterns associated with haplotypes showing homogeneity in stock structure. The population

structure of various lobster species from different parts of the world is compared to the present study and given in Table 47. The results from our study revealed population homogeneity similar to those obtained in other geographic locations for various lobster species like *Panulirus inflatus* (Garcia-Rodriguez and Perez-Enriquez, 2008a), *P. argus* (Naro-Maciel *et al.*, 2011), *Jasus edwardsii* (Ovenden *et al*., 1992) etc.

The mtDNA loci provide a better signal for current or more recent patterns of gene flow (Moritz *et al*., 1987). The genetic similarities among populations suggest high gene flow among lobsters along the Indian coast as evident from the high *Nm* values. High contemporary gene flow was considered the primary process in homogenizing populations in crab *P. crassipes* (Cassone and Boulding, 2006). If equilibrium has not yet been attained, the genetic similarity among populations likely reflects demographic history rather than ongoing gene flow. Partially significant as well as highly significant F_{ST} values indicating restricted gene flow are also reported in crustaceans. Results for pair-wise F_{ST} test showing partially genetic differences have been observed in *Panulirus interruptus* (F_{ST} value of 0.036-0.004; Garcia-Rodriguez and Perez-Enriquez, 2006) and *Nephrops norwegicus* (0.018- Stamatis *et al.*, 2004). Significant differences in F_{ST} pairwise comparison has been noted in lobster species like *P. elephas* (Snn statistic- 0.109; Palero *et al.*, 2008), *Jasus verreauxi* (Gst-0.29; Brasher *et al*., 1992), *H. gammarus* (0.0783-Triantafyllidis *et al*., 2005) and in green crabs (F_{ST} 0.264-0.678; Roman and Palumbi, 2004). Higher values of F_{ST} has been noted using mtDNA markers than nuclear markers like microsatellites (Weersing and Toonen, 2009) probably due to its reduced effective population size, increased rate of genetic drift, high mutation rate etc. (Ballard and Wiltcock, 2004).

The hierarchical AMOVA analysis indicated that most of the variation was within samples for both the species. In *P. homarus homarus,* the variation within populations was 96.06% while only 3.94% was due to variation among populations. Likewise, in *T. unimaculatus*, 95.32% of total variation could be attributed due to variance within populations. The AMOVA indicated panmixia and lack of population structure among populations of both species of lobsters. Similar results were observed in lobster

P. interruptus in which only 0.84% of the variance was observed among localities and 98.97% was due to within population variation (García-Rodríguez and Perez-Enriquez, 2006) indicating a lack of population structure in the Baja California coast and also in *P. argus* in which three way AMOVA indicated 96.77% within population variation (Silberman *et al.*, 1994). Interestingly, evidences are also there in which the very low percentage of within population variation can also be statistically significant imparting genetic structuring in lobsters. The same was observed in *Nephrops norwegicus* (Stamatis *et al.*, 2004), *Jasus lalandii* (Matthee *et al.*, 2008) and in *P. elephas* (Froufe *et al.*, 2010) in which 98.25%, 97.2%, and >98%of variation were due to of variation attributable to within population differences.

Many marine species display genetically unstructured populations or low differentiation, although structuring of this differentiation is often species-specific and may be affected by sampling of non-breeding demes (Nesbo *et al.,* 2000; Ruzzante *et al.,* 2006). Similar findings of genetically unstructured populations with little or no intraspecific genetic differentiation have been reported in other spiny lobsters (Ovenden *et al.*, 1992; Tolley *et al.*, 2005; Cannas *et al.*, 2006; Garcı´a-Rodriguez and Perez-Enriquez, 2006; Garcia-Rodriguez *et al.,* 2008b; Inoue *et al.*, 2007; Naro-maciel *et al.*, 2011; Pampoulie *et al.*, 2011) as well as in Scyllarid lobsters (Froufe *et al.*, 2011; Faria, 2012). The same has been noticed in other marine crustaceans with pelagic larval phase like shrimps (Cui *et al.*, 2007; Roldan *et al.*, 2009; Russ *et al.*, 2010), echinoderms like *Holothuria* (Uthike and Benzie, 2003) and crabs (Gomez-Uchida *et al.,* 2003; McMillen-Jackson and Bert , 2004; Sotelo *et al.*, 2009; Silva *et al.*, 2010). It is suggested that marine species with long larval phases are thought to disperse further, have higher gene flow, larger geographic ranges, and lower levels of genetic differentiation among populations (Féral, 2002; Palumbi, 2003).

In contrast to this, presence of substantial genetic differentiation were also reported with various markers in species with pelagic larvae including lobsters (Brasher *et al.,* 1992; Jørstad *et al.*, 2004; Triantafyllidis *et al.*, 2006; Palero *et al.*, 2011; Chow *et al.*, 2011), crabs (Zhao *et al.*, 2002; Fratini and Vannini, 2002; Cassone and boulding*,* 2006; Liu *et al.*, 2009), shrimps

(Khamnamtong *et al.*, 2009), stomatopods (Zhang *et al.*, 2012)*,* star fishes (Konchius *et al.*, 2009), sponges (Dailianis *et al.*, 2010) and molluscs (Pérez-Losada *et al*., 2007; Cho *et al.*, 2007; Sá-Pinto *et al*., 2010) suggesting that larval dispersal may not be materialized. Shallow genetic structuring was observed in high dispersal species like shrimps (Roldan *et al.*, 2009) and lobsters (*Jasus tristani*- Von der Heyden *et al.*, 2007).

5A.2.2. Demography

Inferences on patterns of demographic history were obtained by using neutrality tests. These statistical tests which are based on either: (1) the frequency of segregating sites [e.g. Tajima's (1989) D] (2) the distribution of haplotypes [Fu's (1997) F_S] or (3) the distribution of pair-wise sequence differences (mismatch distribution; Harpending, 1994) These tests have been compared by Ramos-Onsins and Rozas (2002). They showed that Fu's F_S was the most powerful test for detecting population growth when sample size was large.

Tajima's D statistic and Fu's F_S statistic were calculated in the present study for assessing whether nucleotide polymorphisms deviate from expectations under neutral theory. All statistic were significantly negative for *P. homarus homarus* and *T. unimaculatus* populations for Tajima's D and Fu's F_S ($P<0.01$) tests. This indicated that there is departure from mutation drift equilibrium which can be linked to large number of unique haplotypes or selection as a result of sudden/recent expansion in population after a genetic bottleneck. The high haplotype diversity and low nucleotide diversity in the present study with both the species support the above situation. Studies within other species of lobsters in the Mozambican and South African coasts on mitochondrial DNA variation and gene flow have supported the idea that species and individual populations have undergone recent population expansions (Tolley *et al*., 2005; Gopal *et al*., 2006; Von der Heyden *et al*., 2007b; Neethling *et al*., 2008; Palero *et al*., 2008a; Naro-Maciel *et al*., 2011).

5A.3. Hypothesis for connectivity and panmixia in spiny and slipper lobster populations along the Indian coastline

The genetic structure of marine species is determined by the complex interaction of many factors, including adult mating and pre-spawning behaviour, larval development time and behaviour*,* oceanography, and the latter's seasonal and annual variation (Pringle and Wares, 2007), and must be empirically examined to inform management.

The lobsters *P. homarus homarus* (family Palinuridae) and *Thenus unimaculatus* (family Scyllaridae) form the main stay in the multi-species lobster fishery of India. The genetic homogeneity or lack of population structure may be related with the reproductive biology, life cycle, and larval dispersal of these lobster species. Phillips (2006) has discussed in detail about various aspects of lobsters especially the spiny lobsters. The lobsters in general produce large numbers of small eggs that are incubated for a short period before hatching into the larval phase (phyllosoma) that can last weeks to many months. After weeks or months of development in offshore waters, the phyllosoma return towards the continental shelf where the final stage larvae metamorphose into puerulus in Palinurids and nisto in Scyllarids which swim towards the coast and metamorphose into the first juvenile stage signaling the end of planktonic phase.

Achelata lobsters are characterized by the presence of the phyllosoma larva whose dispersive ability is among the highest found in crustaceans (Booth, 1994; Palero *et al*., 2008a). The larval durations are difficult to estimate in nature and planktonic development in many cases is longer in nature than in culture. The larval development also differs in Palinuridae and Scyllaridae. Phillips (2006) stated that most palinurids for which there are estimated data for larval durations in nature of 6-12 months (but longer for temperate species like *Jasus* -upto 24months) disperse well offshore. There is more variety in the length of larval life (1-9months) and concomitant extent of dispersal among the scyllarids. Many of the small adult scyllarid species have brief inshore development. In both families, the long-

lived phyllosomal larval phase is the key to dispersal of the species (Phillips, 2006).

Unlike the spiny lobsters, which exhibit habitat diversity during their life span, the sand lobsters tend to aggregate in the same grounds, irrespective of their developmental phase. Sekiguchi *et al*. (2007) proposed that the phyllosomas of *Thenus* species may not be widely dispersed even though they possess the potential to be transported large distances from shore. Prasad *et al*. (1975) observed that greater concentration of phyllosoma larvae found in 50m depth near land masses. Scyllarid phyllosoma were numerically most abundant of all phyllosomas collected from coastal waters (McWilliam and Phillips, 1983; Rothlisberg *et al*., 1994; Sekiguchi and Inoue, 2002). Sekiguchi *et al*. (2007) also opined that the short larval durations evolved among many scyllarid species so that fewer larvae are carried far from the coast by currents, allowing them to recruit to their benthic inshore populations. Radhakrishnan *et al*. (2007) observed that during peak spawning period females do not undertake breeding migrations making them vulnerable to trawling. Hence, a population structuring may be expected in the case of *T. unimaculatus* populations.

However, our study could not reveal heterogeneity in stock structure both in spiny and slipper lobster populations. Three possible hypotheses may be put forward for the lack of population structuring of the species along the Indian coast. They are 1) The planktonic larval duration of lobster species in wild; 2) Coastal current pattern of Northern Indian Ocean associated with monsoon currents which coincides with the peak breeding season of species. It can carry planktonic phyllosoma larvae along the coast; 3) Movement behaviour in lobsters.

Population connectivity is mainly determined by the potential dispersal of the species and those with high dispersive ability tend to present more genetically unstructured populations (Palumbi, 2003). The distance marine larvae are physically transported generally increases with larval duration (Largier, 2003; Siegel *et al.*, 2003), thus species with long pelagic larval

durations (PLD) can potentially disperse thousands of kilometers (Butler IV *et al.*, 2011). Understanding the larval behaviour is most critical aspect in determining larval connectivity (Chiswell and Booth, 2008). The dispersal of larvae engaged in vertical migration usually differs from those that drift passively in the sea (Pineda *et al.*, 2007). Prasad *et al*. (1975) and Prasad (1978) observed that there was a greater concentration of larvae of both palinurids and scyllarids at 50 and 100 m which seemed to be closely related to the distribution of the pycnocline values. Kathirvel and James (1990) recorded early larval stages of lobsters beyond 150 km- 450 km and advanced stages more close to shore around Andaman and Nicobar. Along the west coast of India too, Kathirvel (1990) observed the same in mid-water trawls. It was noted that the phyllosoma larvae were more in night hauls and attributed it to the diurnal vertical movements of the larvae. Phyllosomata have little horizontal swimming ability, but their horizontal transport may be modulated by vertical migration between surface and deeper waters (Griffin *et al*., 2001; Jeffs *et al*., 2005). The lobster larvae which can undergo both horizontal and vertical migrations are reported in lobster species like *Panulirus cygnus* (Rimmer and Phillips, 1979), *P. interruptus* (Pringle, 1986), *Jasus edwardsii* (Bradford *et al*., 2005), *Scyllarus cultrifer* (Minami *et al*., 2001) etc.

The role of currents and life history of marine species have been usually considered as key elements in shaping intra-specific population structure (Muss *et al*., 2001). The extended larval period gives potential for widespread dispersal in wind-driven surface currents and in subsurface flows (Sekiguchi *et al*., 2007). The North Indian Ocean comprising of Bay of Bengal of the East Coast and Arabian Sea of the West coast is remarkable in that it is not connected to poles and is tropical (Shenoi, 2010). The frequent coastal currents which include the currents along the east coast of India, called the East India Coastal Current (EICC) the current along the west coast of India, called the West India Coastal Current (WICC) which change direction with seasons, may be the causative factor affecting the pelagic larval dispersal
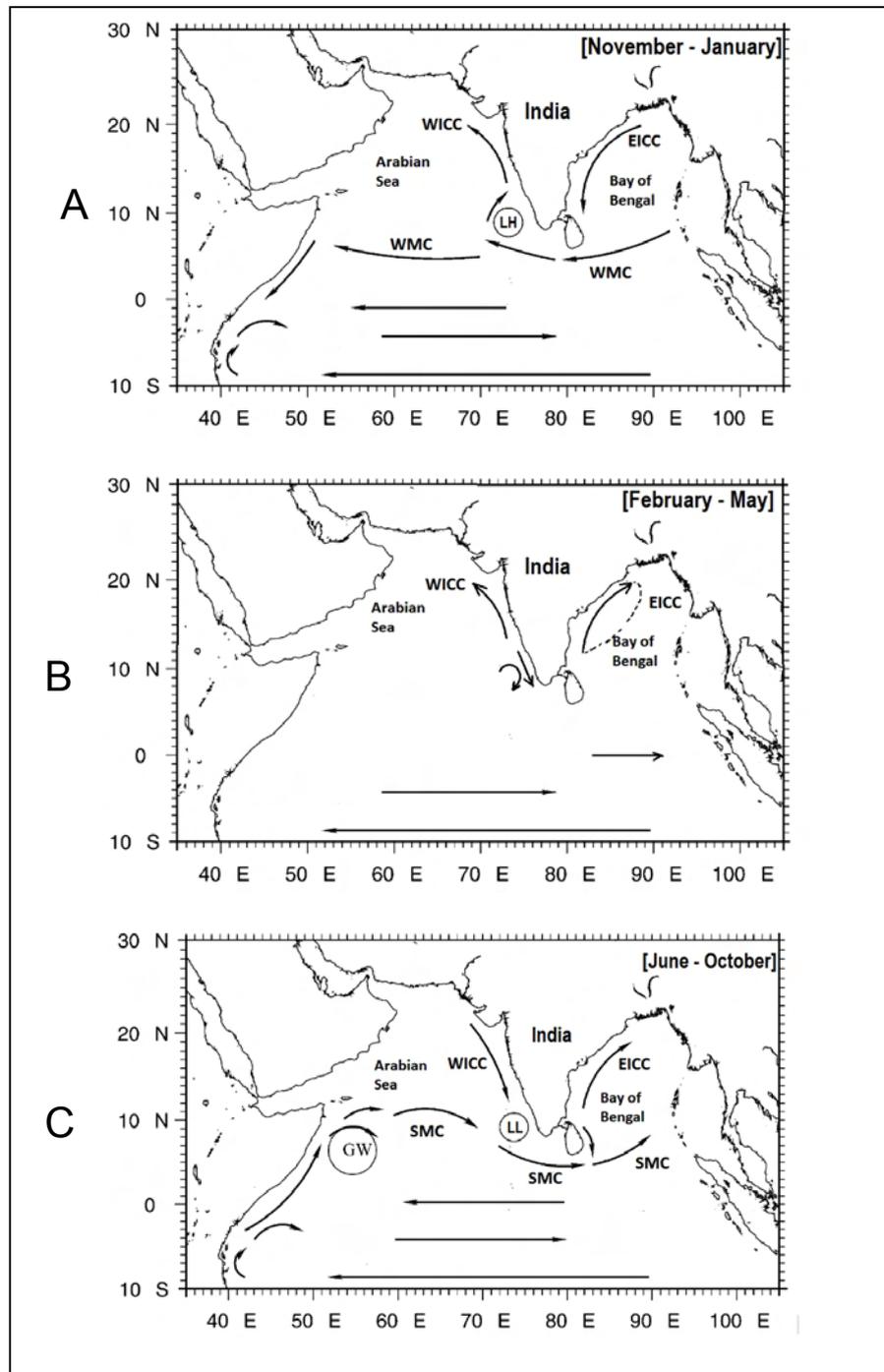
Plate VIII- The schematic of the seasonal cycle of surface currents in the North Indian Ocean.

