RECRUITMENT AND GENETIC POPULATION GENETICS OF SPINY LOBSTERS, *Panulirus ornatus* AND *P. homarus* IN THE SOUTH-EAST ASIAN ARCHIPELAGO

Thesis submitted by

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DECLARATION

I declare that this thesis is my own original work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education.

Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

STATEMENT OF THE CONTRIBUTIONS OF OTHERS

I was involved in the conceptualisation and experimental design of all work presented in this thesis. I performed all laboratory based experiments including DNA extraction, quality checks, quantification and standardisation, and was primarily responsible for data management, integrity, analysis and interpretation. This included variation of *Panulirus ornatus* and *P. homarus* puerulus settlement in Vietnam and correlation to wind stress over ocean currents (Chapter 2); genetic marker development (Chapter 3); genetic population structure of *Panulirus ornatus* in the Southeast Asian archipelago (Chapter 4); population genetic structure of the scalloped spiny lobster, *Panulirus homarus,* throughout the Indo-Pacific region (Chapter 5); and *insilico* development and evaluation of a DNA parentage marker suite for *Panulirus ornatus* to assist future breeding programs (Chapter 6). I was the sole author for all written work throughout the five chapters of this thesis and I am the lead author in all peer-reviewed scientific manuscripts derived from this work, both published, and in preparation. Specific co-author contributions for this thesis are outlined by chapter below:

Chapter	Details of publication(s) on which chapter is based	Nature and extent of the intellectual input of each author and their affiliations
2	Jones, C., Long, N., **Dao, H. T.**, & Priyambodo, B. (2010). Exploitation of puerulus settlement for the development of tropical spiny lobster aquaculture in the Indo-West Pacific. *J Mar Biol Assoc India, 52*, 292-303. **Dao, H. T**., & Jones, C. (2015). Census of the lobster seed fishery of Vietnam. *in: Jones C.M. (ed), Spiny lobster aquaculture development in Indonesia, Vietnam and Australia., Proceedings of the International Lobster Aquaculture Symposium held in Lombok, Indonesia, 22–25 April 2014*(ACIAR Proceedings No. 145), 20-26.	**Jones CM**[1] Funding, sampling support, writing and editing. **Long, N**[2] Statistical advice on census data and editing. **Dao HT**[2] Execution of project in Vietnam and editing. **Priyambodo, B**[3] Execution of project in Indonesia and editing. . **Dao HT**[2,4,5] Execution of project, writing and editing **Jones CM**[4,5] Funding, sampling support and editing.
3	**Dao, H. T.**, Todd, E. V., & Jerry, D. R. (2012). Characterization of polymorphic microsatellite loci for the spiny lobster Panulirus spp. and their utility to be applied to other Panulirus lobsters. *Conservation Genetics Resources, 5*(1), 43-46	**Dao HT**[4,5] Execution of project, writing and editing. **Todd EV**[4,5] Initiation of genome sequencing and statistical advice on genotyping scoring. **Jerry DR**[4,5] Project conception and design, funding, supervision and editing.
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Additional Files 1: Supplementary for the time converter macro in excel file (Chapter 2).

Additional Files 2:Supplementary material such as microsatellite genotypes, raw results from analysis and sampling details for each individual is available from the JCU Tropical Data Hub https://research.jcu.edu.au/researchdata/ (Chapter 5).

ABBREVIATIONS

ACIAR Australia Centre for International Agricultural Research
AFLP amplified fragment length polymorphism
AGRF Australian Genome Research Facility
AIMS Australian Institute of Marine Science
AMOVA analysis of molecular variance
ANOVA analysis of variance

DNA deoxyribose nucleic acid

EACC East African Coastal Current
EGC East Gyral Current
EST expressed sequence tag

FDR false discovery rate
Fst fixation index

gDNA genomic DNA
GW Great Whirl

HWE Hardy-Weinberg Equilibrium

JCU James Cook University

LC Leeuwin Current

MCMC Markov chain Monte Carlo
mtDNA mitochodrial DNA

NEC Northern Equatorial Counter Current
NMC Northeast Monsoon Currents

PCR polymerase chain reaction
PIC polymorphic information content
PLD pelagic larval duration
PNG Papua New Guinea

RAPD random amplified polymorphic DNA
RFLP random fragment length polymorphisms

SNP single nucleotide polymorphism
SMM stepwise mutational model
SEC South Equatorial Current
SECC South Equatorial Counter current
SEMC Southeast Madagascar Current
SC Somali Current
SG Southern Gyre
SMC Southwest Monsoon Currents

SJC South Java Current
SE standard error

USA the United States of America

EXECUTIVE SUMMARY

Spiny lobsters are one of the world's most valuable seafood with high market value and where demand far exceeds supply. Accordingly, tropical spiny lobsters are a very valuable resource for most Southeast Asian countries, although populations of spiny lobsters in the region are currently subject to severe fishing pressure, primarily through the harvest of pueruli for aquaculture grow-out, which is considered a serious threat to the long-term sustainability of stocks. In 2004, a workshop was held at the Institute of Oceanography, Nha Trang, Vietnam, to discuss what was known about the ecology and exploitation of tropical spiny lobsters. This workshop identified several knowledge gaps that, at the time, were affecting the development of sustainable management policy for tropical spiny lobsters, including lack of information on genetic stock structure, where sink or source populations for recruiting larvae were located, and the comparative value of seed as stock recruits. With these knowledge gaps in mind, the overarching aim of the current thesis was designed to gain an increased understanding of recruitment patterns in two commercially important spiny lobsters, *Panulirus ornatus* and *P. homarus*, along with how populations of these two species were genetically structured across a wide range of their distribution. Particular focus was centered on Vietnamese stocks which were observed to exhibit large variations in recruitment prior to 2004.

Chapter 2 first examined patterns in recruitment of the spiny lobster species *Panulirus ornatus* and *P. homarus*. Here long-term data that had been collected over six fishing seasons (2005 to 2011) on the abundance of juvenile seed (pueruli) caught by fishers along the central coast of Vietnam was coalesced.Recruitment data indicated large fluctuations in the monthly and annual pueruli catch of the two species of *Panulirus*, especially for *P. ornatus*. There was a similar pattern of spatial and temporal variation of total monthly catch in both species during the study period, with catch peaks occurring from November to the following February. The

increase of total seed catch of *P. ornatus* was found to be highly correlated with that of southward wind stress(Exponential trendline; R^2 = 0.885, p-value < 0.05), suggesting that recruitment of *P. ornatus* could be predicted for the following season in Vietnam using wind stress as an indicator. This information would be useful to fishery managers, as it will allow them to predict the next season's likely scale of recruitment and the number of wild pueruli that could be sustainably harvested as aquaculture seed.

To understand if spiny lobster recruits throughout the Indo-West Pacific originate from local stocks, or from a common widespread source, Chapters 3, 4 and 5 applied a population genetic approach to determine stock structure of *P.ornatus* and *P. homarus*. Genetic resources (mtDNA control region and nuclear microsatellite markers) were firstly developed in Chapter 3 and subsequently used to reveal the genetic structure of both species. Genetic analyses for*P. ornatus* (Chapter 4) showed no evidence for genetic differentiation among populations over thebroad and oceanographically complex region spanning Australia, Papua New Guinea, Indonesia and Vietnam (mtDNA control region sequences Φ_{ST} = -0.008; microsatellite loci F_{ST}= 0.003). A lack of evidence for regional or localized mtDNA haplotype clusters, or geographic clusters of microsatellite genotypes, reveals a pattern of high gene flow leading to panmixia in *P. ornatus* throughout the Indo-West Pacific. Based on biological and oceanographic data, a dispersal pathway of this species was developed to explain the genetic findings. Simulations incorporating known *P. ornatus* spawning areas, larval pelagic duration, and oceanographic data show that genetic homogeneity of *P. ornatus* can be explained by the oceanography-driven dispersal of the pelagic lobster larvae, which occurs from known spawning grounds in Papua New Guinea, the Philippines and, possibly, Indonesia. Results suggest the existence of a single panmictic population of *P. ornatus* in the Indo-West Pacific for conservation and fisheries management.

In terms of *P. homarus*, the mtDNA control region analyses (Chapter 5) revealed moderate levels of genetic structure among populations(Φ_{ST} = 5.31%; P < 0.05), whilst analyses atsix microsatellites detected significant genetic divergence between populations from Masirah, Oman, and several Southeast Asian Archipelago populations (Φ_{ST} = 7.5% - 19.8%). Low levels of genetic structure were also observed within the Southeast Asian Archipelago. Bayesian individual assignment analysis based on microsatellites showed that lobsters from Masirah, Oman were more homogeneous to a single genetic cluster, which was not revealed by the mtDNA, suggesting the possibility of a one-way migration pattern by females. The findings of the present study suggest regional genetic structuring of *P. homarus* across the Indo-West Pacific, and consequently the assignment of different genetic stocks, or management units, for this lobster species.

Finally, the strength of microsatellite markers as a useful resource for parentage assignment in simulated *P. ornatus* breeding programs was examined(Chapter 6). Results indicated that a marker suite of the five loci with the highest PIC could be used to correctly assign parentage with high confidence.

Overall, the work in this thesis has significantly advanced our knowledge on recruitment patterns and processes, along with genetic population structure, of two of the most important and heavily exploited tropical spiny lobsters in the Indo-Pacific. Data provided by this thesis will have application in the formulation of new management policies for these species, leading to more sustainable harvest of wild pueruli for fisheries and aquaculture purposes.

CHAPTER 1: BACKGROUND AND RATIONALE

1.1 BACKGROUND

1.1.1 Taxonomy, distribution and habitat

Panulirus ornatus and *P. homarus* are two among 19 species of lobster within the family *Palinuridae* (Holthuis, 1991; Ptacek *et al.*, 2001). *Panulirus* species are known as "rock" or "spiny" lobsters, and are found in tropical and subtropical waters of the Indo-West Pacific. The ornate spiny lobster *Panulirus ornatus* was first described by Fabricius (1798), and other synonyms of this species are *Palinurus sulcatus* (Milne Edwards, 1837), *Panulirus sulcatus* (White, 1847), *Palinurus sulcatus* (Pfeffer, 1881), or *Senex ornatus* (Lanchester, 1900). Similarly, the scalloped spiny lobster *Panulirus homarus* was first described by Linnaeus (1758), and later classified under two different species names, *P. buergeri* (de Haan, 1851) and *P. dasypus* (Milne-Edwards, 1837). However, these two 'species' were later confirmed to be morphological variations of the same species, and subsequently reclassified as a single subspecies complex, *P. homarus* (Holthuis, 1946; Barnard, 1950; Gordon, 1953; George, 1963; George & Rao, 1966; Berry, 1971).

Both *Panulirus ornatus* and *P. homarus* are decapod crustaceans belonging to the shallow-water spiny lobster group (George & Rao, 1966; Ptacek *et al.*, 2001; Phillips, 2013). In general, these spiny lobsters have a tough calcified exoskeleton with strong legs and a tail-fan (George, 2005b), with four forward-directed large spines protruding from their antennae which are used for defence (Berry, 1971). The carapace is covered by dense, shorter spines which the lobsters use to wedge themselves into rocky shelters when threatened (Heydorn, 1969; Pollock, 1990). In addition, two short forward-

directed supra-orbital horns protect their elevated eyes (Pitcher, 1993). In *P. ornatus*, the transverse groove of abdominal somites possesses straight margins, while crenulated margins were described in *P. homarus* (Holthuis, 1991). Compared to *P. ornatus*, the morphology of *P. homarus* is varied in terms of colouration and carapace armature. Previous studies have suggested three morphological subspecies (Berry, 1974) and two genetic subspecies (Ptacek *et al.*, 2001) of *P. homarus* across its distribution. According to Holthuis (1991), both *P. ornatus* and *P. homarus* have a wide distribution in the Indo-West Pacific from East Africa to southern Japan, the Solomon Islands, Papua New Guinea, Australia, New Caledonia and Fiji. Despite this, while fisheries for *P. homarus* are spread throughout the species distribution, fisheries based on *P. ornatus* are mainly recorded only from southeast India (Radhakrishnan *et al.*, 2005) and the Southeast Asian Archipelago.

The spiny lobsters *P. ornatus* and *P. homarus* are commonly found in shallow-water habitats. *P. ornatus* exhibits the broadest range of habitats utilised which includes sandy, muddy to rocky bottoms and turbid coastal waters, normally within 1 to 8m depth. Some records have also indicated that this species may be found at depths up to 50m (Holthuis, 1991; Pitcher, 1993). Similarly, most individuals of *P. homarus* are found among rocky substrates from 1 to 5m deep, with a recorded depth limit of 90m (Holthuis, 1991; Kulmiye *et al.*, 2006).

1.1.2 Reproduction and life cycles

Reproduction in spiny lobsters occurs during the summer months as warmer ocean temperatures significantly accelerate the breeding process (Phillips, 2013). Being attracted by sex pheromones released by females, lobsters mate by the males depositing a collection of spermatophores from paired penile projections located at the base of their

fifth walking legs, onto the fourth and fifth sternal plates of females that have developing ovaries. The courtship behaviours in *P. ornatus* (Prescott, 1988) and *P. homarus*(Berry, 1970) have been observed, which first involves the males following and touching the females with their antennules and first three pairs of walking legs. This process might take a few minutes to many hours. Mating occurs in only 5-8 seconds after several attempts by the males to grab and lift the females into the mating position. Polygamous mating behaviour has been observed in both species, corresponding to an individual male's size (Berry, 1970; Pitcher, 1993;George, 2005b). A few days after mating, spiny lobster females extrude several hundred thousand eggs from paired gonopores at the bases of their third walking legs, and fertilize them by scraping the spermatophore packets that males had deposited during mating with special chelae on the dactyli of their fifth walking legs. This is done to release the spermatophores and allow for egg fertilisation. Eggs then develop under the abdomen of the female for about a month prior to hatching and the timing of larval release may be stimulated by the full moon (Dennis *et al.*, 1992). It is also suggested that spiny lobster females, including *P. ornatus*and *P. homarus*, would move to areas with strong currents to maximise larval dispersal (Moore & MacFarlane, 1984).

The life cycle of all *Panulirus* species is complex, with a long oceanic larval phase of 4 to 8 months in *P. ornatus* and 4.5 to 6 months in *P. homarus* (Pitcher *et al.*, 2005; Phillips, 2013). Phyllosoma larvae are poor horizontal swimmers, relying on ocean currents to be carried offshore where they complete their development (Booth & Phillips, 1994; George, 2005b). The phyllosoma stages (about 1-2mm long) go through 11 instars to get about 30 to 50mm in total length before moulting into the puerulus. The puerulus is a non-feeding stage, which then swims towards the coast (Booth & Phillips, 1994; Pitcher *et al.*, 2005; Phillips, 2013). The recruitment of pueruli begins when they

settle onto the benthic environment and moult into pigmented juveniles after a few days or weeks. Small juveniles are often found in shallow coastal reefs, and migrate to deeper water as larger juveniles or adults to complete their life cycles. Knowledge on the recruitment patterns of larvae is of great value to both fishery managers and fishermen, as they provide information which allows for predictions of future recruitment levels into existing populations and fishing grounds(Pitcher, 1993). However, recruitment patterns of larval spiny lobsters are highly variable due to the influences of oceanographic features, which requires examination and analyses of long-term data (Briones-Fourzán *et al.*, 2008).

1.1.3 Dispersal capability

Lobsters have the longest planktonic larval stage compared to other crustaceans (Phillips, 2013; Pollock, 1995), ranging from a few months to two years, depending on the species (Palero *et al.*, 2008). For example, *P. ornatus* and *P. homarus* have a planktonic phyllosoma stage that is intermediate in development duration (4-8 months) compared to some species of the genus *Jasus* where this can extend up to 24 months (Booth & Phillips, 1994; Phillips, 2013). Pelagic larval duration (PLD) may influence the dispersal capability of marine species, as well as their population genetic structure. Various surveys conducted on marine finfish and invertebrates to investigate this showed differing results. While PLD was believed to be related to long-range dispersal (Waples, 1987), other studies could not establish the correlation of PLD with genetic differentiation (Taylor & Hellberg, 2003; Planes *et al.*, 1998). Other observations suggested not only the length of pelagic life, but the combination of larval strategies, distribution and environment may be a good predictor of population genetic structure (Shulman & Bermingham, 1995; Riginos & Victor, 2001). The long-lived larval stage,

combined with a variety of potential modes of transport, results in possibly wide dispersal of lobster larvae (Pollock & Melville-Smith, 1993; Silberman *et al*., 1994; Pollock, 1995; von der Heyden *et al*., 2007). As an example of this, Western Rock Lobster, *P. cygnus*, larvae spend approximately one year offshore after hatching on the Western Australian continental shelf, and then are transported long distances by wind-induced water movements into the southeast Indian Ocean. Some *P. cygnus* larvae have been found more than 1000km offshore, although little is known of their subsequent survival (Phillips & Pearce, 1997).

Physical, environmental, and ecological factors can also influence the dispersal capabilities of larvae. The movement and retention of larvae of many marine species is generally thought to occur through a combination of hydrodynamic processes and active larval behaviour, through horizontal and vertical movements in the water column (Pittman & McAlpine, 2003). Various transport modes including oceanic currents, fronts, gyres, local eddies and wind drift are thought to contribute to the geographical distribution of spiny lobsters (Mohamed & George, 1968; Pollock, 1993; Booth & Phillips, 1994).

Another important way in which marine species disperse is through adult migration(Pittman & McAlpine, 2003; Scheltema, 1986). Sub-adult spiny lobsters generally migrate toward deeper water breeding grounds from shallow nursery areas (Pitcher, 1993). While the literature available on the migration of *P. homarus* sub-adultsis very limited, sub-adults of *P. ornatus* are known to undertake long migrations. Maturing individuals of *P. ornatus* in the Torres Strait were observed to walk as far as 500-600km through the Gulf of Papua (southern Papua New Guinea) to reefs west of

Port Moresbyto participate in spawning events (MacFarlane & Moore, 1986; Dennis *et al.*, 2001).

1.1.4 Markets and supplies

Spiny lobsters (*Panulirus* species) are a valuable commercial resource for most countries bordering the South China Sea and make up an important component of the niche live seafood trade market in China, with Hong Kong, Taiwan and Singapore valuable secondary markets. Malaysia, for example, exports spiny lobsters in various forms (live, fresh, chilled and frozen) amounted to around 97MT (US $1.37million) in 2001, representing some 0.1% by volume and 0.6% by value of Malaysia's total fish exports. In Australia, wild fishery sourced *P. ornatus* fetch a wholesale price of $90 per kg, which equates to A$130 per kg in Hong Kong, which applies a 40% tariff.

As well as the wild fishery, aquaculture of spiny lobsters is also an attractive proposition given the great demand and high prices paid in traditional markets and a static, or decreasing supply from wild-capture fisheries (Booth & Kittaka, 2000; Jones & Shanks, 2009). Lobster farming has developed since the mid-1990s into a US $50–60million per annum industry for Vietnam. The industry is totally reliant on the collection of wild seedstock, with these then being on-grown to a marketable size in 15 to 18 months (Williams, 2004). The source of seed for this production is naturally settling post-larval lobsters, or 'pueruli', and small juvenile lobsters, which are captured using a variety of techniques and equipment. Because of their high market value, spiny lobsters in all stages are under severe fishing pressure and this level of exploitation is a serious threat to the sustainability of the stocks.

