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 megalopodus* 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
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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), failing 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
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(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.
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- 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 ($\pi$)
  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 $\Phi_{ST}$ 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
  populations. 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
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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 \((\pi)\) 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 *Petractus 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.