A- Surface Currents In Nov-Jan; B –Currents In Feb-May; C- Currents In
B- June-Oct.
The abbreviations are as follows: **WMC**, Winter Monsoon Current; **SMC**, Summer Monsoon Current; **WICC**, West India Coastal Current; **EICC**, East India Coastal Current; **LH**, Lakshadweep High; **LL**, Lakshadweep Low.

(Courtesy: D. Shankar *et al*., 2002; Shenoi, 2010)

including of lobsters around Indian coast (Plate VIII- A, B, C). Kathirvel and James (1990) suggested possible emigration and immigration of larval forms through offshore or onshore currents when they noted occurrence of lobster larvae in offshore waters of Andaman. Prasad (1986) suggested that phyllosoma larvae released in shallow inshore areas are typically described as being carried offshore in the particular watermass in which they are released and their return can be taken as indicating the presence of water movements. The role of currents in larval dispersal was evident in many species like *Panulirus cygnus* (Caputi *et al*., 1996) and *Panulirus japonicus* (Yoshimura *et al*., 1999; Sekiguchi and Inoue, 2010).

Ferraris and Palumbi (1996) opined that lobsters (or their larvae) are transferred to water bodies by various oceanic circulations, such as southwestern monsoon. The north Indian Ocean is affected by this circulation (Suryanarayana *et al*., 1992). It is reported that the captive breeders of *P. homarus* spawned four times in an year in India (Vijayakumaran *et al*., 2004) with a maximum of 6, 28,930 eggs (Vijayakumaran *et al*., 2012). The prolonged breeding season with multiple broods in large females conforms to the typical pattern of tropical palinurids. The peak breeding season for the species is in November along the coast, which coincides with the peak-fishing season. The length of larval life for the species is estimated to be 5.5 - 8 months (Phillips and Matsuda, 2011). Since *P. homarus* is a prolonged breeder, the larval transport can happen both by coastal as well as both monsoon currents. The lobster larvae are seen throughout the year along the west coast. Kathirvel (1990) pointed out the possibility of association of *P. homarus* larvae with currents in the west coast.

T. unimaculatus breeds mainly in Oct-Jan along the west coast (Radhakrishnan *et al*., 2007) and Nov-March along east coast (Kizhakudan, 2006b; Subramanian, 2004). The EICC along with WMC and WICC flowing westward during Oct-Feb and there is a strong north-eastward flow during March-April (Shankar *et al*. 1996, Mc Creary *et al*., 1996) (Plate VIII- B).

These may be of more importance for larval dispersal in case of *T. unimaculatus* whose larvae are weak swimmers (Barnett *et al*., 1984). Thus the continuously changing current pattern in the North Indian Ocean may result in the exchange of long-lived planktonic larvae of lobsters along the Indian coast, leading to genetic homogeneity among stocks. However detailed investigation is essential to prove that pelagic larval dispersal happens along with the distribution of coastal currents.

There are many dispersive processes in spiny lobsters at various life stages. Movements can be homing/territorial, dispersive/nomadic and migratory. Migration is common among the shallow-water palinurids (Phillips, 2006). Mass migrations reported in many lobster species like *Panulirus delagoe* and *P. argus* (Cobb and Phillips, 1980), *P. ornatus* (Moore and MacFarlane, 1984) but appear to be absent in some species like *P. elephas* (Follesa *et al*., 2007) or restricted in some like *H. gammarus* (Smith *et al*., 2001). The tagging experiment in *P. homarus* at Muttom (Kanyakumari) by CMFRI (Mohamed and George, 1967) indicated a restricted movement for the spiny lobsters. Prasad and Tampi (1968) based on the tagging experiment study opined that the widespread distribution of *P. homarus* is due to dispersal of the phyllosoma larvae by water movements. Tagging study to investigate movement patterns of *P. homarus rubellus* indicated adult *lobsters* were predominately resident with only 3% of recaptured lobsters moving over 500 m (Steyn *et al*., 2011). This may suggest that the migration may not contribute to the observed panmixia in scalloped spiny lobster along the Indian coast. The larval exchange within the environment may be more likely due to ocean currents. Oceanographic processes are assumed to have role in preventing phyllosoma from being carried vast distances from shore and ineffecting their return towards shore. Even if the adult migration in adults is restricted, the planktonic larvae, which have high dispersal potential can, cause homogeneity in population.

Unlike palinurids, the phyllosomas and nisto of *Thenus* species are weak swimmers with a larval morphology that limit their vertical migrations putting constraints over their wider dispersal off the shelf. Still, in terms of

locomotion, *Thenus* spp. is distinct in Achelate lobsters by virtue of its highly developed swimming ability (Jacklyn and Ritz, 1986). Mobility of the species has been checked by tagging experiment (Jones,1988; Courtney *et al*., 2001) which indicated that *Thenus* spp. can be very mobile capable of moving large distances, but not likely to be migratory. Unfortunately, such experiments are yet to be carried out with the species along Indian coast. However, the relative movement and the larval dispersal through currents may also be the major factors for genetic homogeneity of populations.

5A.4. Comparative assessment of RAPD and mtDNA marker studies in population structure of lobsters

Even though mtDNA phylogenies can provide unique insights into population history (Avise *et al*., 1987; Avise 1994), mtDNA must be used in conjunction with nuclear markers to identify evolutionary distinct populations for conservation (Cronin, 1993) because given a lower effective number of genes (Birky *et al*., 1989) or greater dispersal by males than females, mtDNA can diverge while nuclear genes do not. MtDNA studies provide only a single "gene tree" that might not accurately reflect the 'organismal tree', because the entire mitochondrial genome acts as a single genetic locus (Degnan, 1993). In this study, the mtDNA markers are used along with nuclear type II marker (RAPD) to get a more reliable picture of population structuring. A combination of RAPD and mtDNA RFLP analysis has revealed genetically significant difference between populations for the former marker while the latter marker could not bring out differentiation in *Penaeus monodon* in Thailand (Klinbunga *et al*., 2001). Nevertheless, our study revealed the absence of stock structure in *T. unimaculatus* from both mtDNA and RAPD markers.

In this study, the moderate levels of genetic variability were observed with both species as revealed by nuclear RAPD markers and low nucleotide diversity with mtDNA analysis. However, high haplotype diversity was reported with mtDNA COI in both the species. Populations that experienced bottlenecks can have low levels of diversity despite their large contemporary

population sizes (Canino *et al.*, 2010). The populations of *T. unimaculatus* and *P. homarus homarus* have suffered strong declines due to over-fishing over the years in certain regions of the country (Radhakrishnan *et al.*, 2007). The low fidelity of the mtDNA polymerase and the apparent lack of mtDNA repair mechanisms lead to a higher rate of mutation in the mitochondrial genome compared with the nuclear genome (Budowle *et al.*, 2003).

The results from the nuclear RAPD and mitochondrial markers were concordant and they revealed occurrence of panmictic populations for *P. homarus homarus* and *T. unimaculatus* along the Indian coast which indicates extensive admixture, high levels of population connectivity and gene flow occurring from neighbouring or more distant populations. Despite the relatively long distance that separates the sampling sites of slipper lobsters (2988 km between Veraval and Visakhapatnam), the larval behaviour associated with specific hydrographic patterns can be the major factors that facilitate a high connectivity between populations.

The results, however, deviates from a number of observations based on RAPD (Mishra *et al.*, 2009; Lakra *et al.*, 2010), mtDNA (Kumar *et al.*, 2007) and microsatellite (Mandal *et al.*, 2012) markers in marine shrimps along Indian coast. Such differences can be attributed to the changes in life history traits among speces. However, this was the first study on population genetics of lobsters from Indian waters. In the case of *P. homarus homarus* and *T. unimaculatus*, further examination of the population structure could be carried out using genetic markers like the mtDNA D-loop region or nuclear microsatellite markers with higher sensitivity for the detection of genetic differentiation.

5A.5. Management implications of the present study

The results obtained from this study are of significance in the present context of alarming decrease in landings of lobsters over the years in the country. The decline in landings is an indication of the growing instability of most of the lobster stocks on both the east and west coast of India. Based on

the current landing data, and biological information on the mean size of *P. homarus homarus*, it could be deduced that the stock has been overexploited. The Scyllarid lobster *T. unimaculatus* sustains a fishery of importance along the northwest and southeast coasts where landings are mainly as by-catch of trawlers. This is the only slipper lobster of commercial significance among the rich diversity of Scyllarid lobsters recorded from Indian coast (Radhakrishnan *et al*., 2007) and the only slipper lobsters whose aquaculture potential has been demonstrated (Vijayakumaran and Radhakrishnan, 2011). At present Gujarat reports maximum landings of the species, although the stock shows depletion at faster rate when compared to landings of the previous decades from 148.3 MT (1980-85, Kagwade *et al*., 2001) to 6 MT in 2004 (Radhakrishnan *et al.*, 2005; Vijayakumaran and Radhakrishnan, 2011). The fishery of the same collapsed in the waters off Mumbai due to recruitment overfishing (Deshmukh, 2001; Vijayakumaran and Radhakrishnan, 2011).

Marine species with long larval phases are thought to disperse further, have higher gene flow, larger geographic ranges, and lower levels of genetic differentiation among populations (Féral, 2002; Palumbi, 2003). The lack of obvious physical barriers to dispersal in conjunction with Wright's (1931) mathematical prediction that even rare gene flow (on the order of one migrant per generation) is enough to prevent population divergence by genetic drift leading to high genetic homogeneity among marine populations, with isolation by distance over the broadest scales. The level of genetic homogeneity lobster populations is not surprising due to the more-or-less continuous distribution, high gene flow and the enormous potential for dispersal of the planktonic phyllosoma larval stages, which can last for long in wild. The need of recognizing management measures for such a population structure is that, over-fishing of one of these locations would not significantly reduce the overall level of genetic variation in the species. The low overall level of genetic differentiation among lobster populations does not mean that important inter-population adaptive genetic differences are absent. But the reduced number of stocks reported in some regions advocate for

more effective and adequate regulatory measures in its conservation even though conservation measures like minimum legal size for export as well as participatory management approach has been adopted in the country to regulate lobster stock depletion (Radhakrishnan *et al*., 2005). Because populations of species are found to be panmictic, all management, conservation and restocking efforts must be coordinated at the national level, as over-exploitation in one region will negatively affect the metapopulation and will decrease recruitment across the distributional range.

The current results will be of help for better management of lobster stocks. Given the high value of the resource and decline of the stocks in several areas, all information that improves management should prove useful. Future efforts to find genetic distinctions between various inshore and offshore lobster populations should be attempted with high-resolution molecular markers.

Table 47. The population structure of various lobster species from different parts of the world in comparison to the present study.

Population	Region	Pelagic Larval period in nature (months)	Mitochondrial Marker used	Haplotype Diversity (h)	Nucleotide diversity (π)	Genetic Differences among demes	Reference
Spiny Lobsters							
Panulirus japonicus	Japan	8-14	COI	0.959-0.999	0.009-0.01	No genetic differentiation	Inoue *et al*., 2007
Panulirus penicillatus	Western and Eastern Pacific	>7-8	COI	0.993		High	Chow *et al*., 2011
Panulirus regius	Cape Verde and south-western Africa	……..	COI	High	0.011	No genetic differentiation	Frouse *et al*., 2010
P. mauritanicus	North Western Mediterranean and Atlantic	Short larval period	COI	0.905	0.0045	No genetic differentiation	Palero *et al*., 2008
Panulirus argus	N. Caribbean Sea	4-6	COI	0.942-0.986	0.014-0.023	No genetic differentiation	Naro-Maciel *et al*., 2011
	N. Caribbean Sea	4-6	Control Region	0.984-1	0.008-0.012	No genetic differentiation	
	Caribbean sea and Brazil	…..	Control Region	0.883	0.021	High	Diniz *et al*., 2005
Palinurus elephas	Mediterranean	12	COI	…..	…….	No genetic differentiation	Cannas *et al*., 2006
	Atlantic and Mediterranean	5(Atlantic); 12(Mediterranean)	COI	0.558	0.0016	Present	Palero *et al*., 2008a.
	Azores, Atlantic, Mediterranean, Sagrus	5(Atlantic) -12(Mediterranean)	COI	Low	0.02	Shallow	Frousfe *et al*., 2010
Panulirus interruptus	Pacific-Baja California	8	Mt DNA RFLP	0.867	0.034	No genetic differentiation	García-Rodríguez and Perez-Enriquez, 2006

Palinurus gilchristi	South Africa	4	Control Region	0.843-0.869	0.0038-0.0045	No genetic differentiation	Tolley *et al*., 2005
Palinurus delegogae	SW Indian Ocean (Africa)	……	Control Region	0.957-0.999	0.006-0.009	Shallow	Gopal *et al*. ,2006
Homarus gammarus	Sub-arctic	15-35days	RFLP	0.446-0.944	…..	Medium	Jorstad *et al*., 2004
	Atlantic and Mediterranean	15-35 days (water temp dependant)	RFLP	0.446-0.939	……	Significant (F_{ST} -0.078)	Triantafyllidis *et al*., 2005
Jasus lalandii	South Africa	12-22	16SrRNA	0.64	0.002	Shallow	Matthee *et al*., 2007
Jasus tristani	South Atlantic	8-24	CO II	0.623-0.89	0.0025	Shallow	Von der Heyden *et al*., 2007
Nephrops norwegicus	Northeast Atlantic and Mediterranean	1-2	RFLP	0.93	0.0057	**Shallow** (F_{ST} -0.018)	Stamatis *et al*., 2004
Slipper lobster							
Scyllarus latus	Slipper lobster	11	COI	High	0.01	No genetic differentiation	Froufe *et al*., 2010
Present study							
P. homarus homarus	**Arabian Sea and Bay of Bengal (North Indian Ocean)**	**5.5-8months (estimate only)**	**COI**	**0.8947-0.9368**	**0.0089**	No genetic differentiation (F_{ST} -0.039)	**Present study**
T. unimaculatus	**Arabian Sea and Bay of Bengal (North Indian Ocean)**	**27-45days**	**COI**	**0.778-0.928**	**0.007**	No genetic differentiation (F_{ST} -0.0468)	**Present study**

5B. Barcoding and phylogeny of 11 commercially important lobster species along the Indian coast

Species are the principal currency of biodiversity and usually the focal taxonomic unit of conservation biology, hence accurate, unambiguous, and robust species identifications is of paramount importance. Specifically, COI as a DNA barcoding tool helps to identify an organism based on DNA sequence variability and assignment to a certain species previously described (Lefe´bure *et al.*, 2006) and for classification of potentially new species. (da Silva *et al.*, 2011). However other genes are also required to evaluate the evolution or phylogenetic information contained in the barcode region of COI (De Salle *et al.*, 2005; da Silva *et al.*, 2011). In the present study, species-specific signatures were generated for 11 commercially important lobster species from the Indian coast using mitochondrial (mtDNA) genes like COI, 16SrRNA, 12SrRNA as well as by the nuclear 18SrRNA gene.