Lobster farming is a particularly attractive opportunity for Vietnam, and potentially for other developing countries, because capture of seed lobsters and their grow-out involves simple technology, moderate capital and is ideally suited to village-based enterprises (Hambrey *et al*., 2001). However, the sustainability of such on-growing is of concern because it is based on a natural resource for which there is currently no management (Williams, 2004). Clearly the future for lobster aquaculture will demand technologies that are independent of wild populations, involving hatchery production of seed and that are economically and environmentally sustainable. In the meantime, however, the farming of lobsters using natural settlement of seed is expanding and it should be managed carefully to maximise the benefit to the coastal communities who have embraced it, while remaining sustainable (Jone, CM *et al.*, 2010).

1.1.5 Spiny lobster fishery and management

Understanding stock structure of a resource is an important component of effective fishery management (Cochrane, 2002). For many species of spiny lobster, the number of pueruli caught can provide an index of settlement and a forecast of future stock. This information, therefore, is of great value to both fishery managers and fishermen (Pitcher, 1993). Puerulus recruitment of several spiny lobster species, such as *P. argus* (Witham *et al.*, 1968; Briones-Fourzán, 1994; Acosta *et al.*, 1997; Briones-Fourzán *et al.*, 2008;), *P. japonicus* (Yoshimura *et al.*, 1999; Inoue *et al.*, 2004) and *P. cygnus* (Phillips, 1972, 1986; Caputi *et al.*, 2003; Caputi & Brown, 1993; Pearce & Phillips, 1994; Caputi *et al.*, 2001; Griffin *et al.*, 2001) that support major fisheries around the world has been documented in a number of studies. As an example, the variation in *P. cygnus* puerulus settlement in Western Australia accounts for about 75% of the variability in subsequent catches (Phillips, 1972, 1986; Caputi *et al.*, 2003), which

provides a means of predicting future catches and managing the fishery. It also suggested that the level of *P. cygnus* puerulus settlement in each season has been affected by the environment, such as the effect of wind stress over ocean currents, gyres, or regional eddies (Caputi *et al.*, 2003). However, these studies could not provide knowledge about the sources of these stocks, or the population structure across the species distribution, which is very important information for fishery management.

1.1.6 Population genetics structure

Natural populations of most species can be subdivided into separate subpopulations in which random mating takes place. When there is subdivision, or population structure, genetic variation within the species is partitioned into that which exists within local populations, and that which differs among populations (Phillips, 2013). Population genetics is the study of the genetic composition and evolution of populations (Hallerman, 2003). The discipline is concerned with understanding patterns of genetic variation, or changes in allele frequencies within and among populations, or groups of conspecific individuals (Hedgecock *et al.*, 2007; Klug *et al.*, 2008; Hellberg, 2009). A number of evolutionary processes such as natural selection, genetic drift, mutation and gene flow can lead to changes in population genetic structure. Population genetic structure may change in space (spatial variation), as well as in time (temporal variation; Hellberg, 1994).

For most inshore marine species, life cycles can be complex and are often in three phases, which includes the movement of planktonic eggs and larvae to nursery areas, a range of routine shelter and foraging movements that maintain a home range and spawning migrations away from the home range to close the life cycle(Pittman & McAlpine, 2003). Therefore, dispersal capability can be accounted for during two main

periods of their life cycles: dispersal of eggs (or larvae) and adult migration. The long planktonic stage, wide dispersal capacity and the ability to settle on various habitats during periods of unstable climatic or oceanographic conditions are considered evolutionary strategies that have allowed spiny lobsters to circumvent extinction, and as such, may be the reason contemporary population genetic structure exists (Booth & Phillips, 1994; Thorpe *et al.*, 2000; Cowen & Sponaugle, 2009).

1.1.7 Application of genetic markers in population genetic studies

The results of population structure studies can be influenced by the choice of genetic marker (Hellberg, 2007). However, no single analysis method, or genetic marker is suitable for all questions, species, or spatial scales under investigation. Allozymes, mitochondrial random fragment length polymorphisms (RFLPs), mtDNA sequences, microsatellites and recently, single nucleotide polymorphisms (SNP) have all been used at some time to conduct population genetic studies. Allozymes were the first genetic technique used to understand the correlation of pelagic larval duration with population structure; unfortunately, however, allozymes were not sensitive enough to detect fine-scale spatial structuring (Hellberg, 1994;Doherty *et al.*, 1995; Hellberg, 1996). Methods that utilized RFLPs and mtDNA led to increases in the sensitivity of population genetic analyses for marine species and the detection of fine scale structure (Shulman &Bermingham, 1995;Bay *et al.*, 2006). Finally, studies based on microsatellites have been able to find population variation at even smaller spatial scales in species that have limited dispersal potential (Gutierrez-Rodriguez & Lasker, 2004; Hoffman *et al.*, 2005).

The application of genetic markers has been used previously in spiny lobsters to examine population genetic structure. Genetic studies on the Hawaiian lobster, *P. marginatus*, failed to reveal stock heterogeneity, as did studies with *P. argus* in the

Caribbean Sea (Hateley & Sleeter, 1993; Silberman *et al.*, 1994), and *P. cygnus* along the coast of Western Australia (Thompson *et al.*, 1996). Recently, no evidence of subdivision was found among populations of *P. japonicus* from the Japan Sea (Inoue *et al.*, 2007), or *P. gilchristi* in the deep shelf waters along the southern coast of South Africa (Tolley *et al.*, 2005). Whilst many spiny lobster species do not appear to be strongly genetically structured, others have been found to comprise several genetic stocks. Significant differences were observed between *P. argus* populations over greater distances between the Caribbean Sea and Brazil (Sarver *et al.*, 1998; Diniz *et al.*, 2005), as well as among populations of *P. interruptus* along the west coast of Baja California Peninsula, Mexico (Perez-Enriquez *et al.*, 2001) and in the European spiny lobster,*P. elephas*, between Atlantic and Mediterranean populations (Palero *et al.*, 2008). The application of genetic markers has been used to reveal the genetic population structure in a number of other marine species, and therefore, has important implications for fisheries management of commercially important natural resources (Ward, 2000).

1.1.8 The application of genetics to breeding programs in aquaculture

Breeding programs for genetic improvement of targeted production animals relies on having accurate phenotypic and pedigree data. Pedigree information is essential in estimating genetic parameters in the selective breeding of aquaculture species to minimize the threat of inbreeding. Inbreeding might occur with the use of small numbers of broodstock and high selection intensities due to the high fecundity of most aquatic animals. Consequently, if pedigree information of broodstock is absent, or not well managed, after a few generations of selection the stock risks losing substantial genetic diversity, becoming highly inbred, and losing additive genetic variance (Hulata, 2001). In addition, environmental variables have differential effects on the production

of quantitative phenotypes of animals if aquatic animals are cultured separately. Tagging methods allow researchers to track the performance of family groups in communal tank or pond settings, minimize tank or pond effects and establish accurate breeding values and heritability estimates. Many types of tags and identifying marks have been developed to maintain pedigrees in selective breeding programs and as a way to identify animals (Mahapatra *et al*. 2001). Tagging and marking are also essential techniques in aquaculture which allow for the gathering of a variety of information for aquatic animals. Despite its importance in aquaculture, the tagging of young finfish (or other aquatic species) with physical tags is very difficult and time-consuming. New technologies, therefore, which can provide an alternative to traditional pedigree identification need to be introduced. Recently, molecular parentage analysis using DNA markers has made the reconstruction of pedigrees possible in aquaculture species (Yue & Xia, 2014).

Fish and shellfish often have a higher genetic variance compared to farmed land animals (Gjedrem, 1997; Dunham *et al*., 2000) and hence the application of genetics has been introduced to aquaculture. In 2010, around 8.2% of global aquaculture production was based on genetically improved stocks (Gjedrem *et al*., 2012). It is predicted that global aquaculture production will reach 190 million tonnes in 2020, if 100% fish and shellfish are genetically improved. In addition, a wide range of genetic markers (allozymes, mtDNA, RFLPs, RAPDs, AFLPs, microsatellites, SNPs, and ESTs) have been developed for application in genetic improvement and management, with each having its particular advantages depending on the application in question (Liu & Cordes 2004; Liu *et al*., 2011). However, not all these markers can be easily employed to assign parentage in aquaculture breeding programs. While use of mtDNA, RFLPs, RAPDs, and AFLPs, are not usually applied in aquaculture breeding programs now,

microsatellites and SNP have been demonstrated to be the most effective markers for assigning progeny to their parents. DNA pedigrees help to remove the need for physical tags, increasing potential family numbers that can be evaluated, and can also reduce the cost of maintaining breeding nucleus backup populations. The entire task of DNA marker technologies is to provide the means to reveal DNA level differences of genomes among individuals of the same species and also among various related taxa (Liu and Cordes, 2004; Liu *et al*., 2011).

Studies involving breeding programs of spiny lobsters have been initiated in many countries (Phillips & Matsuda, 2011). However, the success of these in many spiny lobster species was very limited. The difficulties associated with culturing spiny lobster larvae from the egg through to puerulus lieswith the prolonged larval development duration. Therefore, the seed supply for aquaculture of spiny lobsters such as *P. ornatus* and *P. homarus* in many countries is still based on collection of naturally settling pueruli.

1.2 THESIS OVERVIEW

Panulirus ornatus and *P. homarus* are two high-value commercially important species that are exploited throughout the tropical eastern Indian Ocean, South-east Asia, Australia and the Western Pacific. Spiny lobster pueruli (the postlarval stage) are under heavy exploitation as seedstock for aquaculture, particularly in Vietnam and Indonesia. Of concern to managers of this fishery is the fact that large fluctuations in juvenile recruitment have been experienced in recent years, suggesting that the fishery may be subject to collapse in heavily exploited regions. In Vietnam for example, the total catch of *P. ornatus* from eight provinces in the seasons 2006-2007 and 2009-2010 was only approximately 1million pueruli, about half of the catch recorded in other seasons.

Whether the annual removal of 1 to 2million pueruli by fishers is a factor in the absence of any recovery to adult stocks is unknown (Long & Hoc, 2009; Jones C. *et al*., 2010). Recovery of fisheries in these exploited populations will be particularly problematic if recruitment is localized. An investigation into the genetic structure of spiny lobster species will therefore provide valuable data on the resilience and potential of populations to recover from heavy exploitation, as well as providing important information on the sources and sinks for settling seed-stock.

1.2.1 Objective

This study aimed to determine the temporal variation during 7years of puerulus settlement for *P. ornatus* and *P. homarus* in the central Vietnamese coastal region, and to describe the spatial distribution pattern of genetic variation in these two species across a broad part of the two species Indo-West Pacific range.

1.2.2 Specific aims

The specific aims of this study were to:

a) Estimate the variation in puerulus recruitment of *P. ornatus* and *P. homarus* collected along the central Vietnamese coastal region from 2005 to 2011, based on data logged by lobster dealers (who are middlemen between the fishermen and farmers). The correlation between *P. ornatus* and *P. homarus* puerulus catch rates and wind stress variation at the same time intervals was also evaluated to identify if seasonal windstress patterns can be used as a predictive tool of the following season's pueruli recruitment into Vietnam.

b) Investigate the population genetic structure of *P. ornatus* within the South-east Asian archipelago using both mtDNA control-region and microsatellite markers. A potential dispersal pathway map for this species within this region was also developed, based on a synthesis of data on regional oceanography and knowledge of the species' biology to understand the observed patterns of genetic structure.

c) Examine the genetic differentiation among populations of *P. homarus* collected from Australia, Indonesia, Vietnam and Oman using both mtDNA control-region and microsatellite markers. The results of mtDNA control-region assays in this study were merged with results in a previous study by Farhadi *et al.* (2013) in the western Indian Ocean to provide a more comprehensive view on the population structure across the species' distribution.

d) Explore the power of microsatellite markers developed for the population genetic analyses to reconstruct pedigree information in breeding programs using an*in silico* simulation of various broodstock mate allocation models.

PUBLICATIONS

Journal articles produced within this Ph.D. candidature period that have been published, submitted, or are currently in preparation include:

Dao, H. T., Smith-Keune, C., Wolanski, E., Jones, C. M., & Jerry, D. R. (2015). Oceanographic Currents and Local Ecological Knowledge Indicate, and Genetics Does Not Refute, a Contemporary Pattern of Larval Dispersal for The Ornate Spiny Lobster, Panulirus ornatus in the South-East Asian Archipelago. *PloS One, 10*(5), doi.org/10.1371/journal.pone.0124568

Dao, H. T., Todd, E. V., & Jerry, D. R. (2012). Characterization of polymorphic microsatellite loci for the spiny lobster *Panulirus* spp. and their utility to be applied to other Panulirus lobsters. *Conservation Genetics Resources, 5*(1), 43-46.

Dao, H. T., & Jones, C. (2015). Census of the lobster seed fishery of Vietnam. *in: Jones C.M. (ed), Spiny lobster aquaculture development in Indonesia, Vietnam and Australia., Proceedings of the International Lobster Aquaculture Symposium held in Lombok, Indonesia, 22–25 April 2014* (ACIAR Proceedings No. 145), 20-26.

Jones, C., Long, N., **Dao, H. T.**, & Priyambodo, B. (2010). Exploitation of puerulus settlement for the development of tropical spiny lobster aquaculture in the Indo-West Pacific. *Journal of the Marine BiologicalAssociation of India, 52*, 292-303.

CHAPTER 2. VARIATION OF *Panulirus ornatus*AND *P. homarus PUERULUS* SETTLEMENT IN VIETNAM AND CORRELATION TO WIND STRESS OVER OCEAN CURRENTS

2.1 BACKGROUND

Spiny lobsters are the world's most valuable crustaceans. Many countries have an interest in spiny lobster aquaculture, but only a few have produced any farmed lobsters for the market, because they have difficulties in securing a supply of seed. The reason is the difficulty in culturing spiny lobster larvae from the eggs through to the puerulus, due to the protracted larval duration, from four months to more than one year, and the technical difficulties in nurturing and sustaining them. At this time there is no large-scale commercial hatchery production of spiny lobsters anywhere in the world.

Nevertheless, there is a significant lobster aquaculture industry in Vietnam producing more than 1,500 tonnes of cage-raised lobsters annually, and a rapidly developing spiny lobster farming industry in Indonesia (Williams, 2004). The aquaculture in these countries is based on collection of naturally settling pueruli and juveniles. Until a hatchery supply of seed supply can be established, the supply of naturally settling seed is very important to sustain aquaculture production. Little is known about this supply, including the source and the impact of fishing.

Previous studies on marine larvae indicate the key role of hydrographic features, such as the effect wind stress over ocean currents, gyres, or regional eddies, play in influencing planktonic larvae transport and/or fluctuations in larval recruitment and settlement

(Hedgecock, 1986; Scheltema, 1986; White *et al.*, 2010). For example, variation in post-larval recruitment of *P. argus* to the Mexican Caribbean coast (Briones-Fourzán, 1994) and Southern Florida (Acosta *et al.*, 1997; Yeung *et al.*, 2001) is the result of varying counter-currents, coastal eddies, or gyres. Patterns of larval recruitment for *P. japonicus* around Kyushu Island (Yoshimura *et al.*, 1999; Inoue *et al.*, 2007) and *P. cygnus* in Western Australia (Pearce & Phillips, 1988; Caputi *et al.*, 2001; Griffin *et al.*, 2001) were also explained by the influence of the Kuroshio Subgyre and the Leeuwin Current (and westerly winds). Studies on other crustaceans with a planktonic larval phase, such as the blue crab,*Callinectes sapidus*, or the smooth mud crab, *Hexapanopeus angustifrons*, also found that oceanographic features and wind influence the dispersal and recruitment of larvae (Epifanio *et al.*, 1984;Johnson *et al.*, 1984; Johnson, 1985; Johnson & Hester, 1989;Goodrich *et al.*, 1989; Steppe & Epifanio, 2006; Tilburg *et al.*, 2006). To date no studies have explored the relationship between the local recruitment patterns of *P. ornatus*or*P. homarus*and environmental factors.

This chapter quantified *P. ornatus* and *P. homarus* pueruli catch variability in coastal regions of Vietnam over six lobster fishing seasons (2005-2011). Under the assumption of the larvae dispersal pathway developed for *P. ornatus* in Chapter 4, this chapter also examined the correlation between the change of wind stress (the shear stress exerted by the wind on the surface of oceans) and the variation of *P. ornatus* and *P. homarus* pueruli catch along the Vietnamese coast during that time. Vietnam was chosen for the correlation test because of the availability of long term recruitment data on pueruli settlement originating from wholesale lobster seedstock trading (Long & Hoc, 2004). Therefore, Vietnam was considered a good location model to test the correlation between wind stress and puerulus recruitment and to understand how oceanographic features influence the local recruitment of puerulus.

2.2 METHODS

In order to estimate the number of pueruli settling in Vietnam, an annual census of lobster seed catch was conducted in eight coastal south-central provincesfrom 2005 to 2011:Provinces sampled were Da Nang, Quang Nam, Quang Ngai, Binh Dinh, Phu Yen, Khanh Hoa, Ninh Thuan and Binh Thuan (Figure 2.1). This work was funded by the Australian Centre for International Agricultural Research (ACIAR) through project SMAR/2008/021.

Previous studies show that the postlarvae supply had a lunar phase periodicity with higher catch near the new moon (Phillips *et al.*, 1978; Acosta *et al.*, 1997). As lobster seed collection in Vietnam is based on the lunar calendar and due to catch rates showing a strong lunar pattern (Long & Hoc, 2009), all time data used in this chapter were converted to lunar time (see supplementary material for the time converter macro in an excel file).

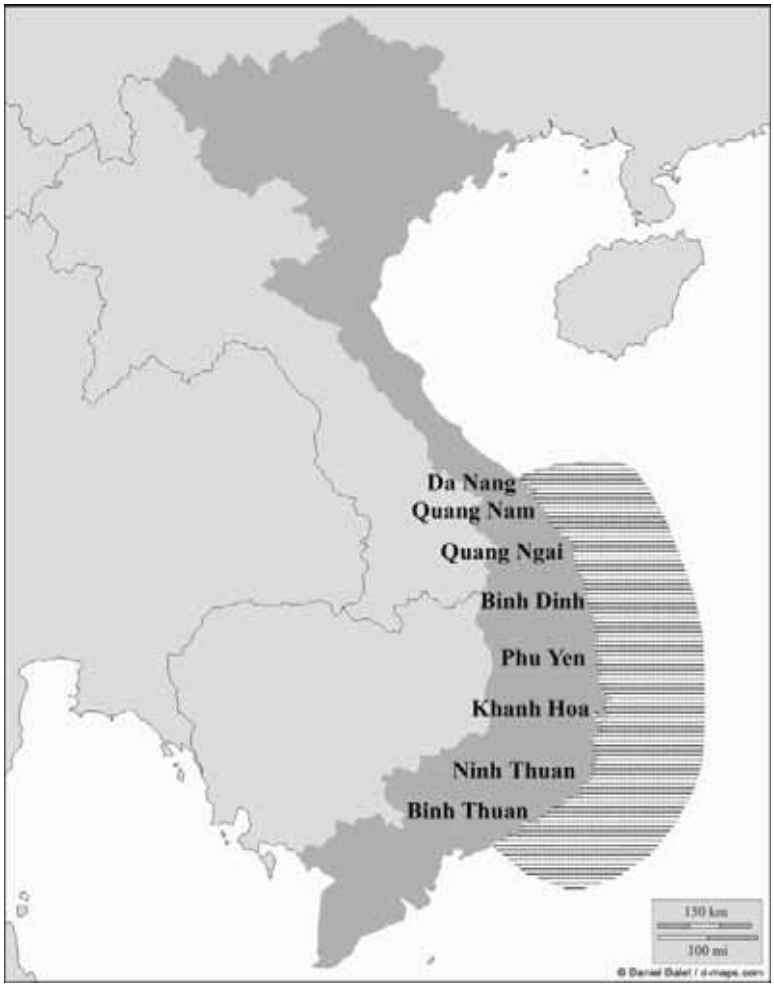

Figure 2.1 Geographical location of the eight coastal south-central provinces of Vietnam from which puerulus census data was collected.