Earlier studies and reports of shovel nosed lobsters of the genus *Thenus* in India were based on the single species – *Thenus orientalis* (Chacko, 1967; Prasad and Tampi, 1968; Rahman and Subramoniam, 1989; Kagwade and Kabli 1996a, b; Deshmukh 2001; Kizhakudan *et al.*, 2004a; Radhakrishnan *et al.*, 2005). However, in view of the species revision of the previously believed monotypic *Thenus* spp., using the COI barcodes, the species of genus *Thenus* distributed and caught widely along the Indian coast was ascertained to be *Thenus unimaculatus* Burton and Davie (2007). The presence a less abundant species, *Thenus indicus* along the east coast could also be confirmed with the above gene. It was also identified that the subspecies of *Panulirus homarus* distributed along the coastline is *P. homarus homarus*. The morphology of the species was also examined and found that all specimens collected from Indian coast had a median interruption in the transverse abdominal grooves and shallow scallops characteristic of the 'microsculpta' form described by Berry (1974). This form is the nominotypical *P. homarus homarus* (Linnaeus, 1758). No other

subspecies could be found in sampling even though Berry (1974) and Holthuis (1991) suspected occurrence of another sub species *Panulirus homarus megasculptus* Pesta, 1915 along the west coast of India.

Mitochondrial ribosomal genes have been used for crustacean molecular phylogenetic work since its inception (Tudge and Cunningham, 2002). Partial sequences of mitochondrial ribosomal genes to infer phylogenetic relationships have been reported in clawed lobsters (Chu *et al.*, 2006; Tshudy *et al.*, 2009; Chan *et al.*, 2009), nephropid lobsters (Tarn and Kornfield, 1998), family Palinuridae (Cannas *et al.*, 2006), genus *Panulirus* (Ptacek *et al.,* 2001), genus *Linuparus* (Tsoi *et al.*, 2011), genus *Palinurus* (Groeneveld *et al.*, 2007), genus *Jasus* (Ovenden *et al.*, 1997) and in slipper lobsters (Yang *et al.*, 2012).

Because different genes may reflect different evolutionary histories (Avise, 2004), use of multiple genetic markers is often recommended (Funk and Omland, 2003; Rubinoff, 2006). Here, phylogenetic relationships are inferred based on an analysis of a combined mtDNA dataset utilizing these genes.

5B.1. Morphological groupings of the species

George and Main (1967) classified species groups in genus *Panulirus* into four based on condition of second and third maxilliped and geographical differences. Phylogenetic studies based on phyllosoma and puerulus morphology (Baisre, 1994; McWilliam, 1995), and the genetic studies (Ptacek *et al.*, 2001; Ravago and Juinio-Menez, 2003) also supported this grouping. They found it useful but concluded that two clear divisions or two lineages, rather than four, better represented the previous findings. Ptacek *et al.* (2001) stated that the first major lineage included all *Panulirus* species of the morphologically based GroupsI (e.g. *P. longipes, P. interruptus, P. argus* etc.) and II (e.g. *Panulirus penicillatus, P. echinatus* etc.); the second lineage contained all species included in Groups III (e.g. *P. polyphagus, P. inflatus* etc.) and IV (e.g. *P. homarus, P. ornatus, P. stimpsoni, P. versicolor* etc.) of

initial classification. George (2006a) assigned the first major lineage to exhibit a habitat preference for clear waters and the second major lineage showing a distinct preference for the turbid waters of coastal habitats. It is generally agreed that Groups I and II represent an early radiation of *Panulirus* species followed by a later radiation, Groups III and IV (Ptacek *et al.*, 2001).

Of the species of *Panulirus* considered here, *P. longipes longipes* and *P. penicillatus* belonged to group I and II respectively as per George and Main (1967) and is included in the first major lineage (Ptacek *et al.,* 2001)*. P. polyphagus* belongs to group III and *P. homarus*, *P. versicolor* and *P. ornatus* to group IV or together in second major lineage. The two deep-sea genera in the present study, *Puerulus* and *Linuparus*, are considered to be the most primitive (George and Main, 1967; Baisre, 1994; George, 2006b) of the family Palinuridae.

The scyllarids *Petrarctus rugosus* and the two *Thenus* species belong to the subfamily Scyllarinae and Theninae of family Scyllaridae.

5B.2. Nucleotide composition comparisons in the sequence data sets

Decapod mitochondrial genome is observed to be A+T biased with highest A+T content in the putative control regions (Lin *et al.*, 2012). Regions rich in A+T nucleotides are indicators of reduced selective constraints (Nigro *et al.*, 1991) and regions which have high A+T content usually are hypervariabe (e.g. non-coding control regions). Nucleotide bias can have drastic effects on phylogenetic reconstructions, especially if the bias is different for individual taxa (Simon *et al.*, 1994). Such bias in phylogenetic analysis can result in the grouping of taxa based on nucleotide composition rather than on shared history (Hasegawa and Hashimoto,1993; Steel *et al.*, 1993) and can reduce the amount of phylogenetic information in the data because of the increased chance of homoplasious changes (Ovenden *et al.*, 1997).

5B.2a. *COI gene*

The A+T content of COI region (58.7%) in the present study is comparable to the 57% for spiny lobster genus *Panulirus* (Ptacek *et al*., 2001), 59.5% reported among species of *Jasus* (Ovenden *et al*., 1997), 56.4% in genus *Linuparus* (Tsoi *et al*., 2011), 57.7% in genus *Metanephrops* (Chan *et al*., 2009) but lower than 65.8% reported in crabs of family Potamonautidae (Daniels *et al*., 2002), 62% in horseshoe crabs (Obst *et al*., 2012), 63.55% in squat lobsters (Machordom and Macpherson,2004) and 63.3% in shrimps (Quan *et al*., 2004).

5B.2b. *16SrRNA*

The AT bias of 66.3% in the 16S gene region observed in the present study is comparable to the 64.2% for spiny lobster genus Panulirus (Ptacek *et al*., 2001), 62% among species of *Jasus* (Ovenden *et al*., 1997), 67.4% in genus *Metanephrops* (Chan *et al*., 2009), 62% in horseshoe crabs (Obst *et al*., 2012), 66.25% in shrimps (Quan *et al*., 2004) but lower than that of 73% in freshwater crabs of Africa (Daniels *et al*., 2002) and 72.22 % in squat lobsters (Machordom and Macpherson,2004).

5B.2c. *12SrRNA*

The 12S gene region in the present study was the most AT rich region with a percentage of 68.6 comparable to the 67.3% in genus *Linuparus* (Tsoi *et al*., 2011) but higher values for the same observed in genus *Metanephrops* (72.9%- Chan *et al*., 2009), grapsoid crabs (75.2 %- Schubart *et al*., 2006) and in African fresh water crabs (71.6% -Daniels *et al*., 2002).

5B.2d. *18SrRNA*

AT content was relatively low and balanced in the 18SrRNA gene (49.2%) comparable to the 49% observed in horseshoe crabs (Obst *et al*., 2012), 48.7% in Onychopoda (Cristescu and Hebert, 2002), 49% in decapod reptantia (Ahyong and O`Meally, 2004) and 51.4% in mysidae (Remerie *et al*., 2004).

5B.3. Parsimony Information from various data sets

The number of parsimony informative sites varied among the genes of ingroup taxa from 118 informative sites in the 18SrRNA sequence data set to 293 informative sites in the 12SrRNA. In this study, as expected for the 16SrRNA gene, fewer variable (230) and parsimony informative (173) characters were obtained in the ingroup taxa compared to the protein coding COI gene (variable = 259; parsimony informative = 253). Similar results of 16S containing fewer variable sites have been observed in phylogeny of genus *Panulirus* (Ptacek *et al*., 2001), *Jasus* (Ovenden *et al*., 1997) and genus *Palinurus* (Groenfield *et al*., 2007). 12SrRNA gene including indels was having higher number of variable (334) and parsimony informative sites (293) compared to the other two. Similar to this, 344 bp out of 547 was found to be variable in 12S region in grapsoid crabs. Out of the variable 344bp, 255 were parsimony informative (Schubart *et al*., 2006). 18SrRNA sequences are found to have lowest number of parsimony informative sites compared to other mtDNA genes similar to a number of studies in crustaceans (Cristescu and Hebert, 2002; Ahyong and O`Meally, 2004).

5B.4. Intra-specific variation in sequence data

Intra-specific variation was revealed in all the data sets. Low intra-specific divergence values of 1.12% for COI region using the same universal primers of Folmer *et al.* (1994) has been observed in genus *Jasus* (Ovenden *et al*., 1997), 0.9- 3.8% in *Petrarctus* (Yang *et al*., 2008), 0.3% in some genera of Galatheidae (Macpherson and Machordom 2005), 0.1-1.1% in genus *Linuparus* (Tsoi *et al*., 2011), 0.74- 3.42% in horseshoe crabs (Obst *et al*., 2012), 0.285 to 1.375% in malacostracan crustaceans (da Silva *et al*., 2011) and 0.46% on average in crustaceans (Costa *et al*., 2007). Hebert *et al*. (2003a) have suggested intra-specific variation for COI of less than 2%.

Similarly intra-specific variation values for 16S as of present study (0.2-0.5%) using universal primers (Palumbi *et al*., 1996) has been reported

in *Jasus* (0.3%-Ovenden *et al*., 1997); 0- 0.2% on average in *Metanephrops* (Chan *et al*., 2009) and 0.4% in Munida (Machordom and Macpherson,2005).

For the 12SrRNA region too, similar to present study, low intra-specific divergence of 0.1-0.6% was observed in *Linuparus* (Tsoi *et al*., 2011) and in *Pseudodromia* (0.31%- Stewart *et al*., 2004). For 18S gene, intra-specific variation of 0.00-0.12% was observed in horse shoe crabs (Obst *et al*., 2012). Even though polymorphism has been reported in *P. homarus* from Indian coast (Mon *et al*., 2011) our study didn`t observe the same from the fairly large number of individuals sequenced for the gene.

5B.5. Inter-specific and inter-generic sequence divergence

5B.5a. *COI region*

In this study, the degree of sequence divergence for the COI gene between the eight species of family Palinuridae was 15.3-27.6% with an average evolutionary divergence of 17.7%. The inter-specific distance was 16.5-23.3% in Scyllaridae with an average of 10.7%. Similar value of 2.1-16.2% COI divergence with mean sequence divergence of 10.6% has been observed for clawed lobster genus *Metanephrops* (Chan *et al*., 2009) , *Jasus* (4.03-25.61%; Ovenden *et al*., 1997), among five species of *Thenus* (2-11.5%- Burton and Davie, 2007), 16.2–19.4% in *Linuparus* (Tsoi *et al*., 2011), 12.5-22.3% in *Petrarctus* (Yang *et al*., 2008) and 6-32.3% in *Panulirus* (Ptacek *et al*., 2001).

The inter-generic distance ranged from 21.5-26.4% in three genera of Palinuridae and 21.4% in Scyllaridae. It ranged from 21.3% (between *Petractus* and *Thenus*) to 26.9% (*Linuparus* and *Thenus*) among 5 genera of lobsters. Inter-generic distance of 3.5-22.53% has been observed for the same gene with same primers in squat lobsters (Machordom and Macpherson, 2004) and upto 26.83% in African freshwater crab fauna (Daniels *et al*., 2002).

5B.5b. *16SrRNA*

The inter-specific sequence divergence observed for 16SrRNA was 4.6 -26.4% in family Palinuridae and 4.9-18.1% in Scyllaridae. The overall divergence in the ingroup taxa ranged from 4.6-32.2% in the analysis. An inter-specific distance of 0.7-24 % has been observed for the gene in genus *Panulirus* (Ptacek *et al*., 2001; Nayak and Umadevi, 2012), 0.2-13.8% in *Metanephrops* (Chan *et al*., 2009) and 0.68-19.46% in *Jasus* (Ovenden *et al*., 1997).

In the present study, the degree of sequence divergence between of Group I/ II was 11% for 16S gene and 20.2% for COI gene which lies well within the limit reported (2.8–19.4% for 16S and 12.4–31.8% for COI) by Ptacek *et al* (2001). Between species in Group III/IV, the values were lower in the present study for 16S (4.6–7.1%) and COI (15.3–17.3%) similar to those reported by Ptacek *et al*., 2001 (5.3–13.2 % for 16S gene; 12.6–19.6% for COI) for the groups.

Inter-generic distance ranged from 19.8-22% in three genera of Palinuridae and 18 % between Scyllaridae. It ranged from 18% (between *Petractus* and *Thenus*) to 32.1% (*Linuparus* and *Thenus*) among 5 genera of lobsters. Inter-generic distance showed a range of 1.65-27.78% in freshwater crabs of the family Potamonautidae (Daniels *et al*., 2002) and 0.39-14.3% in squat lobsters (Machordom and Macpherson, 2004). The inter-generic value for 16S gene from the present study appears to be higher at one end which is probably because of the analysis of data from two families.

5B.5c. *12SrRNA*

The inter-specific sequence divergence for 12S ranged from 5.8 to 38.6% within Palinuridae and 7.9 to 30% within Scyllaridae. Usually the 12SrRNA region is highly conserved (Ballard *et al*., 1992) and 16SrDNA is more variable than 12S rDNA (Hwang and Kim, 1999). Variation is extremely low at the 3`half of the molecule but it is also observed that the third domain

of 12S is A+T rich and phylogenetically informative (Simon *et al*., 1994). Our sequence data was sufficiently long (592 bp) and could resolve the phylogeny at the species level with high divergence between species. There are reports of sequence divergence for 12SrRNA in the range of 0.65%-43.4% in African freshwater crabs of the family Potamonautidae (Daniels *et al*., 2002) that are similar to the figures reported in the present study. Inter-specific values ranging from 7.9–9.5% has been observed among three species of *Linuparus* (Tsoi *et al*., 2011) and 0.31-17.1% in *Pseudodromia* (Stewart *et al*., 2004).