In Vietnam, the fishing season for spiny lobster puerulus usually starts in August, or early September, and ends in April/May of the following year. A total of six fishing seasons from 2005 to 2011 (05-06, 06-07, 07-08, 08-09, 09-10 and 10-11) were monitored for capture data (Table 2.1). The census was conducted through interviewing commercial lobster seed dealers and collating the number of pueruli sold as recorded in their logbooks. Data for lobster seed catch of *P. ornatus* and *P. homarus* were extracted from daily logbook recordings of each fishing boat at each fishing village (Figure 2.2).

Table 2.1 Location and number of local dealers in coastal waters of the south central provinces of Vietnam surveyed for lobster seeds over six seasons 2005-06 to 2010-11.

Season	05-06	06-07	07-08	08-09	09-10	10-11
Number of contacted dealers	71/81	94/97	97/97	99/99	101/102	114/114

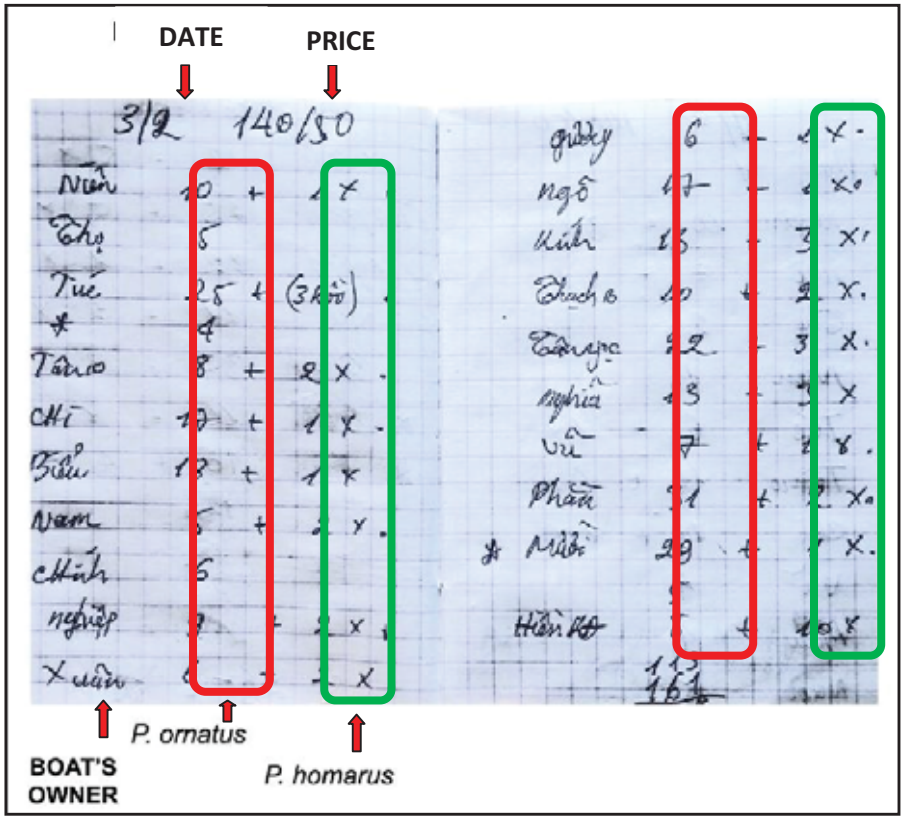

Figure 2.2 Example of a daily record from the logbook of a local dealer who sells *Panulirus* puerulus. The numbers refer to individual pueruli catch that day by each boat.

Table 2.2 Total catch of pueruli of *Panulirus ornatus* (normal font) and *Panulirus homarus* (italics) in each province along the coast of Vietnam over six fishing seasons from 2005 to 2011.

Province/Season	05-06	06-07	07-08	08-09	09-10	10-11	Total
Da Nang	78,820	184,624	165,604	59,278	79,730	182,880	750,936
	877	*102,510*	*25,484*	*31,006*	*31,939*	*72,478*	*264,294*
Quang Nam	91,270	74,834	76,936	61,898	25,275	40,050	370,263
	17,474	*76,000*	*27,616*	*27,434*	*6,282*	*13,593*	*168,399*
Quang Ngai	259,290	102,639	180,584	357,811	90,047	238,156	1,228,527
	36,494	*113,689*	*51,615*	*100,965*	*30,727*	*61,138*	*394,628*
Binh Dinh	453,450	279,623	561,667	438,905	192,863	644,858	2,571,366
	75,790	*220,780*	*123,132*	*105,897*	*59,595*	*118,650*	*703,844*
Phu Yen	414,860	154,383	449,077	571,502	246,885	716,749	2,553,456
	44,482	*96,215*	*65,815*	*131,401*	*79,037*	*145,365*	*562,315*
Khanh Hoa	415,690	110,354	459,077	338,162	148,460	629,143	2,100,886
	140,121	*108,587*	*179,304*	*111,418*	*86,734*	*152,039*	*778,203*
Ninh Thuan	154,330	68,954	228,476	100,332	56,300	452,940	1,061,332
	64,663	*284,888*	*90,218*	*83,256*	*83,724*	*108,428*	*715,177*
Binh Thuan	50,200	23,210	158,868	56,132	61,006	343,542	692,958
	114,264	*326,000*	*166,494*	*125,488*	*128,734*	*161,772*	*1,022,752*
Total	1,917,910	998,621	2,280,289	1,984,020	900,566	3,248,318	11,329,724
	494,165	*1,328,669*	*729,678*	*716,865*	*506,772*	*833,463*	*4,609,612*

For each year, total lobster seed catch for each month and annual catch was calculated based on dealer daily catch reports. These calculations were also repeated for catch in each province. Comparisons of monthly catch within each season and among all seasons were subsequently graphed to examine the yearly and provincial variation of puerulus recruitment.

The wind data at 15 sites in Vietnamese coastal waters were provided by Dr. Nguyen Huu Nhan from the Institute for Coastal and Offshore Engineering of Vietnam (unpublished data; Figure 2.3). This data was gathered in GIS format from NASA Earth Data (www.podaac.jpl.nasa.gov).

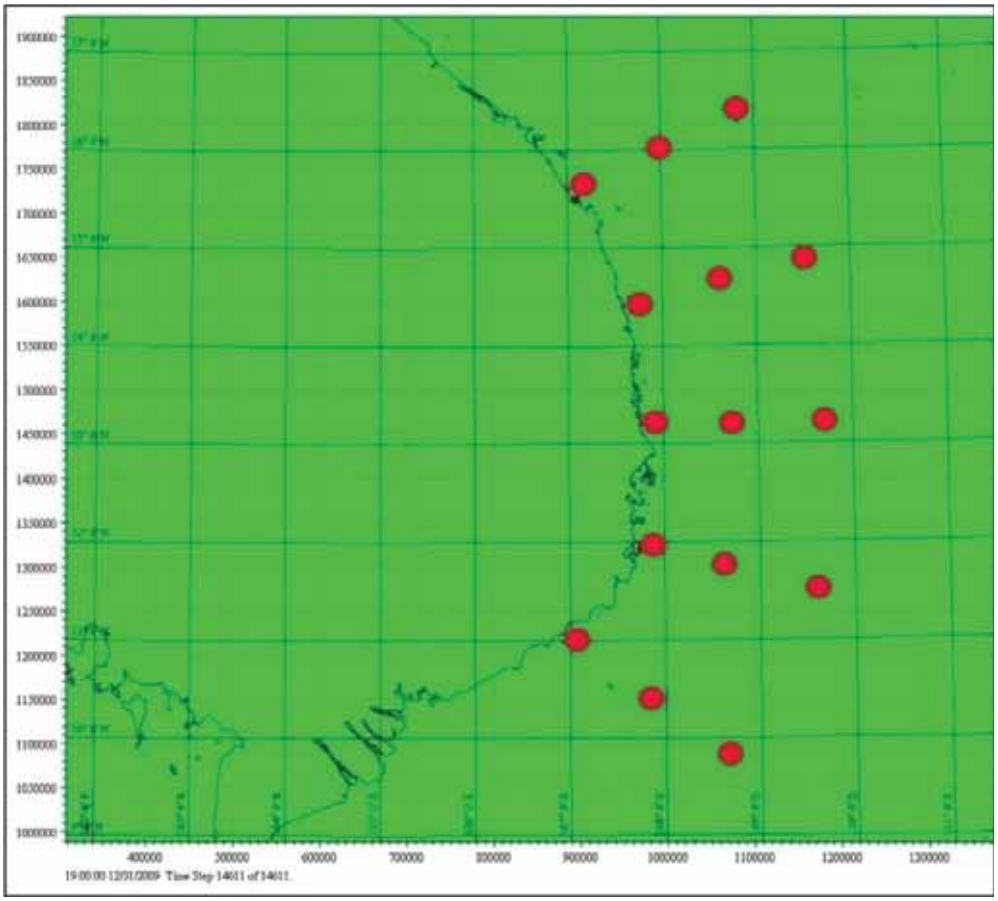

Figure 2.3 The distribution of 15 sites (red dots) along the Vietnamese central coast where wind data was gathered.

Based on wind data (JCU Data Hub), the sea surface wind stress (τ_{wind}) was calculated by the formula:

$$\tau_{wind} = \rho_{air}\, C_D |\boldsymbol{U_h}| U_h \qquad (1)$$

where ρ_{air} is air density (1.3 kg/m^3), C_D is the drag coefficient, and U_h is wind velocity at a standard altitude of 10m (Yelland & Taylor, 1996).

A positive wind stress indicates a northward wind and a negative value a southward wind. The more positive or negative the value the greater the wind stress present in that particular direction. Monthly averages of wind stress were calculated for each season

from 2005 to 2011, and then were compared with the monthly capture data of *P. ornatus* and *P. homarus* juveniles at the same temporal and spatial scale to determine if any correlation between commercial catch data and wind stress existed. In this chapter, the correlation between wind stress and seed catch was only tested during five fishing seasons from 2006 to 2011 due the lack of wind data in the season 2005-06.

2.3 RESULTS

2.3.1 Annual lobster seed catch

There was large variation in the annual total catch of lobster seed, especially for *P. ornatus* (Figure 2.4 and Table 2.2). For *P. ornatus*, the largest number of seeds harvested was about 3 million in the 2010-11season, while in the 2006-07 season and the 2009-10season, only about 1 million and about 0.9 million were harvested, respectively. Similarly, for *P. homarus*, the number of lobster seeds harvested also fluctuated inter-annually, ranging from about 0.5 million in the 2005-06 season to about 1.3 million in the 2006-07season . Except for the 2006-07season, the total catch of *P. homarus* was substantially less than that of *P. ornatus*, whereby 1,328,669 *P. homarus* seeds were collected in 2006-07, while only 998,621 *P. ornatus* seeds were harvested.

The capture rate varied spatially (Figure 2.2. & Table 2.2), whereby there was also a trend of increasing catches of *P. homarus* in the more southerly locations surveyed (Binh Thuan) compared to those in the north (Da Nang). For *P.ornatus* however, the largest number of seeds recruit were caught in the central region of Vietnam, with a tapering in the catches as more northerly and southerly catches were sampled.

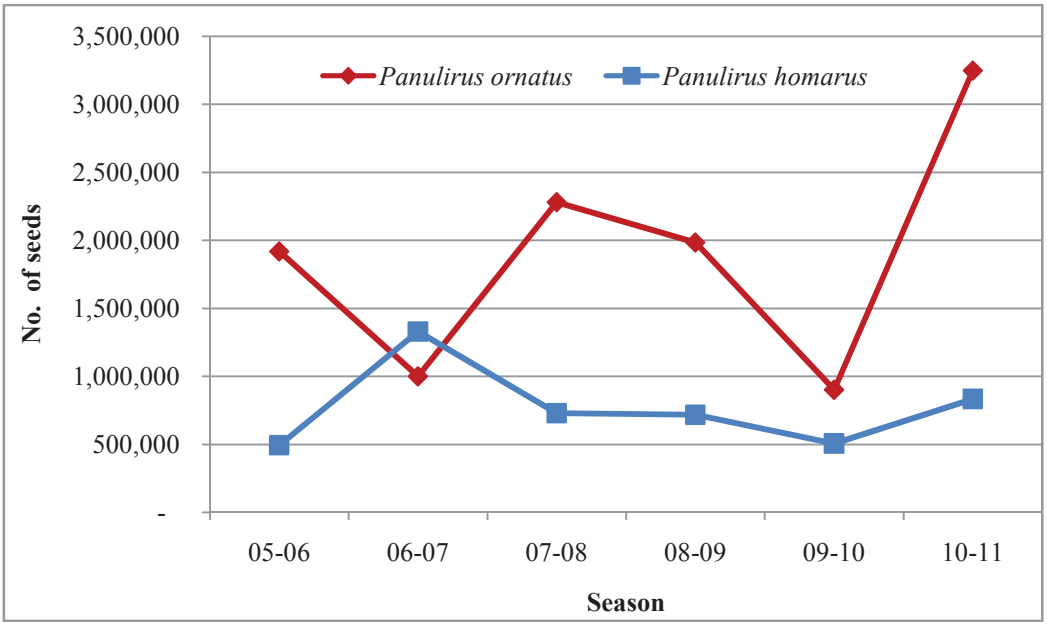

Figure 2.4 Annual total catch (2005-2011)of *Panulirus ornatus* and *P. homarus* pueruli from Vietnam.

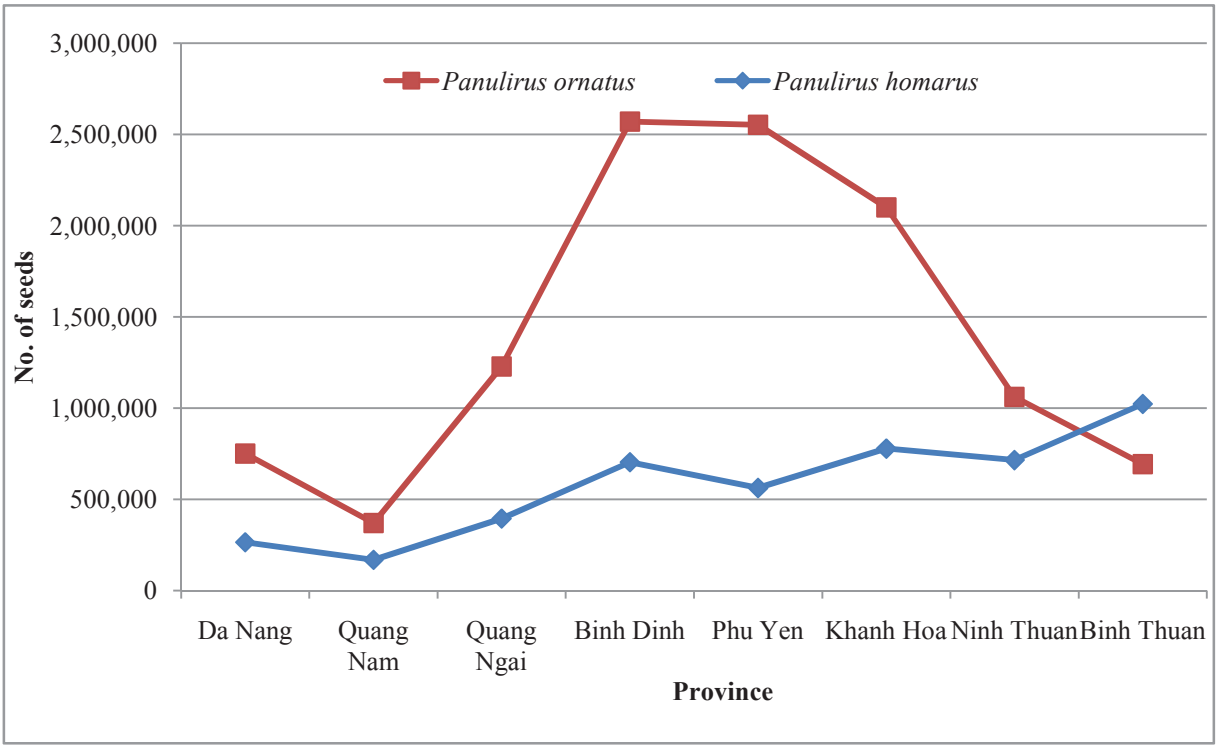

Figure 2.5 Total catch of *Panulirus ornatus* and *P. homarus* pueruli across locations over six seasons from 2005 to 2011 in Vietnam.

2.3.2 Monthly lobster seed catch (Total catch per month)

In general, census data showed that the fishing season for lobster seed could start as early as August (seasons 2006-07 and 2010-11) and went through to as late as July of

the following year (season 2010-2011). However, the main fishing season for both *P. ornatus* and *P. homarus* was over the six-month period between October - March and reached its peak from November to February (Figure 2.6 & 2.7). For*P. ornatus*, in the 2008-09season, the highest catch occurred in January with more than 1 million collected seeds. This was double that in December (461,941 seeds) and about 3.5 times larger than that in February (296,548 seeds). In the season 2010-11, when more than 3 million seeds were recorded, the high catch rates were maintained during 6 months from October of 2010 to March of 2011 (510,550 seeds per month on average). Conversely, monthly total catches for most months in the seasons 06-07 and season 09-10 were very small, with an average value at 161,677 and 148,658 seeds per month, respectively (Figure 2.6 and Table 2.3).