5B.5d. *18SrRNA*

The divergence rates for nuclear 18S gene were found to be very low in the ranges of 0.3-7.8% within Palinuridae and 0.2-0.7% within Scyllaridae. The divergence for 18S gene has been found consistently moderate among species (5.8-7.2%) within decapod suborder Pleocyemata (Toon *et al*., 2009). Englisch and Koenemann (2001) found 1–1.3% 18S differences within the amphipod genus *Bactrurus* and 2.7% between *Gammarus pulex* and *G. troglophilus*.

In summary, the pairwise sequence divergences were highly variable for all four genes. The mean evolutionary divergences in the ingroup taxa of the present study were 3.9% for 18SrRNA, 30% for 12SrRNA, 20.8% for COI, and 17.3% for 16SrRNA. Higher maximum uncorrected divergences between ingroup taxa in amphipod crustaceans (7.86% for 18S, 33.6% for COI, and 36.9% for 16S) have been observed by Hou *et al*. (2007). Munasinghe *et al*. (2003) detected percentage average divergence at generic level in crayfishes to be 23.82% for COI, 21.92% for 12S and 17.21% for 16S. Less divergence was found for the mitochondrial rDNA genes than for the faster evolving protein coding gene COI in Onychopods (Cristescu and Hebert, 2002). But our analysis revealed a higher divergence for 12SrRNA compared to all other genes.

5B.6. Phylogenetic relationships

5B.6.1. Phylogeny based on mtDNA data sets

Phylogenetic information content of the selected gene regions was analyzed. Phylogeny was reconstructed using individual mitochondrial gene and for the combined mtDNA data set which gave concordant trees except for the slight variation in the positioning of species in group III and IV using 16S gene tree by different methods. This variation for 16S gene tree was observed by Ptacek *et al.* (2001) too in genus *Panulirus*. Four major clades were recognized in our analysis.

The monophyly of Palinuridae and Scyllaridae is apparent from genetic datasets. The concatenated mitochondrial gene data set based on 1790 bp resulted in phylogenetic trees with four major clades within it. Representatives of the group III *(P. polyphagus)* and IV (*P. homarus homarus*, *P. versicolor*, *P. ornatus*) clustered together into one clade; species of group I (*P. longipes longipes)* and II (*P. penicillatus)* into second clade, the deep water genera *Linuparus* and *Puerulus* into third clade and the scyllarid lobsters in another fourth clade within which *Thenus unimaculatus* and *T. indius* clustered together into one group and *P .rugosus* formed another. The overall phylogeny was in concordance with the morphological groupings of the species.

Many studies have shown an increase in resolution when multiple genes especially of nuclear and mitochondrial origin are combined in phylogenetic analyses. Combined (concatenated) analysis of nuclear (nucDNA) and mtDNA data is a common practice in phylogenetic studies and is adopted in decapod crustaceans too (Ahyong and O'Meally, 2004; Porter *et al*., 2005; Shull *et al*., 2005; Robles *et al*., 2007; Bracken *et al*, 2009; Obst *et al*., 2012) including lobsters (Patek and Oakley, 2003; Chan *et al*., 2009; Palero *et al.*, 2009; Yang *et al.*, 2012). But in this study we could not concatenate the data due to difference in tree topologies of mtDNA and nucDNA. Fisher-Reid and Weins (2011) opined that slower evolutionary rates

of nucDNA regions such as 18SrDNA may lead to widespread discordance between trees from mtDNA and nucDNA, hence not appropriate for a combined data tree in many situations.

5B.7. Limitations of 18SrRNA in phylogeny reconstruction

Individual gene tree with 18SrRNA gene failed to resolve the phylogeny jn our study while mtDNA genes gave congruent results. Concordant patterns between mtDNA and nuclear DNA are not always observed (Funk and Omland, 2003; Chan and Levin 2005). It is expected that the mitochondrial genome will show either greater or comparable levels of divergence and structuring compared with markers in the nuclear genome (Zink and Barrowclough, 2008). The discordance in nuclear and mitochondrial genealogies and failure of 18S RNA in resolving phylogeny at generic level has been observed in crabs of family Pinnotherodae (Palacios-Theil *et al*., 2009) and horseshoe crab family Limulidae (Obst *et al*., 2012), the reason for which has been explained in the latter as lack of variation in the marker as well as less percentage of parsimony informative characters. The same gene could not resolve inter-generic relationships well with lobsters of family Palinuridae (Patek and Oakley, 2003) and Scyllaridae (Yang *et al*., 2012). There are reports in other crustaceans like brachyuran crabs (Ahyong *et al*., 2007) and anomurans (Macpherson *et al*., 2005) that 18SrRNA is appropriate for reconstructing deep divergences, and resulting topologies are robust. But from the present study, it may be concluded that the genetic variability in the 18SrRNA gene within these genera of lobsters is not high enough to resolve relationships. The slow rate of evolution of nuclear DNA has often been considered a factor limiting its use in intra-specific studies (Zhang and Hewitt, 2003) and mutations tend to accumulate at a low pace in slow-evolving nuclear genes compared to the haploid mtDNA having small effective population size. It may be assumed that slowly-evolving genes may help to infer deep-level phylogeny (family/order level) because of the presence of a higher proportion of phylogenetically reliable characters (Graham and Olmstead, 2000; Wortley *et al*., 2005).

5B.8. Evolutionary history and biogeography of lobster species from the Indian coast

George and Main (1967) put forward the hypothesis of an evolutionary trend in the Palinuridae from the relatively stable conditions of the deeper waters to the more varied and fluctuating conditions of the shallow waters. Studies based on adult similarity and a larval cladistic analysis (George and Main, 1967; Baisre, 1994; McWilliam, 1995; George, 2005, 2006a, b) too supported the hypothesis of deep water to shallow water evolution. Based on phylogenetic analysis, Ptacek *et al*. (2001) agreed that in the genus *Panulirus*, the first major lineage (clear water inhabitants comprising the species groups I and II) evolved earlier than those species in the second major lineage inhabitants having species groups III and IV (turbid water inhabitants). But the hypothesis has been challenged by a number of researchers (Davie, 1990; Tsang *et al*., 2009; Yang *et al*., 2012). Based on their phylogenetic studies, they proposed that the ancestral form of the family initially inhabited shallower waters and then retreated into the deeper region and not the other way round.

Two species included in the present study *Puerulus* and *Linuparus* are deep-sea residents, considered to be the most primitive, (George and Main, 1967; Baisre, 1994; George, 2006b). *Panulirus* are usually shallow-water inhabitants (Lipcius and Eggleston, 2000). Among the candidates of family Scyllaridae, *Petrarctus rugosus* inhabits a wide range of depths ranging from 20-200 m (Holthuis, 1991) usually at 20-60m (Holthuis, 2002) while *Thenus spp.* are usually at depths of 10-50m (Rahman and Subramoniam 1989; Kizhakudan, 2006b; Radhakrishnan *et al*., 2007; Jones 2007; FAO 2010). The shallow water forms are more derived in our gene trees than the deep sea genera (Figs. 34, 36). Hence our gene tree supports the first hypothesis of a deep water origin and subsequent radiation and diversification of lobster species. In our individual gene trees as well as in the combined gene tree, the group III and IV appears to be the most derived with the shortest branch length. *Thenus indicus* and *T. unimaculatus* belonging to sub family Theninae of family Scyllaridae appear to be more derived

compared to the subfamily Scyllarinae represented by a single species *P. rugosus*. Thus, the gene trees from the present study indicate a recent origin of subfamily Theninae compared to sub family Scyllarinae but the number of species is not sufficient to derive a conclusion.

Even though the origin of two families Palinuridae and Scyllaridae cannot be accurately correlated from the results with the limited number of taxa in the present study, our gene trees indicate the family Palinuridae is older than Scyllaridae (Fig. 34, 35, 36). Based on fossil records an earlier origin of Palinuridae compared to Scyllaridae has been suggested (Baisre, 1994; Weber and Booth, 2007). The tendency toward a shorter larval life which is more pronounced in the scyllarids than in the palinurids, may be because scyllarids evolved more recently in coastal waters (Sekiguchi *et al*., 2007). Cladograms of the present study also agree with George and Main's (1967) classification and their opinion that the groups I and II (*P. longipes longipes* and *P. penicillatus* respectively) diverged earlier than group III species *(P. polyphagus)* which diverged earlier than group IV (*P. homarus, P. ornatus* and *P. versicolor*).

In summary, the overall finding of the present study supports previous hypothesis *viz.* an early origin of Palinuridae than Scyllaridae and groupings of species based on both morphological and molecular characters. Further analysis using more nuclear genes may be necessary to enhance our current knowledge on lobsters of Indian coast. The species-specific molecular signatures generated by various markers in the present study can help further investigations regarding the evolution and biogeography of these valuable and declining decapod resources.

Chapter 6
SUMMARY AND CONCLUSION

Lobsters are low volume yet the most valuable highly priced crustaceans which is estimated to constitute 1852 MT (0.34%) of total marine crustacean landings in India during 2011 (CMFRI, 2012). Although the lobster fauna of commercial fishing grounds of the country comprises 14 species of littoral and six species of deep sea forms, only a few belonging to the families Palinuridae and Scyllaridae are significant in fishery, the most important of which were the Slipper/shovel nosed lobster, *Thenus unimaculatus* Burton and Davie, 2007 and Scalloped spiny lobster, *Panulirus homarus* (Linnaeus, 1758) (CMFRI, 2011).

P. homarus is having three recognized sub-species (Berry, 1974; FAO, 1991).The nominotypical form (*P. homarus homarus*) is found throughout the range of the species. The FAO identification sheets (1991) and Berry(1974) reported occurrence of *P. homarus megasculptus* sub-species in the west coast of India along with other places of distribution like the south coast Arabian Peninsula and Socotrea, which is not confirmed by scientific studies. Earlier studies and reports of shovel nosed lobsters of the genus *Thenus* in India were based on the single species – *Thenus orientalis.* The shovel-nosed lobster genus *Thenus* Leach, 1815, long considered monotypic with *Thenus orientalis* (Lund, 1793), was revised by Burton and Davie (2007). They resurrected *T. indicus* Leach, 1815 from the synonymy of *T. orientalis* and described three new additional species *T. australiensis*, *T. unimaculatus* and *T. parindicus*. In view of the species revision and lack of information on species composition and also at intra-species level of shovel-nosed lobsters, there is a need to carry out in-depth analysis on these lines for accurate documentation of lobster diversity in Indian seas.

The lobster landing of the country is on a decline (Radhakrishnan *et al.*, 2005; CMFRI annual reports, 2002-12). The annual landing of *Thenus* spp. resource has also fallen drastically from about 600 MT to about 130 MT over a span of a decade (1991 - 2001) (Kizhakudan, 2006a) and even collapsed by

1994 in Mumbai due to recruitment overfishing (Deshmukh, 2001). At Veraval, there was a drastic decline in slipper lobster fishery from an average of 97.7 MT (1991-2000) to 6MT in 2004 (Radhakrishnan *et al.*, 2007).The recent trends indicate that there will not be any significant increase in the landing from the presently exploited regions.

The management of exploited species requires the identification of demographically isolated populations that can be considered as independent management units (MUs), failuring in which can lead to over -fishing and depletion of less productive stocks. By characterizing the distribution of genetic variation, population sub structuring can be detected and the degree of connectivity among populations can be estimated. The genetic variation can be observed using identified molecular markers of both nuclear and mitochondrial origin. Hence, the present work was undertaken to study the genetic diversity and population/stock structure in *P. homarus homarus* and *T. unimaculatus* from different landing centres along the Indian coast using nuclear (RAPD) and mitochondrial DNA marker tools which will help towards developing management strategies for management and conservation of these declining resources.

To make consistent conservation and fisheries management decisions, accurate species identifications are needed. It is also suggested that it is not always desirable to rely on a single sequence for taxonomic identification. Thus, the feasibility of using partial sequences of additional mitochondrial genes like 16SrRNA, 12SrRNA and nuclear 18SrRNA has also been explored in our study. Phylogenies provide a sound foundation for establishing taxonomy. The present work also attempts to reconstruct the phylogeny of eleven species of commercially important lobsters from the Indian EEZ using molecular markers.

> ➢ Specimens of *T. unimaculatus* (240 nos.) were collected 60 each from four locations along West coast (Veraval, Kollam) and East coast (Chennai and Visakhapatnam). Similarly, *P. homarus homarus* (180 nos.) were collected 60 each from three locations along West coast

(Kollam) and East coast (Chennai and Visakhapatnam) respectively. Sampling was done in two successive years throughout the range of species distribution.

➤ The samples of eleven commercially important lobster species, eight of which (*P. homarus homarus*, *P. versicolor*, *P. ornatus* , *P. longipes longipes, P. polyphagus, P. penicillatus, Peurulus sewelli* and *Linuparus somniosus)* belong to Palinuridae and three of Scyllaridae (*Thenus unimaculatus, T. indicus* and *Petrarctus rugosus*) were collected from their places of abundance along the Indian coast for barcoding and genetic divergence studies. The species were identified as per FAO (1991) and Burton and Davie, 2007.

➤ Total DNA was extracted following the standard phenol-chloroform method (Sambrook and Russell, 2001) with heat shock modification from all the collected individuals.

➤ 100 Operon random primers were screened and the ones giving most polymorphic, reproducible and clear fingerprints were selected for population studies. RAPD profiles were generated from 180 scalloped spiny lobsters, 60 each from one location using eight Operon random primers and for 240 individuals of slipper lobsters, 60 from each location using nine Operon primers.

➤ Partial sequences of fast evolving region of mitochondrial COI gene were amplified by polymerase chain reaction employing Jerry-Pat primers (Simon *et al*., 1994). Twenty individuals each per sampling site for *P. homarus* and 18 each per location for *T. unimaculatus* were sequenced for the study.

➤ In RAPD technique, genetic variability in the *P. homarus homarus* and *T. unimaculatus* populations were estimated from the percentage of polymorphic loci (P), Nei's genetic diversity (h) and Shannon diversity index.

➢ For *P. homarus homarus* populations, level of polymorphism observed in populations ranged from 29.5-32%, genetic diversity values from 0.12-0.15 with the lowest observed in Visakhapatnam population. Shannon Information Index value ranged between 0.18-0.21. Coefficient of genetic differentiation (G_{ST}) among the *P. homarus homarus* populations was 0.0136.

➢ Higher genetic identity values were obtained between populations (0.95-0.96). The values of Nei's unbiased genetic distance 'GD' between populations had an average value of 0.0513. The dendrogram showed two clusters, the Chennai and Visakhapatnam populations of *P. homarus* formed one cluster while the Kollam population formed another, but with weak bootstrap support, indicating very weak genetic structuring of the species.

➢ For *T. unimaculatus*, the level of polymorphism was highest for Kollam population (30.43%) while lower values (15.22%) was observed in Veraval and Visakhapatnam samples. In the present study, genetic diversity values for 'h' was found to be the highest (0.1375) in Kollam populations and lowest were (~0.073) for Visakhapatnam and Veraval samples. For the over all population, Nei's gene diversity value 'h' was 0.1446. Shannon's Information index ranged from 0.10-0.19 between populations. Coefficient of genetic differentiation (G_{ST}) among the shovel-nosed lobster populations was 0.0442.

➢ Many specific bands were obtained for both lobsters which can be used for development of SCAR markers for accurate species identification.

➢ The values of Nei's unbiased genetic distance 'GD' among populations have an average value of 0.077. The dendrogram showed two clusters, the Veraval and Kollam populations of *T. unimaculatus* formed one cluster while the Chennai and Visakhapatnam populations formed

another cluster but with a low bootstrap support. No significant difference was observed between the genetic distance values of populations from the four sampling sites.

➢ From the hypervariable COI region of 60 *P. homarus homarus* samples, 23 different haplotypes were observed. The nucleotide diversity (π) values at three sampling had an overall estimate of 0.0089 and haplotype diversity (*h*) was 0.9226. The F_{ST} values as well as p-values for F_{ST} and Φ_{ST} values were found to be insignificant at 5% level between populations indicating no population subdivision. The AMOVA analysis indicated only 3.94% variation attributed to differences among poulations. The TCS haplotype network indicated no characteristic geographic distribution pattern for the haplotypes.

➢ From the 681–bp fragment of COI region of 72 *T. unimaculatus* samples, 20 different haplotypes were obtained. Unique haplotypes were observed within all populations at low frequencies. The haplotype *Hap3* was found to be the dominant haplotype shared between all populations .The nucleotide and haplotype diversities among four sampling sites ranged from 0.005–0.008 and 0.758–0.928, respectively. Fixation index over all samples (F_{ST}) was 0.0468, and showed no significant differences at 5% level in pair-wise comparisons. AMOVA analysis showed that 95.32% of the total molecular variance was distributed within samples. The haplotype No.3 was centered but the TCS Haplotype network based on statistical parsimony could not find any geographical clustering of particular haplotypes.

➢ High gene flow (*Nm*) was reported from mtDNA analysis of both species.

➢ Tajima's *D* and Fu's F_S tests were carried out for demographic analysis of both species of lobsters. The Tajima's *D* values and Fu's F_S values

were significantly negative which can be an indicative of population expansion after genetic bottleneck.

➢ The moderate level of polymorphism, gene diversity and Shannon's Index within populations in the spiny and slipper lobster species in our study indicated fluctuation in population size from generation to generations as indicated by the decrease in landings over the years.

➢ Population contraction may be the cause of reduced gene diversity by RAPD and low nucleotide diversity in populations. The low genetic distance between the populations of two species of populations indicates that they act as a single interbreeding population, possibly with high levels of gene flow between them due to absence of physical barriers in the open ocean.

➢ The high haplotype diversity (*h*) and low nucleotide diversity (π) values indicates possibility of genetic bottleneck events, with subsequent population expansion and formation of new haplotypes which are found in low frequencies (Grant and Bowen, 1998).

➢ This study using both markers could not reveal heterogeneity in stock structure both in spiny and slipper lobster populations. Three possible hypotheses may be put forward for the lack of population structuring of the species along the Indian coast. They are 1) The planktonic phyllosoma larval duration which lasts in wild for an assumed period of 5.5-8 months for *P. homarus homarus* and 27-45 days for *T. unimaculatus*; 2) Coastal current pattern of Northern Indian Ocean associated with monsoon currents which coincides with the peak breeding season of species. It can carry planktonic phyllosoma larvae along the coast; 3) Movement behaviour in lobsters.

➢ Despite localized intensive overfishing, general features of the life history and reproductive behaviour of the lobsters such as high fertility

and long duration of its planktonic larvae, may contribute to maintain its genetic diversity. The patchiness in their distribution along the coastline for these species even in presence of a high gene flow may be attributed to the tendency of larvae to settle in preferred habitats.

➤ To generate species-specific molecular signatures, partial sequences of mitochondrial DNA regions such as COI, 16SrRNA, were amplified by polymerase chain reaction employing specific universal primers of Folmer *et al*., 1994 and Palumbi *et al*., 1991 respectively. 12SrRNA was amplified using primer pairs developed for *Tigriopus japonicus* (Machida *et al*., 2002). The annealing temperatures and PCR cycles were standardized per primer for both families of lobsters. Partial sequences of nuclear 18SrRNA were amplified by polymerase chain reaction employing primer pairs developed by Whiting (2002) and Carranza *et al.* (1996).

➤ Species-specific molecular signatures were developed for eleven commercially important species of lobsters using mitochondrial COI, 16SrRNA, 12SrRNA and nuclear 18SrRNA genes. This will help in accurate species identification at various stages such as phyllosoma or puerulii which are otherwise difficult to identify by mere visual examination.

➤ Using the COI barcodes, the species of genus *Thenus* distributed and caught widely along the Indian coast was ascertained to be *Thenus unimaculatus* Burton and Davie, 2007. The presence a less abundant species, *Thenus indicus* along the east coast could also be confirmed with the above gene. It was also identified that the subspecies of *Panulirus homarus* distributed along the coastline is *P. homarus homarus.* No other sub-species could be found in sampling.

➤ Phylogenetic and evolutionary relationships among the species as well as genera were analyzed.

➢ With COI gene, sequence divergence between the eight species of Palinuridae ranged from 15.3-27.6% with an average evolutionary divergence of 17.7%. It was 16.5-23.3% in Scyllaridae with an average value of 10.7%. The mean evolutionary diversity of 20.8% in entire dataset. Intergeneric distance ranged from 21.5-26.4% among three genera of Palinuridae and 21.4 % between two genera of Scyllaridae. For the five genera taken together, the value ranged from 21.3% (between *Petractus* and *Thenus*) to 26.9% (*Linuparus* and *Thenus*).

➢ With 16SrRNA, the interspecific sequence divergence observed ranged from 4.6-26.4% in family Palinuridae and 4.9-18.1% in Scyllaridae. The mean evolutionary divergence over sequence pairs was 0.173 for the entire dataset. Intergeneric distance ranged from 19.8-22% in Palinuridae and 18% in Scyllaridae. It ranged from 18% (between genus *Petrarctus* and *Thenus*) to 32.1% (*Linuparus* and *Thenus*) among five genera of lobsters.

➢ The inter-specific sequence divergence for 12SrRNA ranged from 5.8% to 38.6% within Palinuridae and 7.9 to 30% within Scyllaridae. The mean evolutionary divergence over sequence pairs for the entire dataset was 30%. Intergeneric distance ranged from 26.8-36.6% in three genera of Palinuridae and 28.1% in Scyllaridae. It ranged from 26.8% (between genus *Puerulus* and *Linuparus*) to 46.1% (*Linuparus* and *Thenus*) among the five genera of lobsters.

➢ The interspecific sequence divergence ranged from 0.3%-7.8% within Palinuridae and 0.2%-1% within Scyllaridae..The mean evolutionary divergence over sequence pairs was 3.9%.

➢ The combined mitochondrial data set (COI, 16SrRNA and 12SrRNA) was 1790 bp long. In the ingroup taxa, 746 were parsimony informative of 829 variable characters. The interspecific sequence divergence

ranged from 9-34.7% within Palinuridae and 10.2-25.3% within Scyllaridae. The overall divergence value in the ingroup taxa ranged from 9.0-39.4%. The average evolutionary divergence over sequence pairs was 20.9% within Palinuridae and 8.7% within Scyllaridae .It was 25.7% over all sequence pairs. The Intergeneric distance ranged from 22.4% (*Linuparus* and *Puerulus*) to 28.3% (*Linuparus* and *Panulirus*) in family Palinuridae and 22 %(*Thenus* and *Petrarctus*) in family Scyllaridae. It ranged from 22.4% (*Petractus* and *Thenus*) to 33% (*Linuparus* and *Thenus*) among five genera of lobsters.

➢ The evolutionary history was inferred using the Neighbor-Joining and Maximum-parsimony methods for individual gene data and with combined mtDNA data set. Tree topologies from the NJ and MP analysis of mtDNA genes indicated four major clades. *P. homarus homarus, P. versicolor, P. ornatus* and *P. polyphagus* formed clade I, *P. longipes longipes* and *P. penicillatus* formed the second clade, *Linuparus somniosus* and *Puerulus sewelli* formed the third and *Petrarctus rugosus, Thenus unimaculatus* and *T. indicus* formed the fourth clade. *P. versicolor* and *P. ornatus* were found to be sister taxa in the first clade. *T. unimaculatus* and *T. indicus* formed one sub-clade within the fourth clade. *L. somniosus* and *P. sewelli* were grouped together with the Palinuridae with weak to moderate bootstrap support and formed a basal group to the rest of the Palinurid species. Conspecific individuals from different sampling localities were always clustered together and are represented in the tree by only one individual. The overall phylogeny using mtDNA sequences was in concordance with the morphological grouping of the species. The 18SrRNA couldn't resolve the phylogeny, probably because of the very low evolutionary rate compared to the mtDNA sequences.

➢ The present study supports the previous findings of evolutionary relationships of the genus *Palinurus* by George and Main (1967) and

Ptacek *et al.* (2001) and the hypothesis of earlier origin of Palinuridae compared to Scyllaridae (Webber and Booth, 2007).

Conclusion

Genetic identity of scalloped spiny lobster *P. homarus homarus* and slipper lobster *T. unimaculatus* was established through the present study. Single sub-species *P. homarus homarus* was detected from Indian coast based on the presence of shallow scallops as well as prominent median interruption in the transverse abdominal grooves (Plate II-C) and using molecular tools. Genetic stock structure analysis revealed no significant differentiation among the spiny and slipper lobster populations along Indian coast. The results obtained from this study are of significance in the present context of alarming decrease in landings of lobsters over the years from the recorded maximum of 4075 MT in 1985 (Radhakrishnan *et al.*, 2005) to 1715 MT in 2010 (CMFRI, 2012) which is an indication of the growing instability of the lobster stocks along the Indian coast. Based on the current landing data, and biological information on the mean size of lobsters it could be deduced that the stocks have been overexploited. Proper management and conservation are the only options for a species like *P. homarus homarus* whose hatchery technology has not been perfected to-date. Even though the seed production techniques of *Thenus spp.* has been standardized in India (Kizhakudan *et al.*, 2004a) it has been not been taken up to a commercial level. The management importance of recognizing a population structure as revealed by the present study is that, if there is over harvesting, populations will not be replenished by recruitment from elsewhere in a meaningful time period. The absence of genetic structuring in the lobsters suggests a substantial capacity for locally exploited populations to recover from declines through the dispersal of individuals from other nearby populations. The overall level of genetic differentiation among lobster populations is low does not mean that important inter-population adaptive genetic differences are absent. But the reduced number of stocks reported in some regions advocate for more effective and adequate regulatory measures such as marine protected areas. Because populations of both species of lobsters are found to be panmictic, all management and conservation efforts must be coordinated at the

national level, as over-exploitation in one region will negatively affect the metapopulation and will decrease recruitment across the whole distributional range. The current results help in that direction, hopefully aiding better management of lobster stocks. Given the high value of the resource and the decline of the stocks in several areas, all information that improves management should prove useful. In the case of *P. homarus homarus* and *T. unimaculatus*, further examination of the population structure could be carried out using genetic markers with higher sensitivity for the detection of genetic differentiation like the D-loop region of mitochondrial DNA and nuclear VNTRs like microsatellites.

This is the first comprehensive study using molecular markers on lobsters from Indian coast Further analysis using more nuclear genes may be necessary to enhance our current knowledge on lobsters of Indian coast. The species-specific molecular signatures generated by various markers in the present study can help further investigations regarding the larval identification and their migration as well as evolution and biogeography of these valuable and declining decapod resources.

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Appendix1. Reagents required for genomic DNA isolation by phenol-chloroform method

1. High TE:

Stock 0.5 M Tris-Cl (P^H- 8.0) - 20 ml

Stock 0.5 M Na$_2$ EDTA. 2H$_2$O

(pH - 8.0) - 8 ml.

Makeup the solution to 100 ml with distilled water.

Autoclave it.

Cool it down to room temperature.

Store at 4^0 C.

2. 0.5M Tris Cl (pH -8.0)

Tris base   - 3.028 g

Distilled water   - 40 ml

Adjust pH to 8.0 using HCl.

Make up the volume to 50 ml,

autoclave and store at 4^0 C.

3. 0.5 M EDTA (pH 8.0)

Na$_2$ EDTA. 2H$_2$O - 9.34 g

Distilled water - 40 ml

Adjust the pH to 8.0 using 0.5M NaOH.

Make up final volume to 50ml.

Store at room temperature

4. Lysis buffer (100 ml):

Tris (25 mM)   - 0.372 g.

EDTA (10 mM)   - 0.3028 g

KCl (50mM)   - 0.3727 g

Adjust the pH to 8.0 using 1N HCl

5. Proteinase K (20 mg/ml):

Proteinase K   -20 mg

Autoclaved distilled water - 1000 μl

Dissolve Proteinase K in autoclaved distilled water.Store at -20^0 C.

6. 3M Sodium Acetate

24.6 g in 100ml distilled water

 pH   adjusted to 5.5 with glacial ascetic acid- autoclave

7. TE buffer (pH 8)

Tris Cl (pH-8.0)   - 10 mM

EDTA (pH-8.0)   - 1 mM

Prepared in double distilled   water. Autoclave and store at 4^0 C

8. RNAase Buffer:

0.5M Tris-Cl (pH 7.5) -0.2 ml

NaCl (0.292 g in 10 ml) - 0.3 ml

Distilled water - 9.5 ml

Autoclave it.

Cool it down to room temperature; store at 4^0 C.

9. RNAase:

RNAase - 10 mg

RNAase buffer (autoclaved) - 1 ml

Dissolve RNAase in RNAase buffer.

Keep the tube in boiling water for 15 minutes.

Allow to cool at room temperature. Store at -20^0 C

10. 1X TBE buffer

10X TBE   - 10 ml

Distilled Water - 90 ml

11. TBE buffer 10X (pH - 8.0) for

13. Gel loading buffer

100ml

Tris base	- 10.8 g
Boric acid	- 5.5 g
0.02 M EDTA	- 0.75 g

Make up the solution to 100 ml with double distilled water.