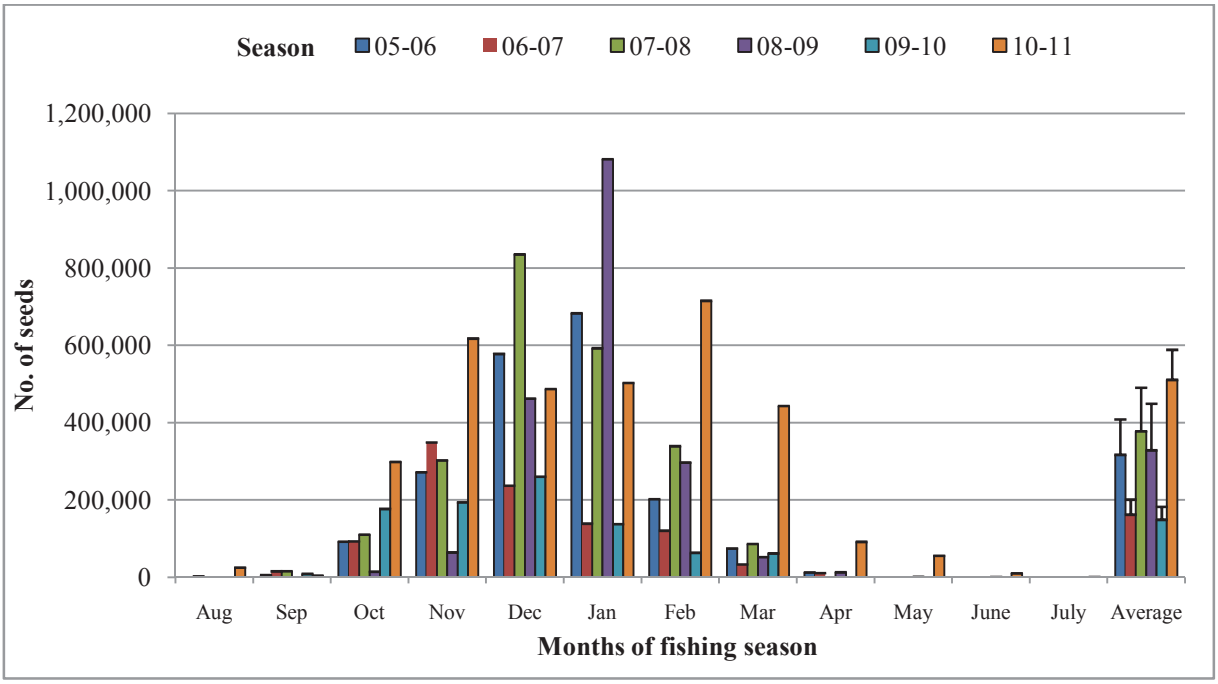

Figure 2.6 Total monthly catch of *Panulirus ornatus* lobster seeds in Vietnam over six seasons from 2005 to 2011.

In terms of *P. homarus*, there was a similar pattern of monthly catch in all seasons to *P. ornatus*, in which most of largest catch rates occurred in December or January (Figure 2.6). Significant peaks in the monthly catch occurred in December (461,545 seeds) and

November (346,000 seeds) of the 2006-07 season and in January of the 2008-09 season (328,872 seeds), with about twice the level of puerlui caught compared to other seasons. Therefore, the average monthly catch during the 6 months of the main season in 2006-07 (207,144 seeds per month), was much higher than the average catch in other seasons (range from 80,755 to 124,733 seeds per month; Figure 2.7;& Table 2.3).

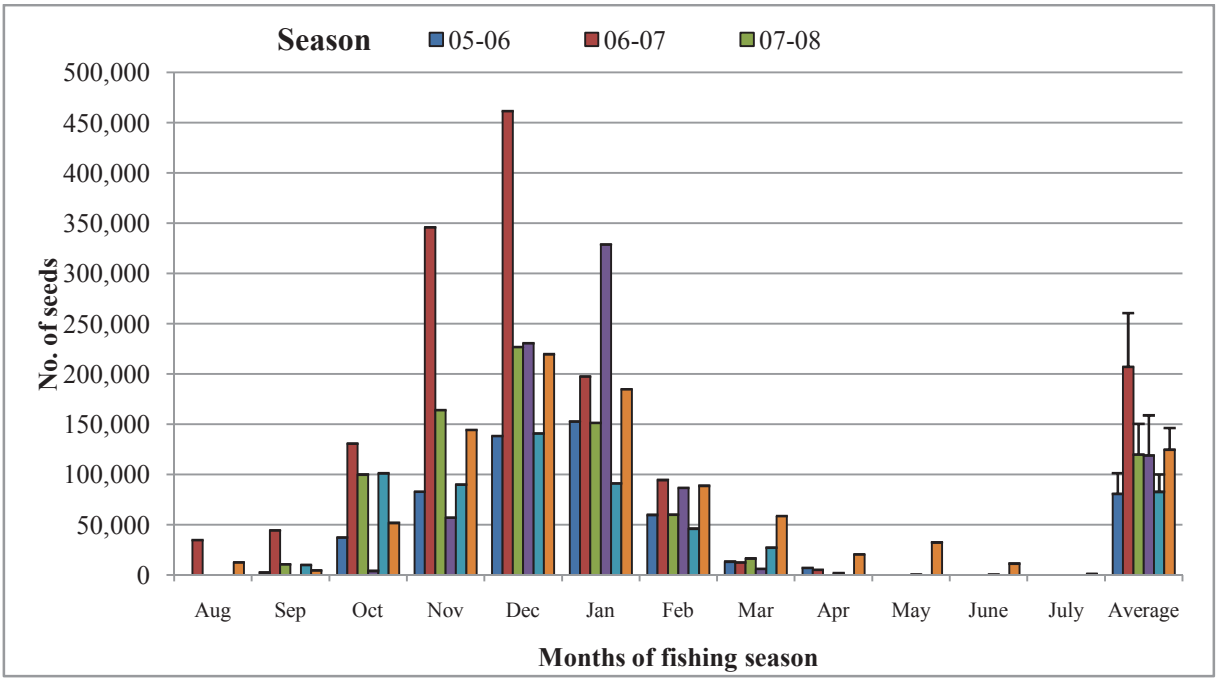

Figure 2.7 Total monthly catch of *Panulirus homarus* lobster seeds in Vietnam over six seasons from 2005 to 2011.

Table 2.3 Monthly catch of *P. ornatus* and *P. homarus* in Vietnam coastal regions from 2005 to 2011. Average value is for the six main months of the fishing season, from October to March of the following year.

		05-06	06-07	07-08	08-09	09-10	10-11
P. ornatus	**Aug.**		2240				24498
	Sept.	5363	15265	15421		8619	3502
	Oct.	91916	92672	109986	14110	176727	298239
	Nov.	271661	348503	302272	64092	193579	617422
	Dec.	577764	236681	835262	461941	259725	486938
	Jan.	682819	138666	592526	1081808	137272	502750
	Feb.	201792	120464	338996	296548	63216	715152
	Mar.	74098	33073	85830	51761	61428	442800
	Apr.	12494	10766		12761		91352
	May				783		55277
	June				216		9818
	July						240
	Average	316,675	161,677	377,479	328,377	148,658	510,550
P. homarus	**Aug.**		34,837				12596
	Sep.	2,532	44,490	10,673		10,048	4658
	Oct.	37,351	130,781	99,997	4,166	101,221	51938
	Nov.	82,860	346,000	164,171	57,162	90,036	144372
	Dec.	138314	461545	226866	230683	140828	219707
	Jan.	152782	197483	151373	328872	91176	184912
	Feb.	59810	94588	60065	86716	46181	88821
	Mar.	13411	12466	16533	6144	27282	58645
	Apr.	7103	5174		2033		20626
	May				576		32526
	June				513		11468
	July						1126
	Average	80,755	207,144	119,834	118,957	82,787	124,733

2.3.3 Wind stress and the correlation to seed catch of *Panulirus ornatus* in Vietnam

In general, southward wind prevailed during the lobster fishing seasons from 2006 to 2011. The total wind stress ranged from -450,089 Pa (06-07season) to -873,838 Pa (2010-11season). There was a similar pattern of variation in total wind stress to the large fluctuation of total seed catch of *P. ornatus* in Vietnam coastal regions (Figure 2.8). As a result, the total catch of *P. ornatus* seeds in Vietnam increased with

increasing wind stress (Figure 2.9; Exponential trendline with R^2 = 0.885, p-value = 0.01,< 0.05).

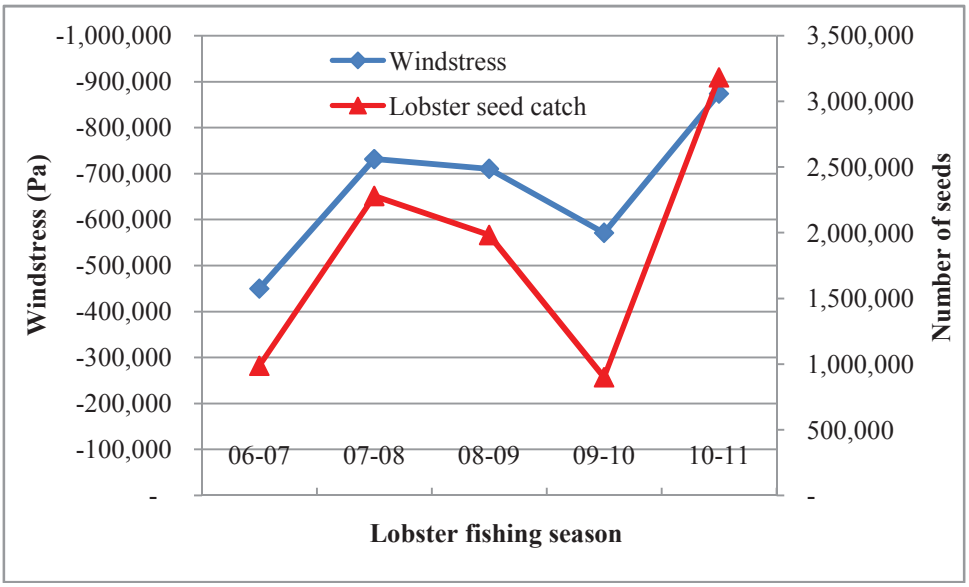

Figure 2.8 The variation of total wind stress and total seed catch of *Panulirus ornatus* during five fishing seasons in Vietnam from 2005 to 2011.

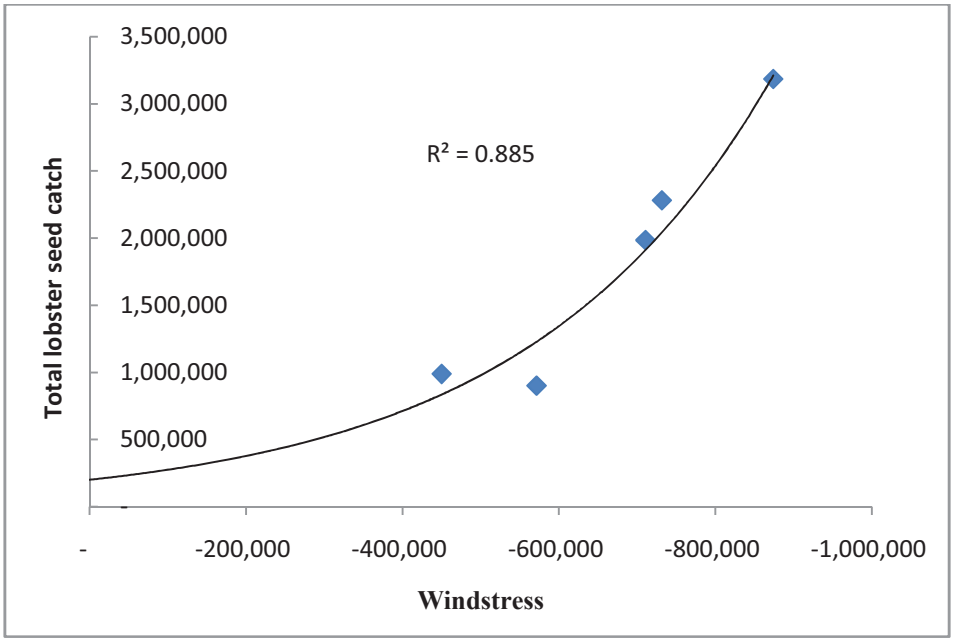

Figure 2.9 Scatter chart showing the correlation between total seed catch of *Panulirus ornatus* and the wind stress value.

According to the time series plot from 2006 to 2011 (Figure 2.10), the seed catch started to increase about one month after the southward wind stress began to become highly

negative (in September or October). The seed catch then peaked two or three months afterward in November to February. For an example, in the 2008-09season, the total wind stress became very negative in October 2008 (-131,280 Pa), while a large number of seeds (461,919 seeds) were caught in November 2008 and peaked in January 2009 with the record of more than 1 million seeds.

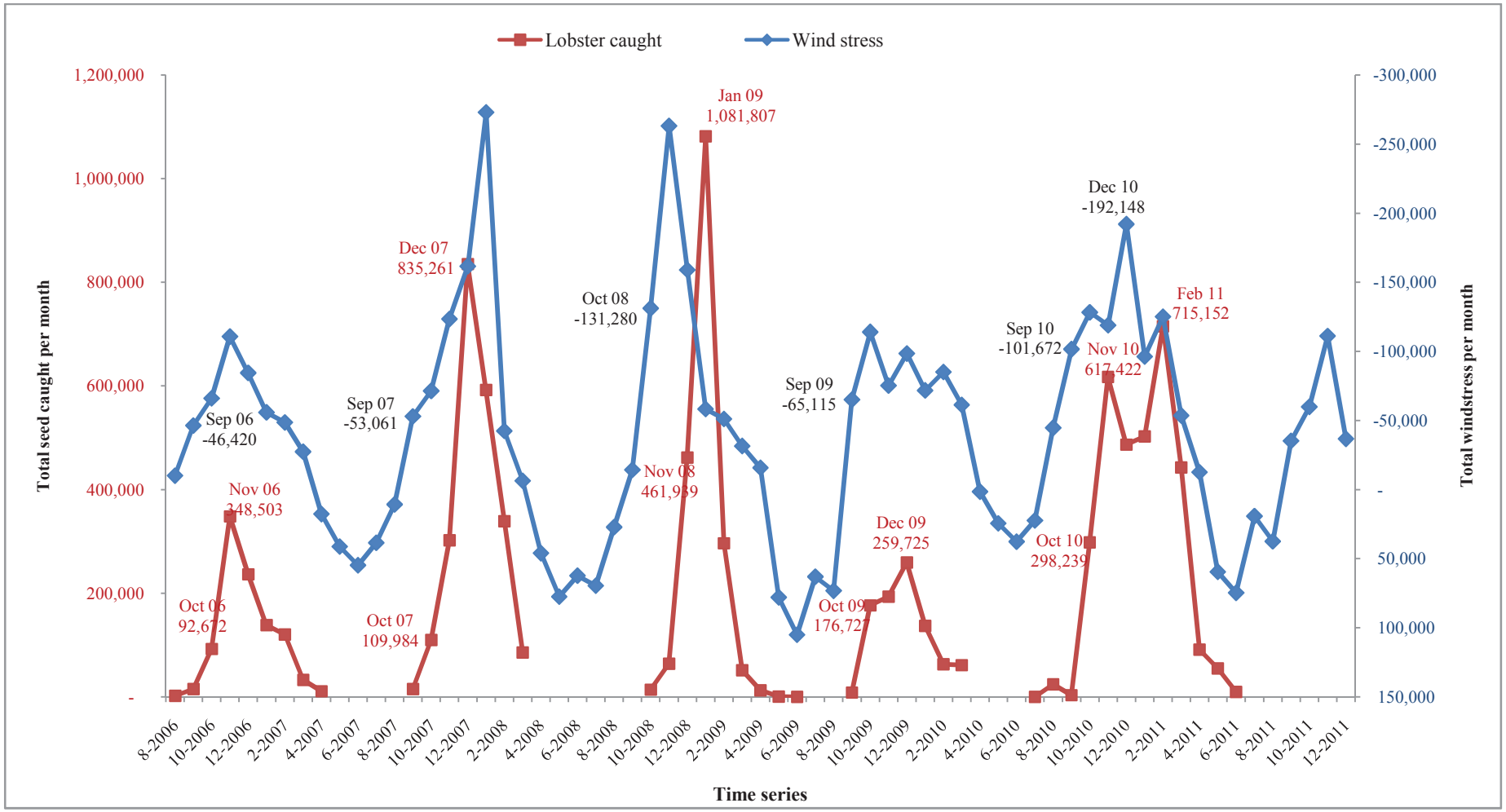

Figure 2.10 Time series plot from 2006 to 2011 of the long shore wind stress over the central Vietnam coastal waters of the South China Sea and of *Panulirus ornatus* puerulus monthly catch. A positive wind stress indicates a northward wind and a negative value a southward wind.

2.3.4 Wind stress and the correlation to seed catch of *Panulirus homarus* in Vietnam

The variation in total wind stress and fluctuation of total seed catch of *P. homarus* in Vietnam from 2006 to 2011 showed an inverse relationship (Figure 2.11). The total catch of seeds decreased with increasing wind stress in the season 2006-07. However, this value increased with wind stress increasing in the season 2010-11 (Figure 2.12; Polynomial trend line with R^2=0.992, p-value = 0.26 > 0.05).

Similar to the pattern of *P. ornatus*, a time series plot from 2006 to 2011 (Figure 2.13) shows that the seed catch of *P. homarus* also started to increase about one month after the southward wind stress started to become highly negative (in September or October) and peaked two or three months afterward in November to February.

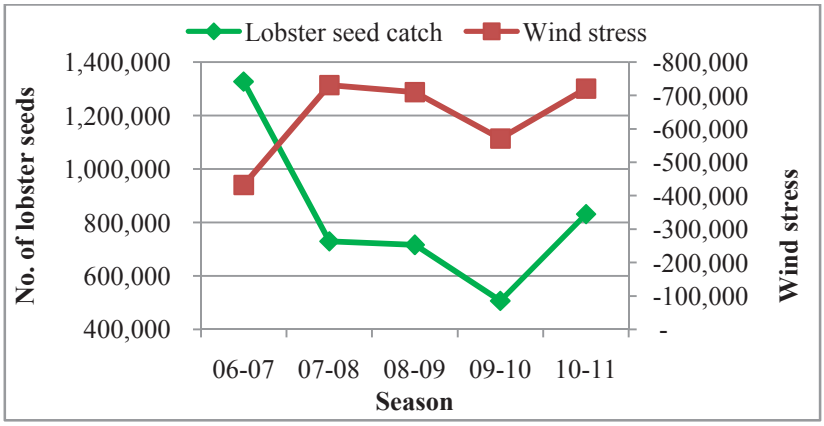

Figure 2.11 The variation of total wind stress and total seed catch of *Panulirus homarus* during five fishing seasons in Vietnam from 2005 to 2011.

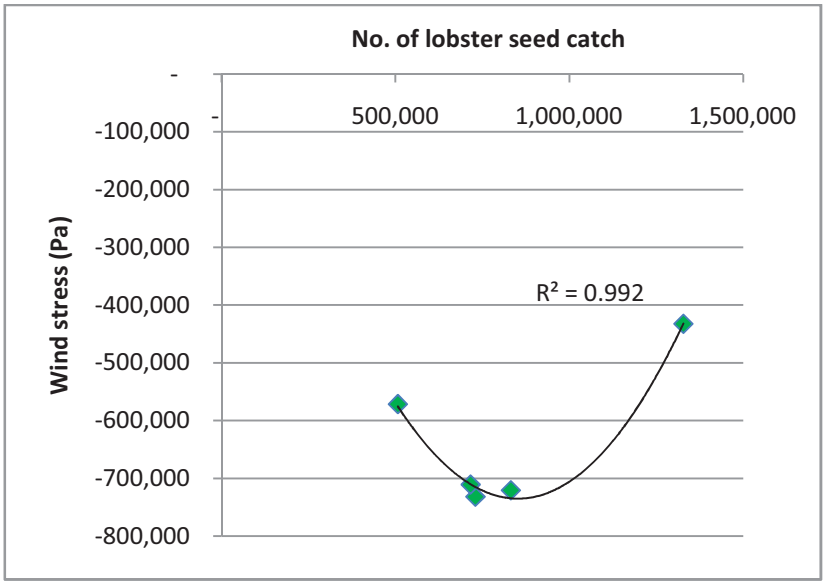

Figure 2.12 Scatter chart showing the correlation between total seed catch of *Panulirus homarus* and the wind stress value.

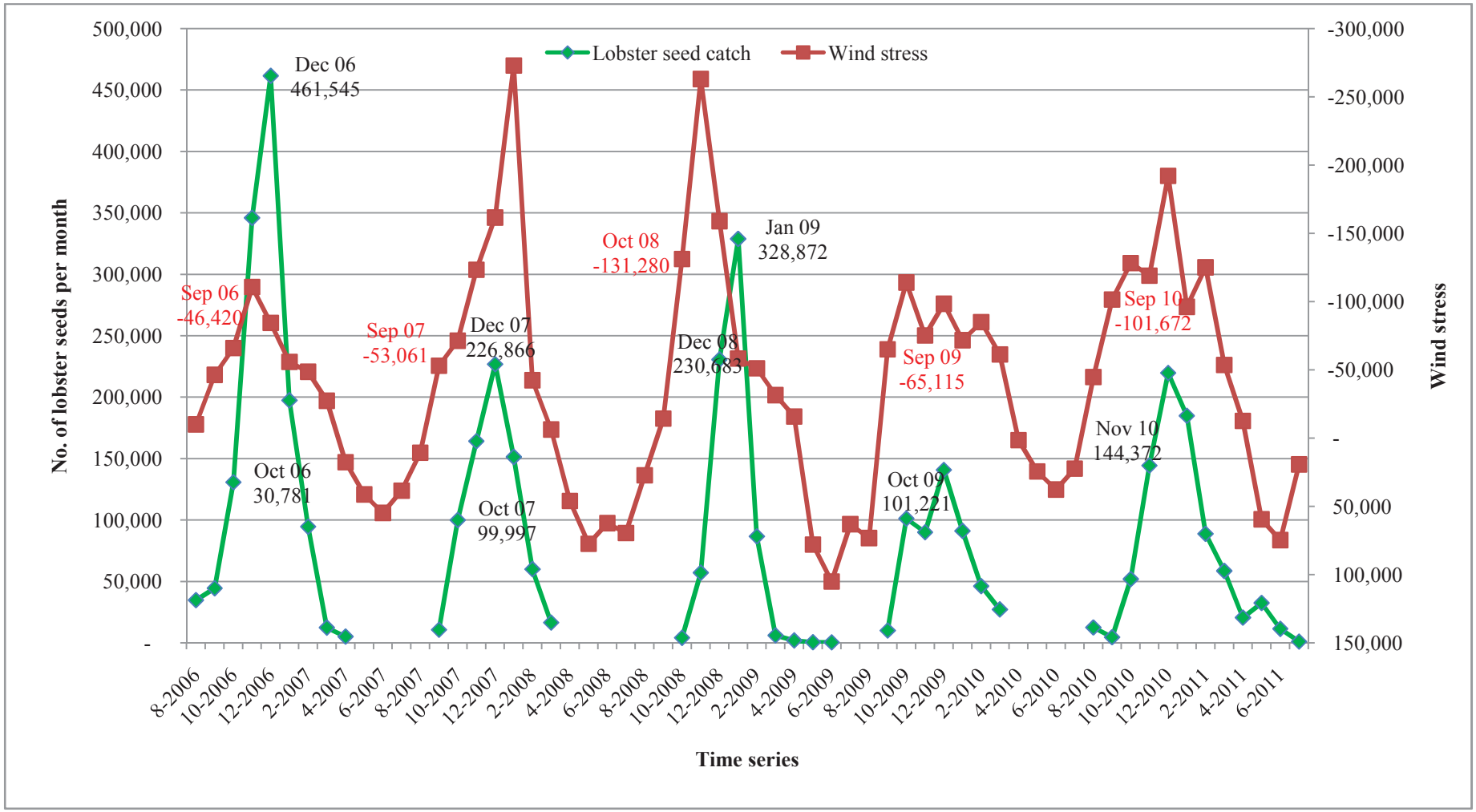

Figure 2.13 Time series plot from 2006 to 2011 of the long shore wind stress over the central Vietnam coastal waters of the South China Sea and of *Panulirus homarus* puerulus monthly catch. A positive wind stress indicates a northward wind and a negative value a southward wind.

2.4 DISCUSSION

The recruitment data collected indicated a large fluctuation in the monthly and annual pueruli catch of the two species of *Panulirus*, especially *P. ornatus*, over six fishing seasons from 2005 to 2011. There were similar patterns of spatial and temporal variation in total monthly catch in both species during the study period. The catch peaks occurred from November to February of the following year. In addition, the increase of total seed catch of *P. ornatus* was highly correlated with the southward wind stress during the study period. Therefore, the recruitment of *P. ornatus* in Vietnamese coastal regions appears to be predictable using wind stress as an indicator.

Previous studies also found a link between the settlement patterns of marine fish and invertebrates to wind-driven surface currents (Johnson *et al.*, 1984; Taggart & Leggett, 1987; Thorrold *et al.*, 1994). In *Panulirus* species, such as *P. argus*, a significant correlation was found (R= 0.32 to 0.34) between postlarval supply to the nearshore Florida Keys (USA) nursery and wind-forcing from the northeast during the winter (Acosta *et al.*, 1997). Similarly, Yeung *et al.* (2001) showed that 50% of the variation in *P. argus* settlement in Long Keys, Florida Bay was explained by wind-driven transport.

The *Panulirus* pueruli commonly found in nearshore waters of Vietnam have been estimated to be about 5 to 8 months of age(Booth & Phillips, 1994; Phillips & Matsuda, 2011). Therefore, these recruitment stocks in Vietnam might originate from spawning events occurring from May to August, and the larvae subsequently being carried to Vietnam by the wind-driven surface waters. However, the sources of recruitment stock of *P. ornatus* in Vietnam are unknown.

In terms of *P. homarus*, the recruitment of seeds could be partly explained by wind stress. The seed catch started to increase about one month after the southward wind stress started to become highly negative and peaked two or three months afterward. However, the correlation between the increase of total seed catch of *P. homarus* and the southward wind stress was very low (Polynomial trendline with R^2=0.992, p-value > 0.05). As a consequence, the recruitment of *P. homarus* in Vietnam coastal regions might be controlled by not only one main environmental factor.

If strong correlations can be established between environmental factors and the scale of recruitment pulses, this information would be useful to fishery managers as it will allow them to predict the next season's likely scale of recruitment and the number of wild pueruli that could be sustainably harvested as aquaculture seed.

CHAPTER 3: GENETIC MARKER DEVELOPMENT

3.1 BACKGROUND

Panulirus spp. are commercially important lobster species, occurring throughout the tropical eastern Indian Ocean, south-east Asia, Australia and the West Pacific, and are a species that is vulnerable to fishery collapse in heavily exploited regions (Long &Hoc, 2009; Jones C. *et al*., 2010). Investigations into the genetic structure of spiny lobster species are therefore essential for resource managers to allow them to effectively regulate the fisheries associated with them.

Several molecular markers are routinely used in genetic studies such as allozymes, restriction fragment length polymorphism (RFLP), mitochondrial DNA sequencing, and microsatellites (Thorpe *et al.*, 2000; Diniz *et al.*, 2005). Each marker provides different aspects of genetic information. Among them, mtDNA and microsatellites are ideal for identifying and quantifying the level of population structure within a species.

Mitochondrial DNA sequencing, particularly of the most rapidly evolving and highly variable control region, has been a useful tool for population genetic studies of many terrestrial and aquatic organisms (Avise, 2012). The control region, which includes the D-loop in vertebrates and is known as the AT-rich region in invertebrates, does not encode a functional protein and is therefore under fewer functional and structural constraints, resulting in a high average substitution rate (Diniz *et al.*, 2005). As it is generally the fastest evolving region in the mitochondrial DNA of vertebrates and invertebrates, the control region is more sensitive than protein loci as a marker of phylogeographic structuring for many organisms (Diniz *et al.*, 2005; Garcia-Rodriguez and Perez-Enriquez (2006) Avise, 2012).

Traditional microsatellite marker isolation protocols are laborious, costly and often inefficient (Zane *et al.*, 2002), thus limiting the accessibility of these powerful markers. Low-coverage whole-genome sequencing is emerging as a fast and cost-effective means of isolating large numbers of markers suitable for population genetic studies and the technology is proving particularly beneficial for conservation biologists from small laboratories working with non-model taxa (Abdelkrim *et al.*, 2009; Csencsics *et al.*, 2010; Saarinen & Austin, 2010). In this chapter, Roche 454 GS-FLXwhole-genome sequencing was used to isolate large numbers of microsatellite markers in *P. ornatus* largely following the techniques of Jones D. *et al.* (2011) and Todd *et al.* (2011). These microsatellite markers once isolated and validated were subsequently used to elucidate the population genetic structure of *P. ornatus* and *P. homarus* in ensuing chapters, as well as testing for their utility for genetic structures in several other species of *Panulirus* lobsters.

3.2 METHODS

Sixty-five samples of *Panulirus* spp. were collected from different locations in Torres Strait (Australia), Gerupuk (Indonesia) and Fiji. They were identified by the author via morphology characteristics and photos provided by fishermen and separated into four species, including 40 *Panulirus ornatus*, 17 *P. homarus*, 4 *P. versicolor* and 4 unknown *Panulirus* specimens (putatively, *P. argus*; Figure 3.1). Genomic DNA was isolated from 3x3mm of pleopod, or abdominal muscle tissues, by a CTAB extraction protocol (Sambrook & Russell, 2001). Genomic DNA then was re-suspended in 50µL of TE (10mM Tris–HCl, 1mM EDTA, pH=8.0). DNA quality was estimated based on gel electrophoresis, whilst quantity was assessed using a ND-1000 Spectrophotometer

(Nano-Drop® Technologies). DNA was diluted to 10–40ng/µLfor use in the ensuing polymerase chain reaction (PCR).

3.2.1 Mitochondrial DNA (mtDNA) control region

Whole and partial genome sequences including mtDNA control regions of *Panulirus ornatus*, *P. gracilis*, *P. stimpsoni*, *P. japonicus*, and *P. inflatus* from the NCBI DNA database were aligned using SEQUENCHER version 4.5 (GeneCode) and primers to amplify 809 base pairs of the mtDNA control region designed based on conserved sites using PRIMER3WEB version 3.0.0 (http://primer3.wi.mit.edu/). These primers were; PO_F2 5' - ATAAAGGTAATAGCAAGAATC - 3' and PO_R1 5' - CAAACCTTTTGTCAGGCATC - 3'.

Extracted DNA from two samples of each species of *P. ornatus* (OR_115; OR_116), *P. homarus* (HO_055; HO_056), *P. versicolor* (VE_002; VE_003) and the unknown species (Unknown_003; Unknown_007) was diluted to 10-40ng/µl for use in a polymerase chain reaction (PCR). The control region was amplified in 20µl reaction volumes containing ~5ng DNA, 1× TM buffer (Qiagen), 1.5mM of $MgCl_2$, 0.2mM of dNTPs, 0.1µM of Tag Red (Qiagen) and 0.3µM of forward and reverse primers. PCR was performed on a BioRadC1000 Thermal Cycler (cycling parameters: 3 min at 95 ºC, followed by 35 cycles of 95 ºC for 45s, 50 ºC for 30s, 72 ºC for 45s, before a final extension step of 72 ºC for 5 min).

PCR products were then run on a 1.5 % agarose gel for quantity and quality verification, and subsequently cleaned to remove excess primers by precipitation with isopropanol (Sambrook & Russell, 2001). A repeat region in the start of the reverse primed sequence resulted in deterioration of sequence. Consequently, only DNA sequences from the

forward primer was used. To verify nucleotide base calls each sample was sequenced twice at the Australian Genome Research Facility (AGRF), Brisbane (Australia).

Sequence data was aligned using Geneious (v. 6.0.5) with default alignment parameters and was checked manually for misalignments. These sequences were then aligned with other reference sequences to confirm the position on the complete mtDNA genome. A Neighbor-joining tree was also built for these sequences and incorporated the mtDNA of other species available on the NCBI database, including *Panulirus ornatus* (mtDNA complete genome, GB.GQ223286.1), *P. homarus* (mtDNA control region, GB.KF715509.1), *P. versicolor* (mtDNA complete genome, GB.KC107808), *P. argus* (mtDNA control region, GB.AY608724.1), *P. gracilis* (mtDNA control region, GB.EF565145.1), *P. inflatus* (mtDNA partial genome, GB.EF645597.1), *P. japonicus* (mtDNA complete genome, NC.004251.1), and *P. stimpsoni* (mtDNA complete genome, GB.GQ292768.1). Neighbor-joining treeswereconstructed using Geneious (ver. 6.0.5) and based on the Tamura-Nei genetic distance model.

3.2.2 Microsatellite markers

A pool of 10 larvae of *P. ornatus* provided by the Australian Institute of Marine Science (AIMS) at Cape Cleveland, Queensland, Australia were used to extract total genomic DNA using a CTAB protocol (Sambrook and Russell, 2001). Sequencing was then performed on Roche 454 GS-FLX instrumentation using Titanium chemistry, at the Australian Genome Research Facility (AGRF) in Brisbane, Queensland, Australia. From more than 100,000 raw sequence reads, thousands of perfect microsatellite repeats (di- to tetranucleotide motifs, with ≥ 10, 8 and 6 repeats respectively) were mined using iQDD (Meglécz *et al.*, 2010) and MSATcommander v.0.8.2 (Faircloth, 2008), with the following primer design parameters also used: a product length of 150-400bps;

annealing temp: 57 – 63°C; a percentage of G and C between 20 – 80%. Sequence reads were then filtered based on distance of identified primers to the beginning and end of microsatellite repeat sequences (>10bp distance between primers and repeat), and to comprise fragments between 150 – 360bp in size. Thirty of the most promising *in silico* mined microsatellites were then kept to be synthesised and trialled via PCR.

Primer pairs for the most promising microsatellites, including four which contained perfect dinucleotide repeats, seven which contained perfect trinucleotide repeats and the remaining 19 with tetranucleotide repeats, were synthesized with the forward primer containing a 5' 17 base pair lambda tag to enable indirect fluorescent labeling (Shimizu *et al.*, 2002).These 30 primer sets were then initially tested for amplification on 4-6 individuals of each of the different *Panulirus* species collected.

Microsatellites were amplified in 10μL reaction volumes containing 20ng DNA, 1× Type-it Multiplex PCR Master Mix (Qiagen), 0.04μM of forward primer, 0.14μM of fluorescently-labelled lambda tag (TET, FAM or HEX) and 0.2μM of reverse primer. PCR was performed on a BioRad C1000 Thermal Cycler (Cycling parameters: 5 min at 95°C, 28 cycles of 95°C for 30s, 58°C for 90s, 72°C for 30 s, and 60°C for 30 min).

After column purification of up to four co-loaded microsatellite loci through Sephadex G-50 resin, DNA fragments were separated on a MegaBACE 1000 capillary sequencer and sized with Fragment Profiler v 1.2 software (Amersham Biosciences), using a 400 base pair DNA ladder as an internal size standard. Genotypes were then run on an Amersham Biosciences Megabase 1000 Capillary Sequencer and scored using Fragment Profiler 1.2 (Amersham Biosciences). From this initial screening, the most polymorphic and easily scored microsatellite loci were chosen and used to genotype 40 *P. ornatus* from Torres Strait (Australia), and 17 *P. homarus*(Gerupuk, Indonesia). Similarly,

polymorphic microsatellite loci were tested on a total of four*P. versicolor* from Gerupuk (Indonesia) and four putative *P. penicillatus* samples from Fiji.

Summary statistics (the number of alleles, as well as observed and expected heterozygosities) for markers characterized for *P. ornatus* and *P. homarus* were calculated in GENALEX v.6.1 (Peakall & Smouse, 2006), which was also used to test for deviations from Hardy-Weinberg Equilibrium (HWE). Genepop on the web (http://genepop.curtin.edu.au/) was used to test for linkage disequilibrium among microsatellite loci. Corrections for multiple comparisons (HWE and linkage disequilibrium) were adjusted using False Discovery Rate (FDR; Benjamini & Hochberg, 1995). Polymorphic information content (PIC) was calculated for each locus in CERVUS v.3.0 (Kalinowski *et al.*, 2007). The presence of null alleles was also checked using Microchecker v.2.2.3 (van Oosterhout *et al.*, 2004).

3.3 RESULTS

3.3.1 Mitochondrial DNA (mtDNA) control region marker

The control region sequences were obtained from a total of 8 *Panulirus* individuals from the four different species. The sequence length was about 649bp (*P. homarus*) to 725bp (*P. versicolor*). According to the Neighbour-joining tree (Figure 3.2), the species identification of *P. ornatus*, *P. homarus* and *P. versicolor* were correct, as they matched 100% to their reference DNA in the NCBI database. It also suggested that the unknown specimen collected from Fiji were *Panulirus penicillatus*.

3.3.2 Microsatellite markers

Genotyping data of 30 microsatellites was obtained from 65 *Panulirus* samples. From this initial screening, 15 loci could be scored reliably. These loci were characterized using a population sample of 40 *P. ornatus* from the Torres Strait, Australia to evaluate their utility in population genetic analyses. Fourteen loci were further tested on 17 *P. homarus* individuals from Gerupuk, Indonesia (Fig. 3.1), nine of which could be scored reliably.

Further analysis showed that in both species of *P. ornatus* and *P. homarus*, no significant linkage disequilibrium was detected, with all microsatellite loci found to be in Hardy-Weinberg equilibrium after FDR correction (Table 3.2). However, null alleles were detected at Orn_05 and Orn_08 for *P. ornatus* and Orn_17 for *P. homarus*. Evidence of an insertion/deletion within the flanking region of Orn_11 was detected in the form of two alleles differing by a single base pair. High allelic diversity (mean Na=9.1 ±1.4) and observed heterozygosity (mean H_o=0.61 ±0.06 and 0.51 ±0.11) was observed for *P. ornatus* and *P. homarus*, respectively.

The 30 microsatellite markers were also tested in four individuals of *P. versicolor* and *P. penicillatus*. A positive control containing DNA from *P. ornatus* was included in each experiment to verify amplification success. Details of cross-species amplification success are summarized in Table 3.2. Ten and two markers were successfully amplified in *P. versicolor* (five of them showed polymorphisms) and *P. penicillatus*, respectively.

3.4 CONCLUSIONS AND DISCUSSIONS

Both mtDNA control region marker and microsatellite markers were developed and validated across different species of *Panulirus*. About 649bp (*P. homarus*) to 725bp (*P.*

versicolor) of themtDNA control region were successful amplified. These sequences perfectly matched the reference mtDNA of each species, which suggested that the control region of *Panulirus* is a good marker in identification at the species level.