Autoclaved and stored at 4^0 C

12. Bromophenol Blue dye:

Bromophenol blue- 2.5 mg

Sucrose   - 40.0 mg

Dissolve in 1 ml distilled water

Autoclave.Store at 4^0 C.

Bromophenol blue   - 0.5%

Glycerol (mol. grade)   - 30%

Prepared in 1X TBE

Store at 4^0 C.

14. Agarose solution (0.7%)

Agarose	- 0.21 g
10X TBE	- 3 ml
Distilled Water- 27 ml	

15. Ethidium bromide solution

Ethidium bromide - 10 mg
Distilled water   - 2 ml

Protocol followed for neutral phenol preparation

Saturation of Phenol with Tris- Cl (pH **8.0)**
▪ If phenol is transparent, added 0.1 % (20 mg) 8-hydroxy-quinoline (to avoid the oxidation of phenol) to 200 ml of water saturated phenol
▪ Cover the flask containing phenol with aluminium foil to avoid light reaction.
▪ Add 200 ml of 0.5 M Tris-HCl.
▪ Stir the solution using magnetic stirrer for 15 minutes
▪ Keep the solution for 30 minutes to allow the phenol to settle.
▪ Decant the supernatant (Tris).
▪ Add 200 ml of 0.1 M Tris-Cl.
▪ Repeat the above four steps once.
▪ Add 200 ml of 0.1 M Tris-Cl to phenol
▪ Store at 4^0 C

Appendix 2. Multiple alignment of the nucleotide sequences of the *COI* gene of haplotypes of 11 lobster species along Indian coast. Only variable sites are reported. The first base in *COI* (655 bp) corresponds to position 56 in the complete mitochondrial genomes of the lobster *H.americanus* (NC_015607.1) and 53 in the cray fish *Cherax destructor* (NC_011243.1) from GenBank. Identity with the nucleotides of outgroup species is indicated by dots

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                                               1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
                            1 1 1 1 2 2 2 2 3 3 3 3 4 4 4 4 5 5 5 5 6 6 6 6 7 7 7 7 8 8 8 8 8 9 9 9 9 0 0 0 1 1 1 1 2 2 2 3 3 3 4 4 4 5 5 5 6
                            2 3 5 8 1 4 7 8 0 3 6 9 2 5 8 9 4 5 7 8 0 3 6 9 2 3 5 8 1 4 7 0 1 3 6 9 2 5 8 1 4 7 0 1 3 6 9 2 5 8 1 2 4 3 6 9 2 5 8 1
P. homarus homarus (JQ229885)  T A T C A A A G T G A G A T T T A C T A T T A A G C C T A A A A C G T A C C A T T T A G A A A C C T T G G C T A T G C T
P. homarus homarus (JQ229916)  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . . . . . . . . . . . .
P. homarus homarus (JQ229911)  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. homarus homarus (JQ229912)  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. homarus homarus (JQ229926)  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. homarus homarus (JQ229922)  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . .
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[Three dense multiple-sequence alignment blocks follow, listing variable-site nucleotides for the species/haplotypes: P. homarus homarus (JQ229885, JQ229916, JQ229911, JQ229912, JQ229926, JQ229922), P. ornatus (HM446347, GQ223286), P. versicolor (JQ229882), P. polyphagus (AF339469, JN418939), P. longipes longipes (JQ229879), P. penicillatus (JQ229881), L. somniosus (JQ229880), P. sewelli (JQ229890), T. unimaculatus (JQ229937, JQ229896, JQ229927, JQ229931, JQ229899, JQ229938, JQ229897, JQ229898), T. indicus (JQ229891, JQ229892), P. rugosus (JQ229889), H. americanus (NC 015607.1), C. destructor (NC 011243.1).]

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                                          4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5
                                          3 4 4 4 4 4 5 5 5 6 6 6 6 6 7 7 7 7 7 7 8 8 8 8 8 9 9 9 0 0 0 1 1 1 2 2 2 2 2 3 3 3 3 4 4 4 5 5 6 6 6 6
                                          7 0 3 5 6 9 2 5 8 9 0 1 2 3 4 7 0 1 2 3 4 6 9 1 2 5 8 9 1 4 7 3 6 9 2 5 8 9 1 2 4 5 7 8 0 1 3 6 9 2 5 6 8 1 4 7 0 3 6 9
P. homarus homarus (JQ229885)             T A A T A T T G A T C T T C A T A A C A T C C G A G A C A T A T G T T T C A T T A C T C A C T T T C A C A T A T T T A T
P. homarus homarus (JQ229916)             . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . .
P. homarus homarus (JQ229911)             . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. homarus homarus (JQ229912)             . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. homarus homarus (JQ229926)             . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. homarus homarus (JQ229922)             . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. ornatus (HM446347)                     C . . . . C . A . . . C . . . C . . . . . T T . A T . G C . A . . C . . . . . . A . T . A . . . T G C G C . . . .
P. ornatus (GQ223286)                     C . . . . C . A . . . C . . . C . . . . T T . A T . G C . A . . C . . . . . . A . T . A . . . T G C G C . . . .
P. versicolor (JQ229882)                  . . . . . . C A . . . C . . . . . . . A T T . C . A T C . C . . C C G . A . C . A . C A . . . A . . . A . C
P. polyphagus (AF339469)                  . T . . . . C A T . . . . . . A . . . . T . . A T . . C . C . . . T . C . . . . C . A C . A T . . C G . . . .
P. polyphagus (JN418939)                  . T . . . . C A T . . . . . . A . . . . T . . A T . . C . C . . . T . C . . . . C . A C . A T . . C G . . . .
P. longipes longipes (JQ229879)           . C G . C . C . T . . . . T C . . . C C T T . G . . . . . G T C . A . . C . . . . T . . C . G T C . . . . . C G .
P. penicillatus (JQ229881)                C C T . T C C A . . . C . . C . . . . G C A . . T A . T . C . C C C C A . . . C T . G . C A A T C . . A . . . C G .
L. somniosus (JQ229880)                   A . . . . C . A C C . A G . C A . . . . T . . C C . C . T G T C . . T C . C C . . C . C C A A T . . A . C C C . A
P. sewelli (JQ229890)                     A C T . . C C A C C . . G . T . G . . T A A . . A T . T C C C C . C T . . C . . A . T T A C A A T . . A . . . A . C
T. unimaculatus (JQ229937)                A . C . T . . . . . . A G . A . . G . A G T . C A T . T C . . C . . A T G C C T . . . T . G . G A . T . C G . . . .
T. unimaculatus (JQ229896)                A . C . T . . . . . . A G . A . . G . A G T . C A T . T C . . C . . A T G C C T . . . T . G . A A . T . C . . . .
T. unimaculatus (JQ229927)                A . C . T . . . . . . A A . . A . . G . A G T . C A T . T C . . C . . A T G C C T . . . T . G . A A . T . C G . . . .
T. unimaculatus (JQ229931)                A . C . T . . . . . . A G . A . . G . A G T . C A T . T C . . C . . A T G C C T . . . T . G . A A . T . C G . . . .
T. unimaculatus (JQ229899)                A . C . T . . . . . . A G . A . . G . A G T . C A T . T C . . C . . A T G C C T . . . T . G . A A . T . C G . . . .
T. unimaculatus (JQ229938)                A . C . T . . . . . . A G . A . . G . A G T . C A T . T C . . C . . A T G C C T . . . T . G . A A . T . C G . . . .
T. unimaculatus (JQ229897)                A . C . T . . . . . . A G . A . . G . A G T . C A T . T C . . C . . A T G C C T . . . T . G . A A . T . C G . . . .
T. unimaculatus (JQ229898)                A . C . T . . . . . . A G . A . . G . A G T . C A T . T C . . C . . A T G C C T . . . T . G . A A . T . C G . . . .
T. indicus (JQ229891)                     A . C . T C . . . . A . . A . . G . A A T . T . T C . C . . C . G C C C T A . T . A . A A T T . A . A . C . .
T. indicus (JQ229892)                     A . C . T C . . . . A . . A . . G . A A T . T . T C . C . . C . G C C C T A . T . A . A A T T . A . A . C . .
P. rugosus (JQ229889)                     A . . . C C . A . . A A . . A . T . T A A . A C A T . T . T . . A A . C C T T A . T T A . C . T T . . C . . C G .
H. americanus (NC 015607.1)               G . T C C . . A . A G A A A . . . . A A . . . A C T . . G A A . . . A A G . C T T A . . . C G . T T T . . . . C
C. destructor (NC 011243.1)               A T G C . C . A . A . A A G . . G . . C A A . . G . T . T . C A A C . A . G G C . . C . . T A C C G T . . C . . . . . A
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                                          5 5 5 5 5 5 5 5 5 5 5 5 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6
                                          7 7 7 7 8 8 8 8 9 9 9 9 0 0 0 1 1 1 2 2 2 2 3 3 3 4 4 4 4 5 5
                                          0 2 5 8 1 4 5 7 0 3 4 6 9 2 5 8 0 1 4 7 0 3 6 7 9 2 5 8 1 2 4 7 0 3
P. homarus homarus (JQ229885)             C T T T T T G C A T A C T C A T A A G A T A A T C T A T C A T A T
P. homarus homarus (JQ229916)             . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. homarus homarus (JQ229911)             . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. homarus homarus (JQ229912)             . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. homarus homarus (JQ229926)             . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. homarus homarus (JQ229922)             . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . .
P. ornatus (HM446347)                     . . C . . . . A . . . G . C T T . G G A T C . . . T . G . T . C G C
P. ornatus (GQ223286)                     . . C . . . A . . . G . C T T . G G A T C . . . T . G . T . C G C
P. versicolor (JQ229882)                  . . A . . C C . T . . . . T . . C A . C C . . A . G . T . . . C
P. polyphagus (AF339469)                  . . C . . . C A . . . C . T . . T A . . T . C T . G C . . . . ?
P. polyphagus (JN418939)                  . . C . . . C A . . . . C . T . . T A . . T . C T . G C . . . . ?
P. longipes longipes (JQ229879)           . . . . . A . . A T C . C T . . . . . . . T . . . . . . C . . . .
P. penicillatus (JQ229881)                . C C C . C . A . T . T . . T . . . . . . . . G C . . . T C . C T . C .
L. somniosus (JQ229880)                   . C C C A . . A T C . . T . T C C . C A . . C C G . . . . C T . C .
P. sewelli (JQ229890)                     . A . C A . C . . C . T . T T C T G . . . . G C . . . . T . . . .
T. unimaculatus (JQ229937)                T A A . A . C T T T . T T . . T C T . . G . T . C T C G . . C C . .
T. unimaculatus (JQ229896)                T A A . A . C T T T . T T . . T C T . A G . T . C T C G . . C C . .
T. unimaculatus (JQ229927)                T A A . A . C T T T . T T . . T C T . . G . T . C T C G . . C C . .
T. unimaculatus (JQ229931)                T A A . A . C T T T . T T . . T C T . . G . T . C T C G . . C C . .
T. unimaculatus (JQ229899)                T A A . A . C T T T . T T . . T C T . A G . T . C T C G . . C C . .
T. unimaculatus (JQ229938)                T A A . A . C T T T . T T . . T C T . A G . T . C T C G . . C C . .
T. unimaculatus (JQ229897)                T A A . A . C T T T . T T . . T C T . A G . T . C T C G . . C C . .
T. unimaculatus (JQ229898)                T A A . A . C T T T . T T . . T C T . A G . T . C T C G . . C C . .
T. indicus (JQ229891)                     . G A C G . C T . C . C T . . . C T T . . T . . . . . . . . . C . .
T. indicus (JQ229892)                     . G A C G . C T . C . C T . . . C T T . . T . . . . . . . . . C . .
P. rugosus (JQ229889)                     T A A . A . . A T C . C T . . . C . . G C C . C T C . . . T . .
H. americanus (NC 015607.1)               T A A . A . . . A T T . . . C T . C T . T . C . G . T C . . T . C G .
C. destructor (NC 011243.1)               . . . A . A . . A T . A . . . . G C . T A G . T G C A . . C T G C . C
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Appendix 3. Multiple alignment of the nucleotide sequences of the *16SrRNA* gene of haplotypes of 11 lobster species along Indian coast. Only variable sites are reported. The first position in reverse complements of *16SrRNA* (541bp) corresponds to position 11397 in the complete mitochondrial genomes of the lobster *H. americanus* (NC_015607.1) and 7524 in the cray fish *Cherax destructor* (NC_011243.1) from GenBank. Identity with the nucleotides of outgroup species is indicated by dots.

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                                        4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5
                                        1 1 1 1 1 1 3 4 4 4 4 5 5 6 6 6 7 7 7 7 7 9 9 0 0 0 0 0 0 0 0 1 1 1 1 1 2 2 2 2 2 2 2 3
                                        1 3 4 5 6 9 8 1 4 6 7 8 7 9 6 8 9 0 1 2 4 7 8 9 0 1 2 4 6 7 8 9 0 1 2 4 7 8 0 2 3 4 5 6 7 9 0
P. homarus homarus (JQ229868)           C G A A T G C A C T T T C A A A A T G T G A G A T C T T G G T G G C T G G C A C T A G A G A
P. homarus homarus (JQ229862)           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. homarus homarus (JQ229866)           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. homarus homarus (JQ229867)           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. homarus homarus (JQ229869)           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. homarus homarus (JQ229870)           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. versicolor (JQ229877)                . . . T C . . . . . . . . . . . . . . . . . . . . . . . . A A . . . . . . . . . . . . T ? G ? G . .
P. ornatus (JQ229863)                   . . . C . . . . . . . . . . . . . . . C . . . . . . . A . . . . . . . . . . . T . G A . . .
P. ornatus (JQ229864)                   . . . C . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . T . G A . . .
P. polyphagus (JQ229873)                . . . C . . . . . . . . . T . . . . . . . . . . . A A . . . . . . . . . . . . T . . A . .
P. longipes longipes (JQ229872)         . . . T C . . . . . C . T . . G G . G . G . . . . A . . . T . . . . T C C A . . . . . . . . . .
P. penicillatus (JQ229874)              . . . T C . . . . . C . T . . G G . G . G . . . . A . . . T . . . T C C A . . . . . A . . .
L. somniosus (JQ229865)                 . . . . . C . . G . . . C T T . G . G A A G . G . T . . . . C A C . . G G . C T G T G . . . . .
P. sewelli (JQ229876)                   T A . . . . . . . . . . C T . . G . G A . A . . . . . . . . C T . . . . G C C C T . . A G . . . .
T. unimaculatus (JQ229902)              T . . . C . . . T C . . . . . . . . G A A C . . . . . . . A A . A A . C A A T . T A . A . . .
T. unimaculatus (JQ229905)              T . . . C . . . T C . . . . . . . . G A A C . . . . . . . A A . A A . C A A T . T A . A . . .
T. unimaculatus (JQ229901)              T . . . C . . . T C . . . . . . . . G A A C . . . . C . . A A . A A . C A A T . T A . A . . .
T. unimaculatus (JQ229908)              T . . . C . T . T C . . . . . . . . G A A C . . . . C . . A A . A A . C A A T . T A . A . . .
T. unimaculatus (JQ229909)              T . . . C . . . T C . . . . . . . . G A A C . . . . . . . A A . A A . C A A T . T A . A . . .
T. unimaculatus (JQ229906)              T . . . C . . . T C . . . . . . . . G A A C . . . . C . . A A . A A . C A A T . T A . A . . .
T. unimaculatus (JQ229907)              T . . . C . . . T C . . . . . . . . G A A C . . . . C . . A A . A A . C A A T . T A . A . . .
T. indicus (JQ229878)                   T . . . C . . . T C . . . . . . . . G A A C . . . . . . . A A . A . . C A A T . T A . A . . .
P. rugosus (JQ229875)                   T . . T G . . . T . . . . . G . . . A A . . . . . . . . . T . . . . T C A A T . T A . A . . .
H. americanus (NC 015607.1)             A A T T . . . T T A . . T . C . . . A A . . . A . . T G C C A . . . . . . A T G T G G A G T .
C. destructor (NC 011243.1)             T . . G . A . T . . . T T T . . . G A . . . A T . T . . T . A A T C C A T . . A . . . A G
```

Appendix 4. Multiple alignment of the nucleotide sequences of the *12SrRNA* gene of haplotypes of 11 lobster species along Indian coast. Only variable sites are reported. The first position of *12SrRNA* (592bp) corresponds to position 12741 in the complete mitochondrial genomes of the lobster *H. americanus* (NC_015607.1) and 14910 in the cray fish *Cherax destructor* (NC_011243.1) from GenBank. Identity with the nucleotides of outgroup species is indicated by dots.

Block 1 — position markers (reading vertically): 1 2 2 2 2 2 3 3 3 3 3 3 4 4 4 4 4 4 4 5 5 5 5 5 5 6 7 7 7 7 7 8 8 8 8 8 8 9 9 9 9 9 9 9 1 0 0 0 0 0 0 0 0 1 1 (tens); 2 4 0 0 2 4 8 9 1 2 3 5 6 9 0 1 2 3 4 6 8 9 4 5 6 7 8 9 6 0 1 6 7 9 1 2 3 6 7 8 9 0 1 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 0 1 (units)