Among the 30 developed microsatellites, 15 loci and 9 loci could be scored reliably for *P. ornatus* and *P. homarus*, respectively. Even though null alleles were detected at a few loci, high allelic diversity (mean Na=9.1 ±1.4) and observed heterozygosity (mean H_o=0.51 to 0.61 ±0.06 and 0.51 ±0.11) for *P. ornatus* and *P. homarus*, respectively, indicate that these microsatellite loci will be useful for studies of population genetic structure, connectivity and mating systems in these taxa. Although the number of individuals of *P. versicolor* and *P. penicillatus* tested was small (4 samples each), some loci were successfully amplified, which indicates that these microsatellite markers originally developed for *P. ornatus* may also be applicable to genetic studies in other species of *Panulirus*.

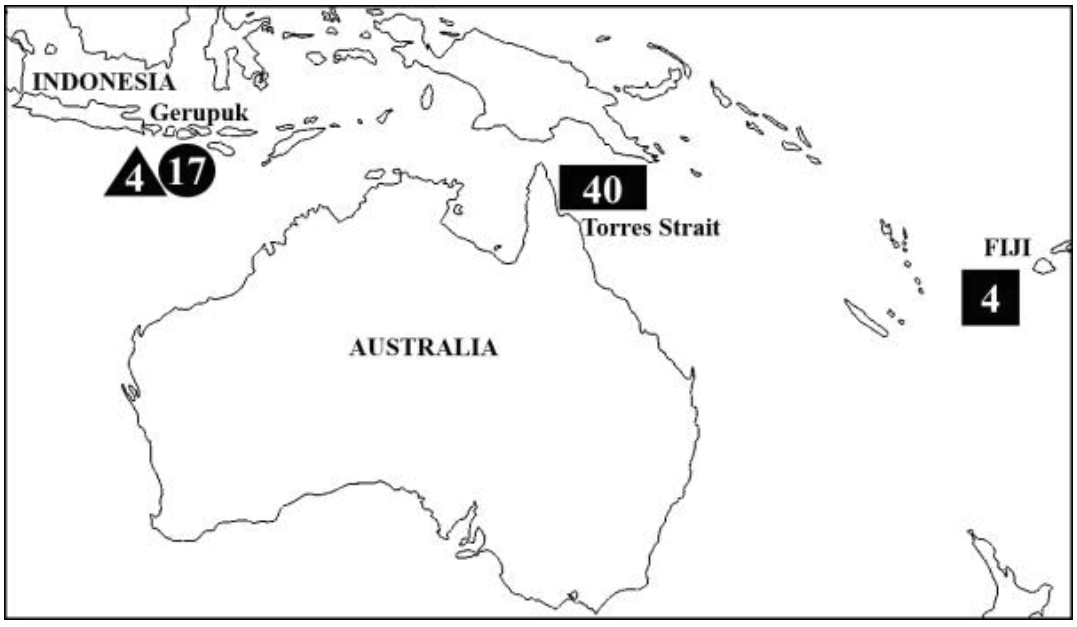

Figure 3.1 Sampling sites and number of samples of *P. ornatus* (rectangle), *P. homarus* (circle), *P. versicolor* (triangle) and the unknown species (*P. penicillatus*? - square).

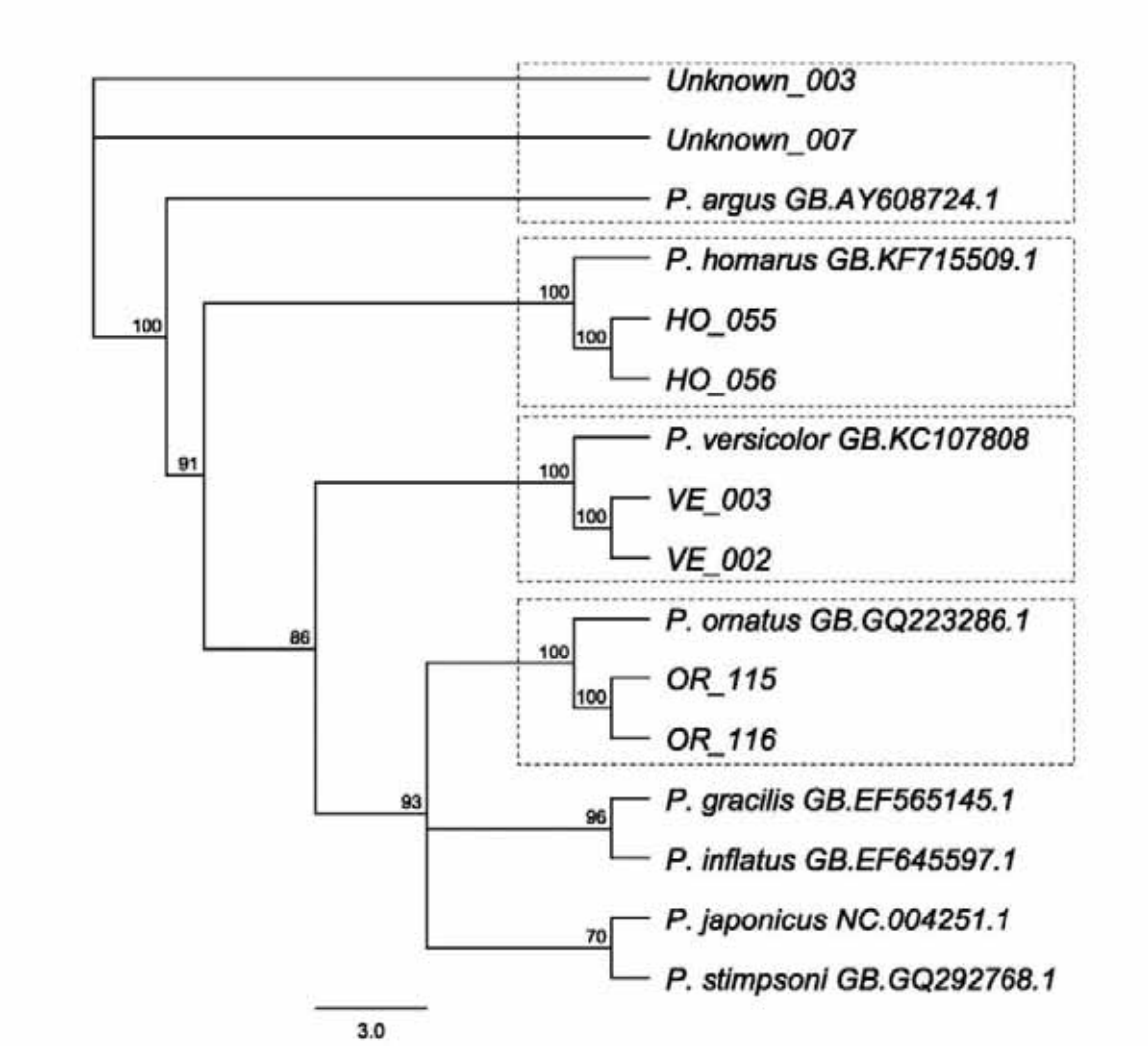

Figure 3.2 Neighbour-joining tree of eight *Panulirus* samples at the mtDNA control region.

Table 3.1 Characteristics of 30 microsatellite loci isolated for *Panulirusornatus* using a Roche 454 GLX whole-genome shotgun sequencing approach. T_A annealing temperature

Primer Name	Product length	Motif	No. of repeats	Primer_Left 5' to 3'	Primer_Right 5' to 3'	T_A (ºC)
Orn_01	137	ATCT	8	GAGCTTATGTTCAGGGGCAG	CTTATTCCCGAGACCAGCAG	58
Orn_02	257	CTTC	6	TGATCCACGAGCAGACACTC	TGGCTTTAACCAAGCAATCC	58
Orn_03	267	ATT	14	CCGCAGTGCTTTGTCTTGG	ATCGGCGTCAGTTCTCCAG	58
Orn_04	354	GCTG	6	TCTCGCCGATTTGGATAAAC	CGATCCATCAACACCACAAG	58
Orn_05	179	AGG	13	CGTCAAGTGTCGTGATGGC	TTGTTACTGATAGCGTTGTGC	58
Orn_06	229	GTT	13	GGGCCTCCAGCTTATACCTC	AACACCTGACGAACCTCGAC	58
Orn_07	161	AGT	13	GCATGCAAAGGGTCTTGCC	CATCGTCGCTACTTCCACG	58
Orn_08	149	ACT	13	TCACTTAACCATCAGTTGTACCC	GACACAGACGGGCCAGATG	58
Orn_09	164	CTT	12	ATTAACCCGGGCGTGGAAG	AGAATTGGGCCAGGTGAGG	58
Orn_10	255	GTT	12	TTCGGGCCATGGTTACGTC	ATCCCAAGTGGCCTCAGC	58
Orn_11	200	CTTT	10	AGGCCTCACTGGACGTTTC	ATTCCTGACGCTACCTCGC	58
Orn_12	297	ACAT	9	CCCTTAGCTGCTGGAAACC	TGCTCCTGTAGACCAGATTG	58
Orn_14	216	AGAT	8	GCCATTGAGAGGACGTTGTT	CCCAATGCTGGTTGAACATA	58
Orn_15	210	ATCT	8	GCTGTACTCGTATGGCAAGC	TCCTAGTGAACGCCTGGTC	58
Orn_16	150	AGAT	7	GGATGGCAGAGGAGGCAAC	ACATCGTGAGGAGGAAGTGG	58
Orn_17	289	AGAT	7	AGTAAGGTCCCAAAGGGAATG	CAGAGTTCTGCAGCTTTGAAATAG	58
Orn_18	341	ACAT	6	AGCCGAACGTTTGATTCCG	CAAATTGGAGGTGGGAGAAC	58
Orn_19	272	ATTT	6	AAGTGTCGAGGGTGGTTCC	GGGTTGGGCCATTCTTTCG	58
Orn_20	314	CTGT	6	TCAGGTGTAGGACATCCGC	CAAGGTTAAGAGACGGGACAAC	58
Orn_21	228	AGGT	6	CTGCACGAGAAAGTCCAGC	TGACGGGTGGTAAAGTGGG	58
Orn_22	148	ACAG	6	TCGCATAAGTGGGAGGCTG	AGACTGCTGAGGCACCTTG	58
Orn_23	115	AAGG	6	GTCTGGGACGAGGAACCTG	TACCCTGTGCATAAACGCC	58
Orn_24	330	ATTT	6	GTTGGGCCATCCTTTCGTC	CTTGCGGTACCTCGCAAAC	58
Orn_25	231	ATCT	6	CGTAGCTAGGACGCCATTTG	TTGAGCCAACGTCCCAGTC	58
Orn_26	96	ACAT	6	CAGCGGTGCAAGGAAACAG	GATAGGGAGCTGTGGTTTCG	58
Orn_28	198	TG	27	CTCTTTTGTGAAGGAGCGATG	TCCTACCGTTCGTCTCTTCTG	58
Orn_29	200	AC	19	AAACGAAACACGGCAAGTTC	CTATCACACCCCTTCCCCTC	58
Orn_30	120	CA	19	CGGCAGCTCGTTACTATGAAG	GGAGGGTTTCGTTTCTAGCC	58
Orn_31	121	TC	10	CGACGAAGAAAAGGAAGTCTG	ACCGCTGGAGCTAAGTATGG	58
Orn_32	241	AGAT	6	AGACTTGCCCTCTGCTAGTG	AGTGTCCTCATGTGTTGGC	58

Table 3.2 Genetic indices for microsatellites characterized in *P. ornatus* and other *Panulirus* species. *N*= sample size, *NA* = number of alleles, H_O = observed heterozygosity, H_E = expected heterozygosity, P HWE = Hardy-Weinberg equilibrium significance value at P<0.05 after FDR correction, ns = non-significant, * = significant, PIC = polymorphic information content, Emty spaces = no amplifications.

		Orn_01	Orn_02	Orn_04	Orn_05	Orn_08	Orn_11	Orn_12	Orn_14	Orn_15	Orn_16	Orn_17	Orn_18	Orn_20	Orn_21	Orn_22	Orn_23	Orn_25	Orn_29	Orn_32
P. ornatus	*N*	40	40	40	40	40	40	40			40	40	40	39	40		39	40		40
	N_A	8	4	3	14	11	17	23			9	8	9	8	7		2	8		3
	Size range	139-176	258-278	372-378	187-225	150-186	175-235	312-396			163-193	288-317	352-370	325-362	240-264		232-236	184-212		249-261
	H_O	0.78	0.63	0.38	0.68	0.7	0.83	0.98			0.75	0.45	0.7	0.41	0.73		0.03	0.73		0.63
	H_E	0.72	0.63	0.42	0.86	0.83	0.9	0.94			0.77	0.54	0.68	0.47	0.82		0.03	0.74		0.51
	PIC	0.69	0.56	0.34	0.85	0.81	0.89	0.94			0.74	0.51	0.66	0.45	0.8		0.03	0.7		0.41
	P HWE	0.86	0.41	0.82	0.01	0.76	0.51	0.22			0.44	0.96	0.97	0.2	0.27		0.94	0.81		0.15
	HWE sig	ns	ns	ns	ns	ns	ns	ns			ns	ns	ns	ns	ns		ns	ns		ns
P. homarus	*N*			17	17		17	17			17	16		17	17					17
	N_A			4	6		10	11			6	5		4	1					1
	Size range			364-374	179-191		167-199	300-344			159-177	289-361		327-339	233					253
	H_O			0.53	0.71		0.82	0.82			0.76	0.38		0.59	0					0
	H_E			0.51	0.65		0.84	0.87			0.72	0.71		0.55	0					0
	PIC			0.53	0.58		0.85	0.9			0.75	0.66		0.48	0.1					0.22
	P HWE			0.71	0.55		0.48	0.92			0.99	0		0.89						
	FDR sig			ns	ns		ns	ns			ns	*		ns						
P. versicolor	*N*	4		4			4		4	4	4					4		4	4	4
	N_A	1		1			4		1	2	3					1		5	6	1
	Size range	136		387			166-187		181	219-246	164-172					116		204-220	147-169	255
P. penicillatus	*N*			1															2	
	N_A			1															1	
	Size			365															143	

CHAPTER 4. GENETIC POPULATION STRUCTURE OF *Panulirus ornatus*IN THE SOUTHEAST ASIAN ARCHIPELAGO

4.1 BACKGROUND

The Ornate Spiny Lobster, *Panulirus ornatus*,is denizen to tropical waters of the Indo-West Pacific, where the species is of significant commercial importance supporting local capture fisheries and developing aquaculture operations. *Panulirus ornatus* pueruli (lobster post-larval stage) are heavily exploited as seed-stock for aquaculture in South-East Asia, particularly in Vietnam and Indonesia, where wild pueruli are collected from the ocean in large numbers (Jones C. *et al.*, 2010). Of concern to fisheries managers are the large fluctuations in juvenile recruitment that have been repeatedly experienced in recent years and which are taken as early indicators that the fishery may be on the verge of collapse in heavily exploited regions. As an example of this apprehension, in 2006-2007 and 2009-2010, pueruli wild harvest in Vietnam was only ~50% of that caught in other years (Jones C. *et al*., 2010; Long & D. Hoc, 2009). This high variability in puerulus settlement raised concerns as to whether the annual removal of 1-2 million pueruli by fishers in Vietnam was significantly impacting the demography of the species, particularly that of adult populations (Jones C. *et al.*, 2010; Long & D. Hoc, 2009). However, very little is understood about the population distribution and dynamics of *P. ornatus* and it is not known where the pueruli being harvested originate from. An investigation into the connectivity among spiny lobster populations is therefore needed to provide data on the resilience and sustainability of heavy exploitation, as well as to provide information on larvae sources and sinks.

As adults, *P. ornatus* individuals occupy diverse habitats from shallow (1 to 8m depth) to deep waters (>50m), sandy or muddy substrates, coral reefs, rocky bottoms and even turbid coastal waters near river mouths (Holthuis, 1991). Adults are known to migrate hundreds of kilometers to form large spawning aggregations, with some tagged adult *P. ornatus* from the northern Torres Strait, Australia, for instance, being shown to migrate up to 500km to a presumed common spawning ground in the Gulf of Papua (Booth & Phillips, 1994; MacFarlane & Moore, 1986; Moore & MacFarlane, 1984). Furthermore, *P. ornatus* larvae have a long planktonic phase of 4.5 to 7 months before settlement as pueruli (Booth & Phillips, 1994; Phillips & Matsuda, 2011). *Panulirus ornatus*, therefore, is a species with life-history attributes indicative of long distance dispersal capability and consequently, the potential for high rates of gene flow between populations.

Several approaches are available to evaluate the connectivity between *P. ornatus* populations, including genetic markers (e.g. mitochondrial DNA or microsatellites), geochemical markers (e.g. microchemical signatures in shells), and/or the utilization of high-resolution biophysical models. Whilst the use of any one of the above approaches can provide information on connectivity, their use in combination helps to evaluate more accurately a species' dispersal potential by providing estimates for both evolutionary and contemporary time scales. For example, combining genetic studies and oceanography-driven larval dispersion modelling can provide information on genetic structure that has formed over eons, as well as elucidating contemporary physical connectivity opportunities among populations (Gilg & Hilbish, 2003; Cowen & Sponaugle, 2009; Feutry *et al.*, 2013). The genetic structure of populations is strongly influenced by gene flow, which is the result of effective migration and/or dispersal (Hartl & Clark, 1997; Hallerman, 2003). For marine species with complex life cycles,

dispersal typically occurs during the earliest life history stages (spore, egg, or larva) where the organism is dispersed, often passively by oceanic currents, until it settles to the benthic adult phase. Active dispersal at later stages (juveniles and adults) may also occur for some species (Cowen & Sponaugle, 2009). For those marine species with long pelagic larval phases, extended larval development durations potentially provide the opportunity for larvae to be transported long distances by surface ocean currents, thus, enhancing gene flow (Radtke *et al.*, 1988; Keith, 2003;Cowen & Sponaugle, 2009; Domingues *et al.*, 2010).

The long pelagic larval duration (PLD) of *P. ornatus* suggests that dispersal over very large distances (thousands of km) across the South-East Asian archipelago may be possible if oceanographic features do not impose a barrier to dispersal. However, the South-East Asian archipelago is a region with numerous surface ocean currents and complex oceanography and, as yet, there is no fine-scale oceanographic model encompassing the whole archipelago to inform understanding of genetic structure, or connectivity of marine populations. There were a number of fine-scale models focusing on restricted areas within the archipelago (e.g. Schiller et al., 2008), and a few medium-scale models of the whole domain (e.g. Gaspar et al., 2012). These models, however, have a grid size too coarse to resolve the current fluxes through the Philippines Straits and, as a result, they largely ignore the connectivity between the Philippine Sea, the South China Sea and the western Pacific Ocean (Gordon, 2005). Accordingly, oceanographic models are currently not suitable for studying the spatially explicit connections among populations of *P. ornatus* within the South-East Asian Archipelago and improved models are needed.

The present study used molecular genetic techniques to elucidate for the first time the genetic population structure of *P. ornatus* within the South-East Asian Archipelago. It then developed an oceanographic dispersal model based on incorporating oceanographic datasets and aspects of lobster reproductive biology (including known sites and seasonal times of spawning, larval migration, and known arrival times of pelagic larvae at specific locations) to explain the evident patterns of genetic structure.