```
P. homarus homarus (JQ229843)   CAACCTAGTAAACCTTTACTATGTTAAAAGAATTCATTATTTACCTAAATATAATCCGTT
P. homarus homarus (JQ229845)   .........................T..................................
P. homarus homarus (JQ229841)   .........................T..................................
P. homarus homarus (JQ229842)   .........................T..................................
P. homarus homarus (JQ229846)   .........................T..................................
P. homarus homarus (JQ229847)   .........................T...............C..................
P. ornatus (JQ229850)           .........................T.................C.C.....G........
P. ornatus (JQ229851)           .........................T.................C.C.....G........
P. versicolor (JQ229858)        .........................T...............GC.......C...C.....
P. versicolor (JQ229859)        .........................T...............GC.......C...C.T...
P. polyphagus (JQ229852)        .........................T.................CCC.T...G........C.
P. polyphagus (JQ229853)        .........................T.................CCC.T...G........CC
P. longipes longipes (JQ229849) ..............T.T........GCT.......A...C.....C.G.T.C.G......A.C.
P. penicillatus (JQ229854)      ...........C.TT...G....CACC........A.....A..CA.T..TG.....T....C.
L. somniosus (JQ229840)         ....TC....CC....C.GG.C.C.CC........ACA.TT...C.G...A.TCGC
P. sewelli (JQ229857)           ....T.......TTCCA.GA.C...........G..A..AGAGT..CTTCT.T....CG
T. unimaculatus (KC951871)      ....T....CTCCTTA.A..GA...C.C..T.TA.TC...C.TA.ATT..ACTTATAGC....AA
T. indicus (JQ229861)           ....T....CTGCT.A.A.GA...C...T.TA.CC...C.C..ATT.ACT.ATAGC....AA
T. indicus (JQ229860)           ....T....CTGCT.A.A.GA...C...T.TA.CC...C.C..ATT.ACT.ATAGC....AA
P. rugosus (JQ229856)           ..GTT.......CT.AAATAA..TCA.TGTA..C...A.AACT.A.TT.ACGT....AA
P. rugosus (JQ229855)           ..GGTT......CT.AAATAA..TCA.TGTA..C...A.AACT.A.TT.ACGT....AA
H. americanus (NC 015607)       T.GTA....T...AAAAGG...C..TTT.AC.C..T.A.AA..T...TT.TC.....
C. destructor (NC 011243)       ..GTT.TT.C...TG...A...T.C.C..A.TC....GCAA.CT.AGTCAC.....A....
```

Block 2

```
P. homarus homarus (JQ229843)   ACTTAACTCTATATTCTTGATTACTTGGTTCACCTTACTTATAATCAAACGTATTAACAA
P. homarus homarus (JQ229845)   ............................................................
P. homarus homarus (JQ229841)   ............................................................
P. homarus homarus (JQ229842)   ..........................................................G.
P. homarus homarus (JQ229846)   ............................................................
P. homarus homarus (JQ229847)   ............................................................
P. ornatus (JQ229850)           ...C.........G.C........T..C....GG..A..T..........A.......G...
P. ornatus (JQ229851)           ...C.........G.C.........C...GG..A..T..........A.......G...
P. versicolor (JQ229858)        .........C..GA.........A.CCG.TA..G..........A............
P. versicolor (JQ229859)        ........C..GA.........A.CCA.TA..G..........A............
P. polyphagus (JQ229852)        .TC....T....G.C.C......A.CT..A..G........C.....C........
P. polyphagus (JQ229853)        .TC....T....G.C.C......A.CT..A..G........C.....C........
P. longipes longipes (JQ229849) TT.....T..A........A...A......A..TC.A..T...T.T..A....AC...GG...
P. penicillatus (JQ229854)      T.CA..T.C.G.C.C..CA...A.......AAC.A..C....T.AG....AC...GG.G.
L. somniosus (JQ229840)         GG..CTT..C....AA......CA.AAATT..CT.C..TC.T..G........
P. sewelli (JQ229857)           GG.ACT...C..GC..AA.......AAA.TTTA..CTC.T.TCGAT.G........
T. unimaculatus (KC951871)      .A....T..A....C.AGA.GA.T..A..ATCTACCC...C..TCA.....A.TA...TTC
T. indicus (JQ229861)           .A....T..G...CC.AGA.GA.T..A..AGCTACCC...T..TCA.C..A.T...TTG
T. indicus (JQ229860)           .A....T..G...CC.AGA.GA.T..A..AGCTACCC...T..TCA.C..A.T...TTG
P. rugosus (JQ229856)           .A...GT....GC...A..GA.T..AAACA.TA.....C.GCAA.T...CT...G.CC
P. rugosus (JQ229855)           .A...GT....GC...A..GA.T..AAACA.TA.....C.GCAA.T...CT...G.CC
H. americanus (NC 015607)       TAA...TT.TC...CTCA.T.A.T..AA..TTAA.CC....CTT.TC..TA.C.A.....
C. destructor (NC 011243)       TAA..TT..CTCGCCTAA.T.A.TC..AC.T.AA.....CTCC.AT...A.C.A....
```

Block 3

```
P. homarus homarus (JQ229843)   GAATAATTATGTTGTAAGCATAGGGTTAAAATTAATATACTACAACTATGCTATTAGAGA
P. homarus homarus (JQ229845)   ............................................................
P. homarus homarus (JQ229841)   ............................................................
P. homarus homarus (JQ229842)   ............................................................
P. homarus homarus (JQ229846)   ............................................................
P. homarus homarus (JQ229847)   ............................................................
P. ornatus (JQ229850)           ...C...C....................................................G
P. ornatus (JQ229851)           ...C...C....................................................G
P. versicolor (JQ229858)        ...C...........G..............A...........T..............
P. versicolor (JQ229859)        ...C...........G..............A...........T..............
P. polyphagus (JQ229852)        ...C........................................TT..G........
P. polyphagus (JQ229853)        ...C........................................TT..G........
P. longipes longipes (JQ229849) ...C...C.........T.T.......C...CA..AG..TA..CG..TAAT.T....T..
P. penicillatus (JQ229854)      ..G.G.C.........C.T......T..CA......TAC.C..G.AT.TC...T..
L. somniosus (JQ229840)         ...............AAA..TGGCCCGC.CCT.C.TCT.CAA.CCCCG.T..
P. sewelli (JQ229857)           ..G.......A......T...AAA..TGGCA..A..TT.C.TCT......G.T.G
T. unimaculatus (KC951871)      ..GC..A....AGA...TG.GAAAC..C..C....TT.T..TT.T.CACT..G.TA.
T. indicus (JQ229861)           ..GC..A....AGA...TGCGAAAC..C..C....TT.T..TT.T.CGCTC...TA.
T. indicus (JQ229860)           ..GC..A....AGA...TGCGAAAC..C..C....TT.T..TT.T.CGCTC...TA.
P. rugosus (JQ229856)           A.GC..A..C.A.A....T..AAACC.C..C....T.CA.CT..CAACC.G.TA.
P. rugosus (JQ229855)           A.GC..A..C.A.A....T..AAACC.C..C....T.CA.CT..CAACC.G.TA.
H. americanus (NC 015607)       AG....T.CG.T.....GAT.A.AAA..T..GAAT.A.C.T..T.TTAT.A..CCAGAT..
C. destructor (NC 011243)       A.GC.T.CT.TAG.C.G.T.A.AAA.....GC.CGA.C....T.CA.TAT..C.A..TAG
```

Block 1 (positions 4068–4456)

Taxon	Sequence
P. homarus homarus (JQ229843)	CGACGGCCTACTAAATTTCTAGCTAATTTAAGTCAATAAAACTTCATTCATTAATAAACA
P. homarus homarus (JQ229845)	....
P. homarus homarus (JQ229841)	....
P. homarus homarus (JQ229842)	....G.................
P. homarus homarus (JQ229846)	....
P. homarus homarus (JQ229847)	....
P. ornatus (JQ229850)	......T..................................TG...T..T.......C.......C.....
P.ornatus (JQ229851)	......T..................................TG...T..T.......C.......C.....
P. versicolor (JQ229858)	...................C..CA......C....CG..G...C.GC..........C........
P. versicolor (JQ229859)	......T............C..CA......C....CG..G...C.GC..........C........
P. polyphagus (JQ229852)	............C........C.G...C......GC..G..G.................G....
P. polyphagus (JQ229853)	............C........C.G...C......GC..G..G.................G....
P. longipes longipes (JQ229849)	.......T..A.......AC.TCT...CG..CC..T.....TCTCTTT.ATGC.T.G.......T...
P. penicillatus (JQ229854)	........A..T..G.AC...AC....C....CC.TG..G..CT..CC..GTAGA..C.T..A......C...G
L. somniosus (JQ229840)	T....A..T.A...CG.G..AC...TA.C...AAA...A.T.TC..C....C..AGAA.TG....CCT..T.
P. sewelli (JQ229857)	T..G..A...A...C......CCC.CTA..C......AG..A..A.CCCT...TA..TCC....G...CCC...T.
T. unimaculatus (KC951871)	........TTAC.C....A..AACA...T.ACC.CAATT.TTTCT..AG.A.....A.C...CT..T.
T.indicus (JQ229861)	........TTAC.C..G.G..AACA.C.T.ACCGCCACTCTTCCT..AA..A.TGACC...CT..T.
T. indicus (JQ229860)	........TTAC.C.G..G..AACA..C.T.ACCGCCACTCTTCCT..AA..A.TGACC...CT..T.
P. rugosus (JQ229856)	........TAG.C..G..CC..AGCA.....T..AC...AA....A..C.......A..ACT.AC.TCG...
P. rugosus (JQ229855)	........TAG.C..G..CC..AGCA.....T..AC...AA....A..C.......A..ACT.AC.TCG....
H. americanus (NC 015607)	T....A..T.A...CTGGCACTACAT...TC...T..T..T.TACT.GT...A....T.A....C...T.T
C. destructor (NC 011243)	.A.T.TTTA...T........TC.A..CC....A..TT..A..CCCC...TC.A....T........C

Block 2 (positions 4978–5378)

Taxon	Sequence
P. homarus homarus (JQ229843)	AATCTTATTATTATATTGAAAATTTTTTAACTATCTACCTACTTTTACCCACAGGACATC
P. homarus homarus (JQ229845)	....C...........T..........................
P. homarus homarus (JQ229841)	....
P. homarus homarus (JQ229842)	....C...........T..........................
P. homarus homarus (JQ229846)	....C...........T..........................
P. homarus homarus (JQ229847)	....C....................................
P. ornatus (JQ229850)	...............G...........................T..................G.................
P.ornatus (JQ229851)	....T..................G.................
P. versicolor (JQ229858)	GG...C....G.............GC.C.A.......T.............C.....T.....A.ATC.
P. versicolor (JQ229859)	GG...C....G.............GC.C.A.......T.............C.C.....T.....A.ATC.
P. polyphagus (JQ229852)	.................C.....GCACCCC.G..C.CT.............T..C.........
P. polyphagus (JQ229853)	.................C.....GCACCCCG...C.CT.............T..C.........
P. longipes longipes (JQ229849)	.....CCC.......AC..A.G..A.AAGA.CT.........ATA..ATT.........C.T..
P. penicillatus (JQ229854)	.....C.C......AC..A.GT.ACGGAA..C..G.......GCA.AA..TTT.G....C.T..
L. somniosus (JQ229840)	.....C.T...GC..A..A.AGC..C....CA.TT..C.....TACA..A..TTA..T.AC..T.T
P. sewelli (JQ229857)	.....C.T..GG...A..G....GG..ACA.T...T...TACA..C..TTT...C..C.CCT
T. unimaculatus (KC951871)	T...TCCT....AGGATA.A..T.GAAAAAATTA...AT...TGTA.A....TT...T...C.CCT
T.indicus (JQ229861)	C...T.CT....AC.GCACA.T..AAAAAATTA...AT...TGTA.A.....TT...T..C.CCT
T. indicus (JQ229860)	C...T.CT....AC.GCACA.T..AAAAAATTA...AT...TGTA.A.....TT...T..C.CCT
P. rugosus (JQ229856)	.....CC...C..A.CCGC..AGT.G..AAA..ATTT...A..A..TATA.AA...AT..TCA.C..C.T
P. rugosus (JQ229855)	.....CC...C..A.CCGC..AGT.G..AAA..ATTT...A..A..TATA.AA...AT..TCA.C..C.T
H. americanus (NC 015607)	....TC.T.......A..A......C..AACA..T..AT...TGTA.AA.....TT..TAAT.T..A
C. destructor (NC 011243)	..CTC.GCC....A..CA........AACA...A.CTATA..C.CGAA.T.T.T..ACTT.T

Block 3 (positions 5890–5913)

Taxon	Sequence
P. homarus homarus (JQ229843)	GAGT
P. homarus homarus (JQ229845)	....
P. homarus homarus (JQ229841)	....
P. homarus homarus (JQ229842)	....
P. homarus homarus (JQ229846)	....
P. homarus homarus (JQ229847)	....
P. ornatus (JQ229850)	....
P.ornatus (JQ229851)	....
P. versicolor (JQ229858)	...C
P. versicolor (JQ229859)	...C
P. polyphagus (JQ229852)	...C
P. polyphagus (JQ229853)	...C
P. longipes longipes (JQ229849)	A..C
P. penicillatus (JQ229854)	A..C
L. somniosus (JQ229840)	TG.A
P. sewelli (JQ229857)	A..G
T. unimaculatus (KC951871)	TTAA
T.indicus (JQ229861)	TTAA
T. indicus (JQ229860)	TTAA
P. rugosus (JQ229856)	T..A
P. rugosus (JQ229855)	T..A
H. americanus (NC 015607)	A..G
C. destructor (NC 011243)	A..C

Appendix 5. Multiple alignment of the nucleotide sequences of the *18SrDNA* gene of haplotypes of 11 lobster species along Indian coast. Only variable sites are reported. The first position of *18SrDNA* (1723bp) corresponds to position *Appendix 5.* 12741 in the complete mitochondrial genomes of the lobster *H. americanus* (AF235971) and 14910 in the cray fish *Cherax quadricarinatus* (AF235966) from GenBank. Identity with the nucleotides of outgroup species is indicated by dots.

The following species (haplotypes) are aligned:

- *P. homarus homarus* (JQ229940)
- *P. ornatus* (JQ229942)
- *P. versicolor* (JQ229948)
- *P. polyphagus* (JQ229943)
- *P. longipes longipes* (JQ229941)
- *P. penicillatus* (JQ229944)
- *L. somniosus* (JQ229939)
- *P. sewelli* (JQ229947)
- *T. unimaculatus* (JQ229950)
- *T. indicus* (JQ229949)
- *P. rugosus* (JQ229945)
- *H. americanus* (AF235971)
- *C. quadricarinatus* (AF235966)

Appendix 6. **List of allotment of NCBI Accession Numbers from the present study.**

Sl. No.	Genbank Accession Number	Definition
1	JQ229883	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-CH1 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
2	JQ229884	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-CH2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
3	JQ229885	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-KL1 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
4	JQ229886	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-KL2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
5	JQ229887	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-Vzg1 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
6	JQ229888	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-Vzg2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
7	JQ229910	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-CH3 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
8	JQ229911	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-CH4 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
9	JQ229912	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-CH5 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
10	JQ229913	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-CH6 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
11	JQ229914	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-CH7 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
12	JQ229915	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-CH8 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
13	JQ229916	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-KL3 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
14	JQ229917	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-KL4 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
15	JQ229918	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-KL5 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
16	JQ229919	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-KL6

		cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
17	JQ229920	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-KL7 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
18	JQ229921	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-KL8 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
19	JQ229922	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-Vzg3 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
20	JQ229923	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-Vzg4 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
21	JQ229924	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-Vzg5 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
22	JQ229925	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-Vzg6 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
23	JQ229926	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-Vzg7 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
24	JQ229862	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-KL5 16S ribosomal RNA gene, partial sequence; mitochondrial.
25	JQ229866	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-CH3 16S ribosomal RNA gene, partial sequence; mitochondrial.
26	JQ229867	*Panulirus homarus homarus* voucher NBFGR-CHN-PH-Ch4 16S ribosomal RNA gene, partial sequence; mitochondrial.
27	JQ229868	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-KL4 16S ribosomal RNA gene, partial sequence; mitochondrial.
28	JQ229869	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-Vzg4 16S ribosomal RNA gene, partial sequence; mitochondrial.
29	JQ229870	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-Vzg5 16S ribosomal RNA gene, partial sequence; mitochondrial.
30	JQ229871	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-Vzg6 16S ribosomal RNA gene, partial sequence; mitochondrial.
31	JQ229841	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-CH1 12S ribosomal RNA gene, partial sequence; mitochondrial.
32	JQ229842	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-CH2 12S ribosomal RNA gene, partial sequence; mitochondrial.
33	JQ229843	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-KL1 12S ribosomal RNA gene, partial sequence; mitochondrial.
34	JQ229844	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-KL2 12S ribosomal RNA gene, partial sequence; mitochondrial.
35	JQ229845	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-KL3 12S ribosomal RNA gene, partial sequence; mitochondrial.
36	JQ229846	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-Vzg1 12S ribosomal RNA gene, partial sequence; mitochondrial.
37	JQ229847	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-Vzg2 12S ribosomal RNA gene, partial sequence; mitochondrial.
38	JQ229848	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-Vzg3 12S ribosomal RNA gene, partial sequence; mitochondrial.
39	JQ229940	*Panulirus homarus homarus* voucher NBFGR-CHN-PHH-Vzg7

		18S ribosomal RNA gene, partial sequence.
40	JQ229863	*Panulirus ornatus* voucher NBFGR-CHN-PO3 16S ribosomal RNA gene, partial sequence; mitochondrial.
41	JQ229864	*Panulirus ornatus* voucher NBFGR-CHN-PO4 16S ribosomal RNA gene, partial sequence; mitochondrial.
42	JQ229850	*Panulirus ornatus* voucher NBFGR-CHN-PO1 12S ribosomal RNA gene, partial sequence; mitochondrial.
43	JQ229851	*Panulirus ornatus* voucher NBFGR-CHN-PO2 12S ribosomal RNA gene, partial sequence; mitochondrial.
44	JQ229942	*Panulirus ornatus* voucher NBFGR-CHN-PO3 18S ribosomal RNA gene, partial sequence.
45	JQ229882	*Panulirus versicolor* voucher NBFGR-CHN-PV3 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
46	JQ229877	*Panulirus versicolor* voucher NBFGR-CHN-PV3 16S ribosomal RNA gene, partial sequence; mitochondrial.
47	JQ229858	*Panulirus versicolor* voucher NBFGR-CHN-PV1 12S ribosomal RNA gene, partial sequence; mitochondrial.
48	JQ229859	*Panulirus versicolor* voucher NBFGR-CHN-PV2 12S ribosomal RNA gene, partial sequence; mitochondrial.
49	JQ229948	*Panulirus versicolor* voucher NBFGR-CHN-PV1 18S ribosomal RNA gene, partial sequence.
50	JQ229873	*Panulirus polyphagus* voucher NBFGR-CHN-PPhgs3 16S ribosomal RNA gene, partial sequence; mitochondrial.
51	JQ229852	*Panulirus polyphagus* voucher NBFGR-CHN-PPhgs1 12S ribosomal RNA gene, partial sequence; mitochondrial.
52	JQ229853	*Panulirus polyphagus* voucher NBFGR-CHN-PPhgs2 12S ribosomal RNA gene, partial sequence; mitochondrial.
53	JQ229943	*Panulirus polyphagus* voucher NBFGR-CHN-PPhgs1 18S ribosomal RNA gene, partial sequence.
54	JQ229881	*Panulirus penicillatus* voucher NBFGR-CHN-PPncl2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
55	JQ229874	*Panulirus penicillatus* voucher NBFGR-CHN-PPncl2 16S ribosomal RNA gene, partial sequence; mitochondrial.
56	JQ229854	*Panulirus penicillatus* voucher NBFGR-CHN-PPncl 12S ribosomal RNA gene, partial sequence; mitochondrial.
57	JQ229944	*Panulirus penicillatus* voucher NBFGR-CHN-PPncl 18S ribosomal RNA gene, partial sequence.
58	JQ229879	*Panulirus longipes longipes* voucher NBFGR-CHN-PLL2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
59	JQ229872	*Panulirus longipes longipes* voucher NBFGR-CHN-PLL2 16S ribosomal RNA gene, partial sequence; mitochondrial.
60	JQ229849	*Panulirus longipes longipes* voucher NBFGR-CHN-PLL2 12S ribosomal RNA gene, partial sequence; mitochondrial.
61	JQ229941	*Panulirus longipes longipes* voucher NBFGR-CHN-PLL3 18S ribosomal RNA gene, partial sequence.
62	JQ229880	*Linuparus somniosus* voucher NBFGR-CHN-LS2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
63	JQ229865	*Linuparus somniosus* voucher NBFGR-CHN-LS2 16S ribosomal RNA gene, partial sequence; mitochondrial.
64	JQ229840	*Linuparus somniosus* voucher NBFGR-CHN-LS2 12S ribosomal RNA gene, partial sequence; mitochondrial.

65	JQ229939	*Linuparus somniosus* voucher NBFGR-CHN-LS2 18S ribosomal RNA gene, partial sequence.
66	JQ229890	*Puerulus sewelli* voucher NBFGR-CHN-PSwli2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
67	JQ229876	*Puerulus sewelli* voucher NBFGR-CHN-PSwli2 16S ribosomal RNA gene, partial sequence; mitochondrial.
68	JQ229857	*Puerulus sewelli* voucher NBFGR-CHN-PSwli 12S ribosomal RNA gene, partial sequence; mitochondrial.
69	JQ229947	*Puerulus sewelli* voucher NBFGR-CHN-PSwli 18S ribosomal RNA gene, partial sequence.
70	JQ229889	*Petrarctus rugosus* voucher NBFGR-CHN-PRgs2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
71	JQ229875	*Petrarctus rugosus* voucher NBFGR-CHN-Prgs2 16S ribosomal RNA gene, partial sequence; mitochondrial.
72	JQ229855	*Petrarctus rugosus* voucher NBFGR-CHN-PRgs1 12S ribosomal RNA gene, partial sequence; mitochondrial.
73	JQ229856	*Petrarctus rugosus* voucher NBFGR-CHN-PRgs2 12S ribosomal RNA gene, partial sequence; mitochondrial.
74	JQ229945	*Petrarctus rugosus* voucher NBFGR-CHN-PRug1 18S ribosomal RNA gene, partial sequence.
75	JQ229946	*Petrarctus rugosus* voucher NBFGR-CHN-PRug2 18S ribosomal RNA gene, partial sequence.
76	JQ229890	*Puerulus sewelli* voucher NBFGR-CHN-PSwli2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
77	JQ229891	*Thenus indicus* voucher NBFGR-CHN-TI1 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
78	JQ229878	*Thenus indicus* voucher NBFGR-CHN-TI 16S ribosomal RNA gene, partial sequence; mitochondrial.
79	JQ229860	*Thenus indicus* voucher NBFGR-CHN-TI1 12S ribosomal RNA gene, partial sequence; mitochondrial.
80	JQ229861	*Thenus indicus* voucher NBFGR-CHN-PH-TI2 12S ribosomal RNA gene, partial sequence; mitochondrial.
81	JQ229949	*Thenus indicus* voucher NBFGR-CHN-TI 18S ribosomal RNA gene, partial sequence.
82	JQ229893	*Thenus unimaculatus* voucher NBFGR-CHN-TU-CH1 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
83	JQ229894	*Thenus unimaculatus* voucher NBFGR-CHN-TU-CH2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
84	JQ229895	*Thenus unimaculatus* voucher NBFGR-CHN-TU-KL1 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
85	JQ229896	*Thenus unimaculatus* voucher NBFGR-CHN-TU-KL2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
86	JQ229897	*Thenus unimaculatus* voucher NBFGR-CHN-TU-Ve1 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
87	JQ229898	*Thenus unimaculatus* voucher NBFGR-CHN-TU-Ve2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
88	JQ229899	*Thenus unimaculatus* voucher NBFGR-CHN-TU-Vzg1 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.

89	JQ229900	*Thenus unimaculatus* voucher NBFGR-CHN-TU-Vzg2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
90	JQ229927	*Thenus unimaculatus* voucher NBFGR-CHN-TU-CH3 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
91	JQ229928	*Thenus unimaculatus* voucher NBFGR-CHN-TU-CH4 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
92	JQ229929	*Thenus unimaculatus* voucher NBFGR-CHN-TU-CH5 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
93	JQ229930	*Thenus unimaculatus* voucher NBFGR-CHN-TU-CH6 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
94	JQ229931	*Thenus unimaculatus* voucher NBFGR-CHN-TU-CH7 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
95	JQ229932	*Thenus unimaculatus* voucher NBFGR-CHN-TU-CH8 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
96	JQ229933	*Thenus unimaculatus* voucher NBFGR-CHN-TU-KL3 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
97	JQ229934	*Thenus unimaculatus* voucher NBFGR-CHN-TU-KL4 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
98	JQ229935	*Thenus unimaculatus* voucher NBFGR-CHN-TU-KL5 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
99	JQ229936	*Thenus unimaculatus* voucher NBFGR-CHN-TU-KL6 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
100	JQ229937	*Thenus unimaculatus* voucher NBFGR-CHN-TU-KL7 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
101	JQ229938	*Thenus unimaculatus* voucher NBFGR-CHN-TU-Vzg3 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial.
102	JQ229901	*Thenus unimaculatus* voucher NBFGR-CHN-TU-CH3 16S ribosomal RNA gene, partial sequence; mitochondrial.
103	JQ229902	*Thenus unimaculatus* voucher NBFGR-CHN-TU-KL3 16S ribosomal RNA gene, partial sequence; mitochondrial.
104	JQ229903	*Thenus unimaculatus* voucher NBFGR-CHN-TU-KL5 16S ribosomal RNA gene, partial sequence; mitochondrial.
105	JQ229904	*Thenus unimaculatus* voucher NBFGR-CHN-TU-Ve3 16S ribosomal RNA gene, partial sequence; mitochondrial.
106	JQ229905	*Thenus unimaculatus* voucher NBFGR-CHN-TU-KL4 16S ribosomal RNA gene, partial sequence; mitochondrial.
107	JQ229906	*Thenus unimaculatus* voucher NBFGR-CHN-TU-Ve1 16S ribosomal RNA gene, partial sequence; mitochondrial.
108	JQ229907	*Thenus unimaculatus* voucher NBFGR-CHN-TU-Ve2 16S ribosomal RNA gene, partial sequence; mitochondrial.
109	JQ229908	*Thenus unimaculatus* voucher NBFGR-CHN-TU-Vzg1 16S ribosomal RNA gene, partial sequence; mitochondrial.

110	JQ229909	*Thenus unimaculatus* voucher NBFGR-CHN-TU-Vzg2 16S ribosomal RNA gene, partial sequence; mitochondrial.
111	KC951871	*Thenus unimaculatus* 12S ribosomal RNA gene, partial sequence; mitochondrial.
112	JQ229950	*Thenus unimaculatus* voucher NBFGR-CHN-TU-Ch6 18S ribosomal RNA gene, partial sequence.

GENETIC DIVERGENCE IN LOBSTERS (CRUSTACEA: PALINURIDAE AND SCYLLARIDAE) FROM THE INDIAN EEZ

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