4.2 METHODS

4.2.1 Tissue collections and DNA extraction

From October 2009 to March 2011, a total of 216 *Panulirus ornatus* individuals were sampled from two sites in Vietnam, three in Indonesia, and one in Australia (Figure 4.1). Specimens from Vietnamese populations were all pueruli, while those from Indonesia were both adults and pueruli. Vietnamese lobster specimens came from the central coastal waters of the Da Nang and Binh Thuan provinces, while Indonesian samples were collected from North Sumatra, Lombok and West Timor. Australian samples were collected from wild-caught Torres Strait juveniles. All samples (pleopods from adults or abdominal muscle tissue from juvenile lobsters) were preserved immediately in a DMSO-salt preservative solution (Dawson *et al.*, 1998). Genomic DNA (gDNA) from all lobster samples was extracted from 4mm^2 pleopod clips or from the abdominal muscle tissue of juveniles using a modified CTAB protocol (Adamkewicz & Harasewych, 1996).

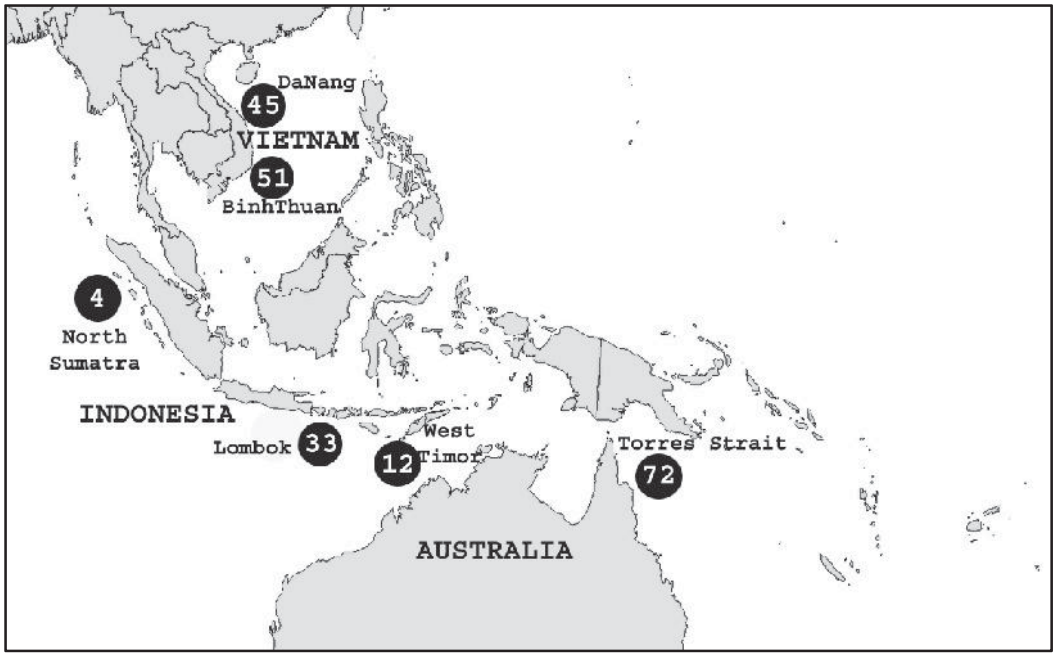

Figure 4.1 Sampling sites and number of *Panulirus ornatus* specimens collected from across the tropical waters of the Indo-West Pacific region. The number of individuals sampled at each location are indicated within the black circles.

4.2.2 Mitochondrial DNA (mtDNA) control region

Whole and partial genome sequences including mtDNA control regions of *Panulirus ornatus*, *P. gracilis*, *P. stimpsoni*, *P. japonicus* and *P. inflatus* from the NCBI database were aligned using SEQUENCHER v.4.5 (GeneCode) and primers to amplify 809 base pairs of the mtDNA control region designed based on conserved sites using PRIMER3WEB version 3.0.0 (http://primer3.wi.mit.edu/). These primers were; PO_F2 5' - ATAAAGGTAATAGCAAGAATC - 3' and PO_R1 5' - CAAACCTTTTGTCAGGCATC - 3'

Extracted DNA from samples was diluted to 10-40ng/µl for use in a polymerase chain reaction (PCR). The control region was amplified in 20µl reaction volumes containing ~5ng DNA, 1× TM buffer (Qiagen), 1.5µM of $MgCl_2$, 0.2µM of dNTPs, 0.1µM of Tag Red (Qiagen) and 0.3µM of forward and reverse primers. PCR was performed on a

BioRadC1000 Thermal Cycler (cycling parameters: 3 min at 95 ºC, followed by 35 cycles of 95 ºC for 45s, 50 ºC for 30s, 72 ºC for 45s, before a final extension step of 72 ºC for 5 min). PCR products were then run on a 1.5% agarose gel for quantity and quality verification, and subsequently cleaned to remove excess primers by precipitation with isopropanol (Sambrook & Russell, 2001). A repeat region in the start of the reverse primed sequence resulted in deterioration of sequence. Consequently, only DNA sequence from the forward primer was used. To verify nucleotide base calls each sample was sequenced twice at the Australian Genome Research Facility (AGRF), Brisbane, Australia.

The statistical packages MEGA v.4.1 (Kumar *et al.*, 2008), DNAsp v.5.1 (Rozas *et al.*, 2003) and ARLEQUIN v.3.11 (Excoffier *et al.*, 2005) were used to perform calculations of nucleotide diversity, haplotype diversity and Φ_{ST}. For calculations of the statistical significance of Φ_{ST} values, a significance test with 10,000 permutations was undertaken with ARLEQUIN v.3.11 (Excoffier *et al.*, 2005). The median-joining network (Bandelt *et al.*, 1999) for the haplotypes was constructed using NETWORK v.4.6.1.0 (http:\\www.fluxus-engineering.com).

4.2.3 Microsatellite markers

Ten highly polymorphic microsatellite markers (Chapter 3; Dao *et al.*, 2013) were used for population genetic investigations. DNA was diluted to 10–40ng/µl for use as template in a polymerase chain reaction (PCR). Microsatellites were individually amplified in 10µl reaction volumes containing ~20ng DNA, 1× Type-it Multiplex PCR Master Mix (Qiagen), 0.04µM of fluorescent labelled forward primer (TET, FAM or HEX), and 0.2µM of reverse primer. PCR was performed on a BioRadC1000 Thermal Cycler (cycling parameters: 5 min at 95 ºC, followed by 28 cycles of 95 ºC for 30s, 58

ºC for 90s, 72 ºC for 30s, before a final extension step of 60 ºC for 30 min). The PCR products then were checked for consistent amplification by visualization on a 1.5% agarose gel. After this step, PCR products were pooled according to size, fluorescent label, and product quantity and the pooled products were purified using Sephadex G-50 resin, before loading onto a Megabace 1000 Capillary Sequencer for size separation of alleles (Amersham Biosciences). Alleles were scored on the basis of fragment size using Fragment Profiler 1.2 (Amersham Biosciences).

Summary statistics such as the number of alleles, as well as observed and expected heterozygosity, were calculated for microsatellites in GENALEX v.6.1 (Peakall & Smouse, 2006), which was also used to test for deviations from Hardy-Weinberg Equilibrium (HWE). GENEPOP on the web (http://genepop.curtin.edu.au/) was used to test for linkage disequilibrium among microsatellite loci. Corrections for multiple comparisons (HWE and linkage disequilibrium) were adjusted using the False Discovery Rate (FDR) method (Benjamini & Hochberg, 1995). Polymorphic Information Content (PIC) was also calculated for each locus with CERVUS v.3.0 (Villanoy, 2004). The presence of null alleles and scoring errors were checked using MICROCHECKER v.2.2.3 (van Oosterhout *et al.*, 2004).

The examination of population genetic structure of *P. ornatus* based on microsatellite markers was conducted in two ways. First, the stepwise mutational model (SMM) was used to partition genetic variation within (F_{IS}) and among (F_{ST}) populations (Weir & Cockerham, 1984; Neigel, 2002). The SMM calculates mutations due to the gain or loss of single repeat units (Kimura & Ohta, 1978). To test for the appropriateness of the SMM, BOTTLENECK v.1.2.02 (Piry *et al.*, 1999) was used. In addition, the level of genetic structure was analysed using an Analysis of Molecular Variance (AMOVA)

with 10,000 permutations, as well as calculating pairwise F_{ST} comparisons between populations, both of which were carried out with ARLEQUIN v. 3.5 (Excoffier *et al.*, 2005).

Further to these analyses, the Bayesian clustering algorithm implemented in STRUCTURE v.2.2.3 (Pritchard *et al.*, 2000) was used to determine spatial genetic discontinuities by inferring the highest probable number of genetic clusters present within the dataset with prior knowledge of the individual's origin. Individuals are placed in K predetermined sub-groups based on their likelihood of belonging to that sub-group calculated using allele frequencies of multiple loci. K was chosen in advance and ranged from 1 to 10 and the populations were assumed to be admixed (an individual could belong to any population) in origin. Burn-in and run length were set to 100 000 MCMC (Markov chain Monte Carlo) repetitions and each run was iterated 10 times. This approach implements a model-based clustering method for inferring population structure and assigning individuals to the most probable genetic sub-group or population. Structure Harvester (http://taylor0.biology.ucla.edu/structureHarvester/) was used to determine optimum number of clusters in this analysis. CLUMPP (http://www.stanford.edu/group/rosenberglab/clumpp.html) also was used to average across the replicate run and outputs were put through DISTRUCT (http://www.stanford.edu/group/rosenberglab/distruct.html) to graph average q values.

4.2.4 Larval dispersal and connectivity

In order to understand *P. ornatus* population genetic structure throughout the South-East Asian Archipelago, both physical and biological data was integrated to develop a larval dispersal model. Here, firstly a literature review was undertaken and expert

opinion from relevant fisheries scientists sought to identify spawning ground locations within the archipelago. One spawning ground is known to be located in the southeast of the Gulf of Papua, Papua New Guinea (PNG), where Torres Strait lobsters spawn during the summer months from November to March (Moore & MacFarlane, 1984; Dennis *et al.*, 2001; Turnbull *et al.*, 2004; Williams, 2004). A second cluster of spawning grounds has been identified from the Philippines, where lobsters spawn from May to August (Juinio-Menez & Gotanco, 2004; Villanoy, 2004). Other cues that were incorporated into the model included the observation that 3 month old larvae appear in May-June at the southern tip of PNG (Dennis *et al.*, 2001; Pitcher *et al.*, 2005), and on the eastern side of the Gulf of Papua also by May-June. Also, the central coast of Vietnam receives arrivals of pueruli in September-December in the North (15^{o} N) and November to January in the South (12^{o} N; Tan Dao, unpublished data; Jones C. *et al*., 2010). Another cohort of pueruli arrives to northern Sumatra in the Indian Ocean in November-December (Jones & Priyambodo, unpublished data). Finally, Lombok, Indonesia receives two cohorts of *P. ornatus* pueruli at different times of the year, namely one cohort arriving in December-February and a second cohort arriving in August-November (Figure 4.4; Jones C. *et al.*, 2010). From Lombok, the larvae must reach and settle in Torres Strait by February during the northern winter monsoon season (November – February; Figure 4.4; Qu & Meyers, 2005) before March-April when the wind reverses and generates a westward current that would inhibit the arrival of larvae (Wolanski *et al.*, 2013). In the model the maximum length of dispersal time for each generation of *P. ornatus* was based on pelagic larvae duration of the species, which ranges from 4.5 to 7 months, equal to about 135 to 210 days (Dennis *et al.*, 2001; Phillips, 2013).

This biological data was then merged with oceanographic data. A monthly map of the mean surface water circulation in the South-East Asian Archipelago was constructed, focusing on different months for different areas based on the known age of lobster larvae reportedly found at that time of year and at that geographical point. The focus of simulations was on surface currents, because in Vietnam fishermen find the larvae or pueruli primarily within the top 15m of the water column, which is similar to findings in Dennis *et al.* (2001). The main source of surface current data was derived from the ARGO program (http://www.aoml.noaa.gov/phod/argo/introduction_argo.php) that provides the most reliable synoptic distribution of the observed currents, but had the limitation of providing only a few data points from the South China, Philippines and Indonesian Seas; for those regions the results of other field studies were integrated (listed in Table 4.1), together with the results of the regional oceanographic models of Forbes & Church (1983); Manh & Yanagi (2003); Liang *et al.,* (2008);Schiller *et al.,* (2008);Potemra & Qu (2009);Mayer *et al.,* (2010); Metzger *et al.,* (2010); Condie (2011); Cravatte *et al.,* (2011); and, for the South China Sea only, Farshid Daryabor (unpublished data). These studies were used to derive the monthly distribution of the speed and direction of surface currents throughout the South-East Asian Archipelago, from which streamlines were identified for connectivity between sites where lobster data was available. A map was developed to visualize transport pathways of the larvae from spawning grounds using Microsoft Visio software v.2003. Another map of the water circulation was also created by combining different current modelling figures with the current vectors at a certain time.The length (L) between two sites was measured by Distance Calculator in Google Maps (http://www.mapdevelopers.com/distance_finder.php)

Table 4.1 Field studies providing data of monthly surface currents for the South-East Asian Archipelago. ADCP = Acoustic Doppler Current meter.

Author	Data source	Data Period
ARGO (2013)	Ocean drifters	2000-2013
Caravatte *et al.* (2011)	Shipborn ADCP	1985-2007
Condie (2011)	NCEP-NCAR40-year Reanalysis dataset	1982-1997
Forbes & Church (1983)	National Aeronautics and Space Administration (NASA)	1978-1979
Liang *et al.* (2008)	Shipborn ADCP	1997-2001
Manh & Yanagi (2003)		
Mayer *et al.* (2010)	Data of the World Ocean Atlas	1970-2006
Metzger *et al.* (2010)	Digital Bathymetric Data Base 2 (DBDB2)	2004-2006
Potemra & Qu (2009)	National Oceanic and Atmospheric Administration (NOAA)	
Schiller *et al.* (2008)	ARGO data	1992-2006

The estimated time for the larvae to reach different locations was calculated by (Fischer,1979),

$$t = L/u$$

where L is the length between two sites and is calculated not as a straight line, but as the length of the streamline of the flow field joining these two sites, and u is the average speed of surface ocean current along that streamline during that period.

4.3 RESULTS

The Indonesian samples are a mix of adults and pueruli. Therefore, different analyses were conducted for separated age groups, as well as one mixed group. These analyses found no genetic differences between the different sample types and as a result they were pooled as a single mixed sample for population genetic analyses.

4.3.1 Genetic variation of the mtDNA control region

Nucleotide sequences of the control region were determined for 168 *P. ornatus* individuals. A small number of samples for which DNA was extracted failed initial quality control checks and were not successfully sequenced. From the 168 individuals sequenced successfully a total of 140 haplotypes were detected, with 84 polymorphic sites (13.6 % of 648bp) identified (Table 4.2).

Table 4.2 Genetic indices for mtDNA control region characterized in *Panulirus ornatus* from six sample sites/collections. *N* = sample size; *H* = number of haplotypes; *hd* = haplotype diversity; *Pi* = nucleotide diversity.

Population	*N*	*H*	*hd* ± SD	*Pi* ± SD
Torres Strait	54	51	0.998 ± 0.004	0.098 ± 0.009
West Timor	11	10	0.982 ± 0.046	0.094 ± 0.028
Lombok	27	26	0.997 ± 0.011	0.113 ± 0.018
North Sumatra	4	4	1.000 ± 0.177	0.079 ± 0.020
Binh Thuan	43	42	0.999 ± 0.005	0.099 ± 0.012
Da Nang	29	26	0.993 ± 0.011	0.094 ± 0.016
Mean	28	26.5	0.997 ± 0.001	0.099 ± 0.006

The *P. ornatus* mtDNA control region was found to exhibit an extremely high mutation rate, resulting in high haplotypic diversity whereby almost every lobster individual possessed a unique haplotype (Table 4.2). Haplotype diversities ranged from 0.982 to 1.000 within populations, while nucleotide diversities indicating the degree of polymorphism within a population/ sample collection ranged from 0.079 (North Sumatra) to 0.113 (Lombok).

No significant population subdivision was detected among the populations sampled, with a non-significant unbiased fixation index evident (Φ_{ST} = -0.008; P = 0.922 ±0.003). All of the genetic variation measured with mtDNA occurred within populations, with no detectable among-population variance (Table 4.3). In addition, no evidence of genetic structure was detected across the wide geographical range sampled from the Torres Strait of Australia to Vietnam and Indonesia, with negligible and non-significant

pairwise Φ_{ST} values between populations being very low (from -0.076 to 0.004, P >0.05;Table 4.4). As further evidence for widespread gene flow and lack of genetic structure the haplotype network tree showed no clustering of haplotypes into geographical regions, or location based groups, with the majority of haplotypes being single or unique units (Figure 4.2). Therefore, mtDNA based on the control region provided no evidence for genetic population structure in *P. ornatus* across the geographical range sampled.

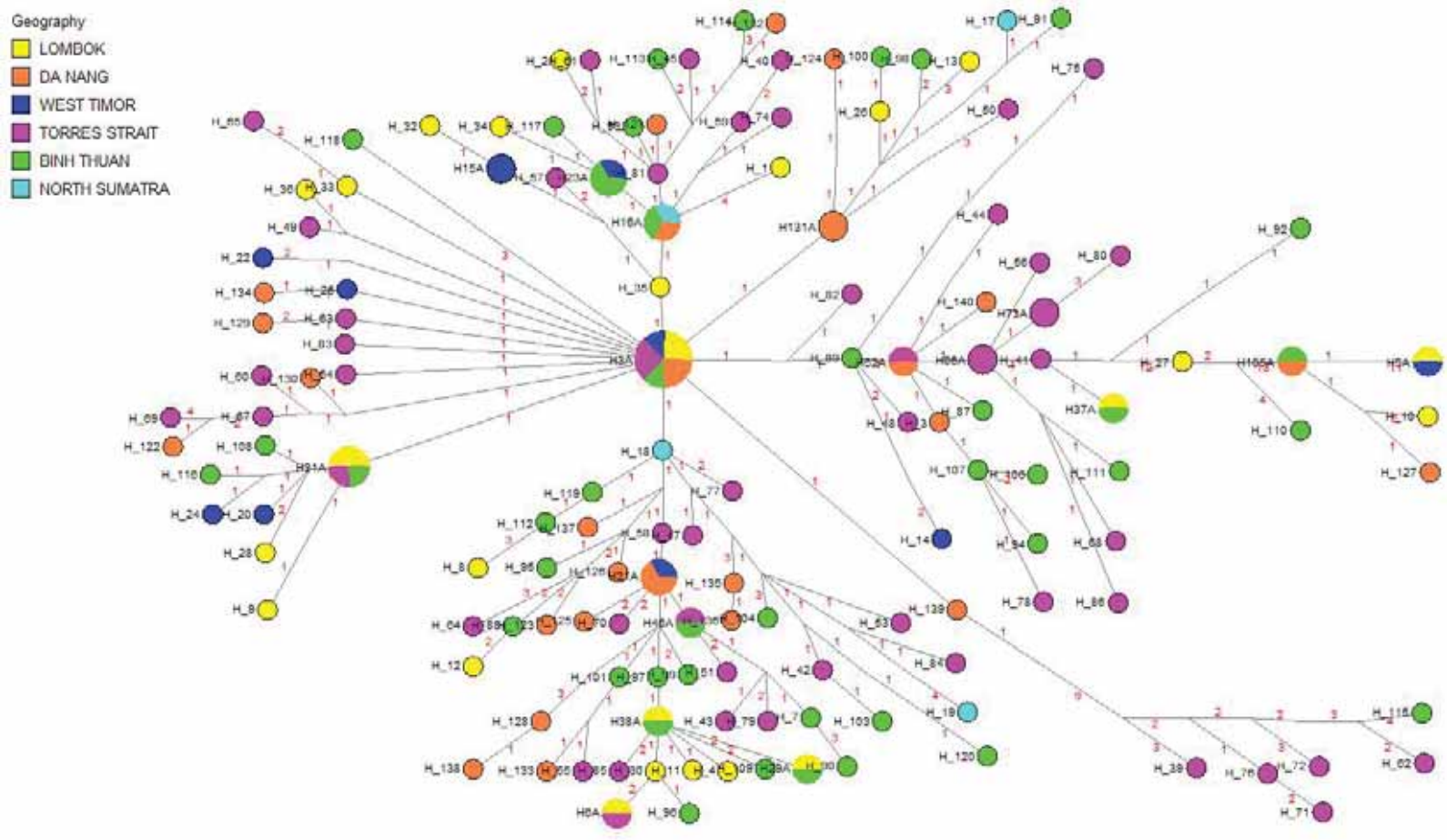

Figure 4.2 Haplotype network of *Panulirus ornatus* control region sequences from six collection locations from the South-East Asian Archipelago. Each circle represents a haplotype, whose diameter is proportional to the number of individuals with that haplotype. The numbers on the connecting lines are the number of mutations between haplotypes.

Table 4.3Summary table of analysis of molecular variance (AMOVA) describing the partitioning of genetic variation for six *Panulirus ornatus* populations based on both mtDNA control region sequences and 10 microsatellite loci.

	Source of variation (%)			
	Among population	**Within population**	Φ_{ST}/F_{ST}	**P ± SD**
mtDNA-control region	-0.80	100.80	-0.008	0.922±0.003
Microsatellites	0.26	99.74	0.003	0.195±0.004

Table 4.4 Genetic differentiation between *Panulirus ornatus* from collection locations using pairwise Φ_{ST} for mtDNA-control region (upper value) and for microsatellite loci (lower value). No significant F_{ST} value was found after correction using FDR.

Localities	**Torres Strait**	**West Timor**	**South LomBok**	**Binh Thuan**	**Da Nang**
Torres Strait		-0.011	0.002	0.000	0.004
West Timor	0.006		-0.038	-0.018	-0.004
LomBok	-0.003	0.008		-0.015	-0.002
Binh Thuan	0.001	0.002	0.004		-0.007
Da Nang	0.000	0.009	0.001	0.002	

4.3.2 Genetic variation of microsatellite markers

Ten polymorphic microsatellite markers were successfully amplified (Table 4.5) and PCR products of all 216 samples of *P. ornatus* were genotyped for subsequent population genetics analyses. A total of 143 alleles were observed, ranging from five (Orn_01) to 29 (Orn_11) alleles per locus. Significant departures from HWE were observed for two loci in the Lombok and Binh Thuan populations (Orn_01 in Lombok and Orn_17 in Binh Thuan) and the dataset was reanalysed with and without these markers in these two populations to test if they were significantly influencing results obtained. No differences were found in genetic structure indices when they were included, so the complete dataset of markers were analyzed and is presented here. No linkage disequilibrium was detected among the ten loci genotyped.

Table 4.5Genetic indices for 10 microsatellites characterized in *Panulirus ornatus* at six sample sites/collections. *N*= sample size, N_A= number of alleles, H_O= observed heterozygosity, H_E= expected heterozygosity, P_{HWE}= Hardy-Weinberg equilibrium significance value at P<0.05 after FDR correction, *ns*= non-significant, *bold text* = significantand *PIC*= polymorphic information content.

		Microsatellites									
Pop		**Orn 01**	**Orn 02**	**Orn 11**	**Orn 12**	**Orn 16**	**Orn 17**	**Orn 18**	**Orn 20**	**Orn 21**	**Orn 25**
Torres Strait	***N***	72	72	71	71	71	70	72	69	71	70
	N_A	8	4	20	23	9	10	9	10	7	9
	H_O	0.72	0.54	0.85	0.94	0.79	0.5	0.74	0.42	0.79	0.71
	P_{HWE}	0.04	0.01	0.04	0.01	0.04	0.04	0.04	0.03	0.01	0.04
West Timor	***N***	12	12	12	11	12	12	12	12	12	12
	N_A	6	3	13	9	6	7	9	7	6	7
	H_O	0.75	0.58	0.92	0.91	0.75	0.42	1	0.67	0.83	0.83
	P_{HWE}	0.02	0.02	0.02	0.04	0.01	0.02	0.03		0.03	0.02
LomBok	***N***	32	32	32	32	32	31	32	30	32	32
	N_A	10	3	20	19	8	7	10	7	8	7
	H_O	0.69	0.41	0.88	1	0.84	0.45	0.69	0.3	0.72	0.63
	P_{HWE}	**0.00**	0.03	0.02	0.04	0.04	0.03	0.03	0.04	0.01	0.04
North Sumatra	***N***	3	3	3	3	4	3	3	3	3	3
	N_A	3	3	5	4	5	2	5	1	3	3
	H_O	0.33	0.67	1	1	0.5	0.67	1	0	0	1
	P_{HWE}	0.02	0	0.01	0.03	0.03	0	0.01	0.03	0.02	0.03
Binh Thuan	***N***	49	49	49	48	49	51	10	49	51	50
	N_A	8	4	22	21	7	6	6	9	7	9
	H_O	0.69	0.67	0.9	0.94	0.76	0.33	0.7	0.55	0.73	0.74
	P_{HWE}	0.01	0.01	0.01	0.03	0.02	**0.00**	0.01	0.04	0.01	0.03
Da Nang	***N***	40	40	44	44	44	43	0	45	44	45
	N_A	8	3	24	21	8	10	0	8	8	10
	H_O	0.75	0.65	0.91	0.91	0.82	0.67	0	0.47	0.75	0.73
	P_{HWE}	0.01	0.02	0.03	0.01	0.05	0.05		0.05	0.02	0.05
Mean	***N***	34.67	34.67	35.17	34.83	35.33	35.00	21.50	34.67	35.50	35.33
	N_A	7.3	3.5	17.2	16.0	7.3	6.8	6.3	7.0	6.33	7.3
	H_O	0.70	0.60	0.91	0.89	0.74	0.49	0.69	0.39	0.69	0.77
	P_{HWE}	0.21	0.51	0.77	0.05	0.99	1.00	0.11	1.00	0.53	1.00
	PIC	0.69	0.55	0.92	0.93	0.72	0.54	0.71	0.44	0.81	0.69
Total	***N***	208	208	211	209	212	210	129	208	213	212
	N_A	11	5	29	26	11	16	11	14	8	12
	Allele size range (bp)	139-176	258-278	175-242	304-400	163-195	260-321	352-372	318-362	240-264	184-216

As for the mtDNA control region, no significant population genetic structure was evident between the six sites when genotyped with the 10 microsatellite loci. F_{ST}

estimates of population structure were again negligible and non-significant (AMOVA, F_{ST} = 0.003; P=0.195 ±0.004; Table 4.3). The microsatellite data indicated that less than 1% of genetic variation was present among populations. A similar lack of population genetic structure was evident in pairwise population comparisons across the Indo-West Pacific region (F_{ST} ranged from -0.003 to 0.031; Table 4.4). The pairwise F_{ST} comparison between North Sumatra and other sampling sites were the highest observed (from 0.017 to 0.034), but were all insignificant after FDR correction (P > 0.05). Due to the small sample size collected from Sumatra the higher sample F_{ST} values involving this population are likely a result of random sampling effects and small sample size.

Individual based Bayesian assignment tests supported the lack of population genetic structure indicated by non-significant and small pairwise F_{ST} estimates. Although Structure Harvester suggests K = 2 from multiple simulations runs at values of K from 1 to 10, visual examination of individual bar plots for K = 2 indicates an inability of the STRUCTURE algorithm to reliably assign any of the individuals to a distinct cluster, with assignment probabilities for each of the two populations of ~50% for all individuals sampled in each of the 10 replicate runs (Figure 4.3). The inability to assign individuals using post-hoc plots if the true K<2 has been discussed in Evanno *et al.* (2005). Therefore, Bayesian analysis using STRUCTURE, also suggests panmixia or lack of genetic structure among the six populations examined, despite the widely spaced regional sampling employed here.

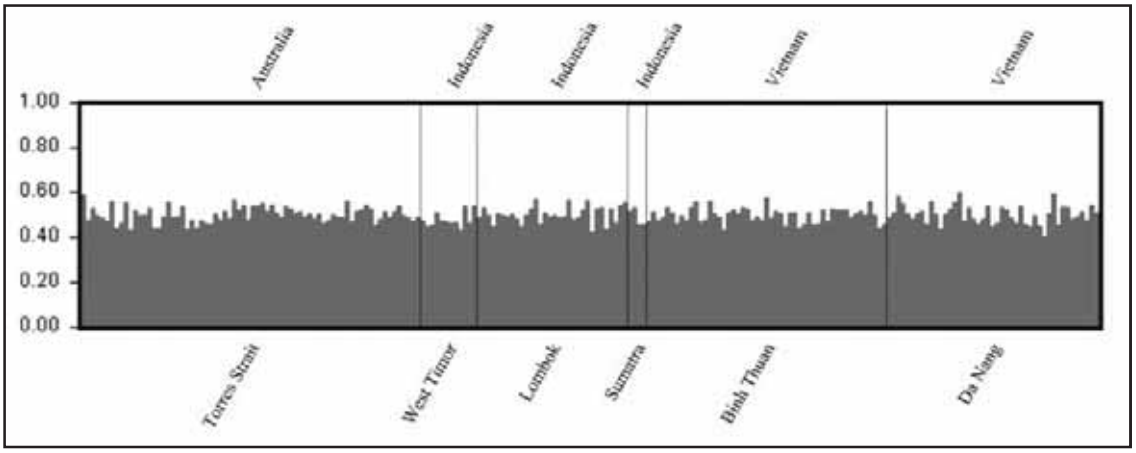

Figure 4.3. Bayesian individual assignment analysis for K = 2 for *Panulirus ornatus* genotyped at ten microsatellites across six Indo-Pacific sampling sites. Colours (grey or white) represent probability of individuals being assigned to each genetic cluster(y-axis). Lobsters were sampled from sampled from Torres Strait, West Timor, Lombok, North Sumatra, Binh Thuan (Vietnam), Da Nang (Vietnam). Sampling locations were used as priors.

4.3.3 Larval dispersal throughout the South-East Asian Archipelago

The distribution of surface currents in the South-East Asian Archipelago during known larval transport periods are provided in Figure 4.4, while estimated time that larvae of *P. ornatus* would reach different locations in the South-East Asian Archipelago was calculated in Table 4.6. From these seasonal-linked ecological data, the suggested dispersal connectivity network as it relates to surface currents are showed in Figure 4.5. According to this dispersal model, the high levels of gene flow leading to genetic panmixia in this tropical lobster species across the broadly sampled South-East Asian Archipelago are explained by current-mediated larval transport that connects lobsters among spawning populations across a 2-3 generation period. The predictive model of this dispersal (Figure 4.5) occurs as follows. Starting from the spawning ground in the Gulf of Papua in PNG, with spawning by adults from November to March, the outflow of *P. ornatus* larvae from the spawning grounds has the potential to form two larval plumes, one plume which would perform a loop in the northwest Coral Sea to return to the Torres Strait, while the other plume exits the Coral Sea through the Vitiaz Strait to

enter the Bismarck Sea and travel along the north-eastern coast of PNG in July to September (Schiller *et al.*, 2008; Cravatte *et al.*, 2011; Figure 4.5). Larvae within this second plume avoid being swept eastward into the Pacific Ocean by the Northern Equatorial Counter Current (NEC) at that time of the year, and arrive in the southernmost Philippines where they disperse widely from September to October with the complex currents through the straits in the Philippines. A separate portion of this larval plume travels southward towards the Java and Banda Seas, although there is no field data to confirm this. With spawning grounds both on the east and west coasts of the Philippines, and spawning occurring from May to August, larvae most probably disperse in three plumes. The eastern Philippines plume could cross the Luzon Strait and join with the western Philippines plume of larvae released from the Northern Philippines, and disperse into the South China Sea to reach the central coast of Vietnam three to four months later (Dennis *et al.*, 2001; Manh & Yanagi, 2003; Liang *et al.*, 2008; Potemra & Qu, 2009). The timing of the arrival of pueruli matches catch data from Vietnam (Figure 2.13 & 4.6), showing that spiny lobster larvae are only present during north-east winds during the north-east monsoon, when a long-shore southward current exists along the Vietnamese coast. This current is most likely to bring lobster larvae that were spawned in the Philippines to Vietnam (Figure 4.5). Indeed, the monthly catch increases with increasing monthly cumulative wind stress ($r^2 = 0.83$, Hoc Tan Dao, unpublished data), indicating increasing advection to Vietnam of lobster larvae, presumably from the Philippines, with increasing currents and wind (Figure 4.6, Hoc Tan Dao, unpublished data). This larval plume would then be transported southward to Indonesia to reach Lombok from 70-95 days later (Liang *et al.*, 2008). Some larvae sourced from northern Luzon are also presumably adverted northward towards Taiwan (Villanoy, 2004). From the southern spawning ground near eastern

Samar of the Philippines, during the northern summer monsoon (from May to August) in the south-east of the Philippines, newly-spawned *P. ornatus* larvae then would rapidly travel with the Mindanao Current southward to Lombok which they would reach in about 73-113 days (Liang *et al.*, 2008; Schiller *et al.*, 2008; Potemra & Qu, 2009; Metzger *et al.*, 2010). From here, the larvae could divide into two plumes. The first plume would travel with the Indonesian Throughflow to arrive in the Indian Ocean and ultimately reach Western Sumatra in November - December (Schiller *et al.*, 2008; Potemra & Qu, 2009). These larvae may then be advected back towards southern Lombok by the northern winter monsoon (from November to February; Qu and Meyers, 2005) before settling. The second larvae plume from northern Lombok would follow the surface current to the Banda Sea (Schiller *et al.*, 2008; Mayer *et al.*, 2010; Metzger *et al.*, 2010) and the northern Arafura Sea (Forbes & Church, 1983; Schiller *et al.*, 2008;Condie, 2011) during the northern winter monsoon to reach reefs in the Torres Strait by February. These larvae could spend their post larval, juvenile, and adult stages in the Torres Strait before migrating towards spawning grounds in the Gulf of Papua in South-East PNG two years later. Based on the timing of the arrival of pueruli cohorts in Lombok and the time expected for dispersal of lobster larvae from Lombok to Torres Strait (Figure 4.5), a third spawning ground may also exist in Indonesia, possibly at Lombok, but no field data is currently available to confirm this.

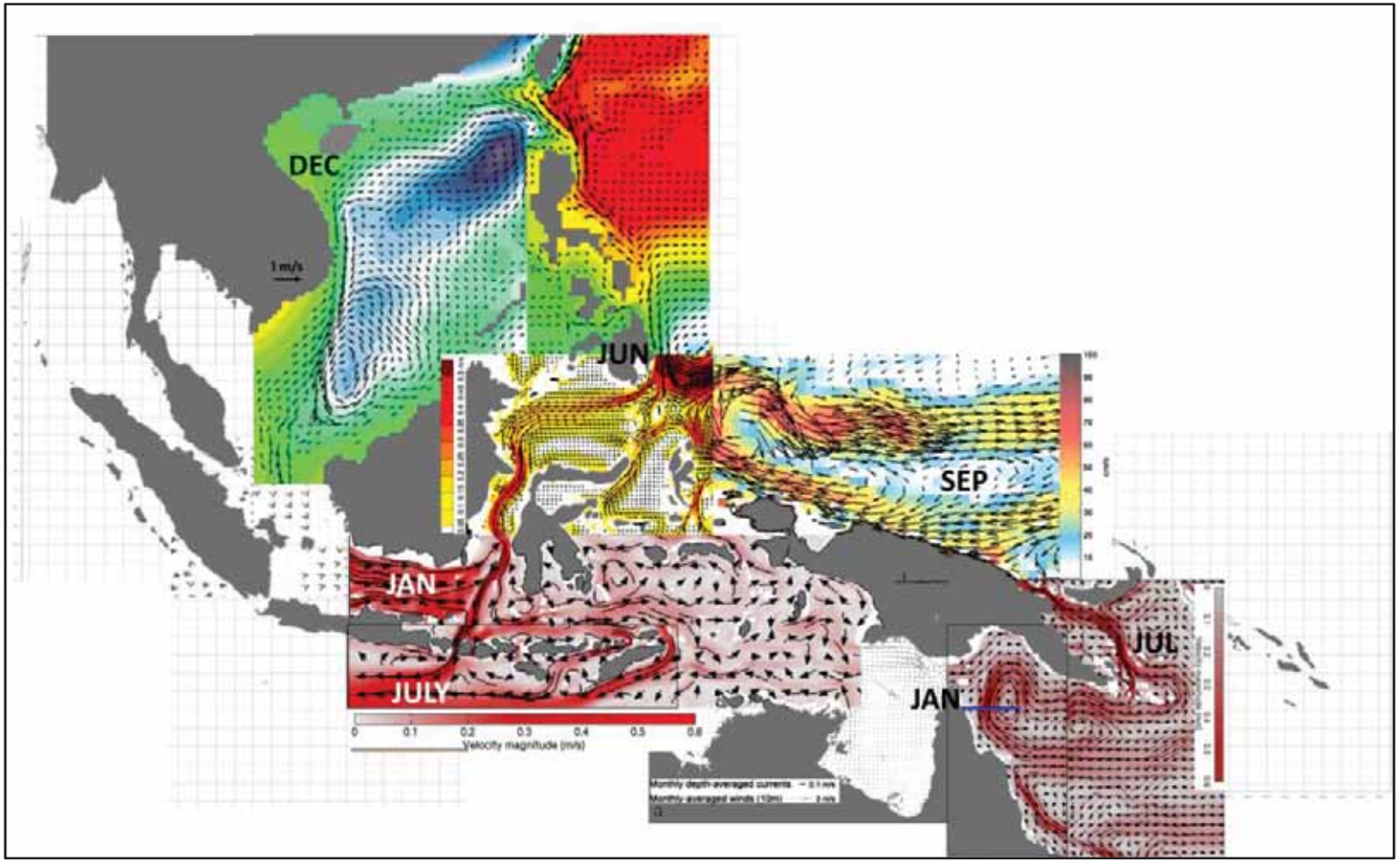

Figure 4.4 Seasonal surface ocean currents in the South-East Asian Archipelago at the times when *Panulirus ornatus* larvae are travelling between the various sites shown in Figure 4.5, based on studies of Liang *et al.* (2008); Schiller *et al.* (2008); Potemra and Qu (2009); Metzger *et al.* (2010); Condie (2011)and ARGOS data (http://www.aoml.noaa.gov/phod/graphics/dacdata/seasonal_wpac.gif).

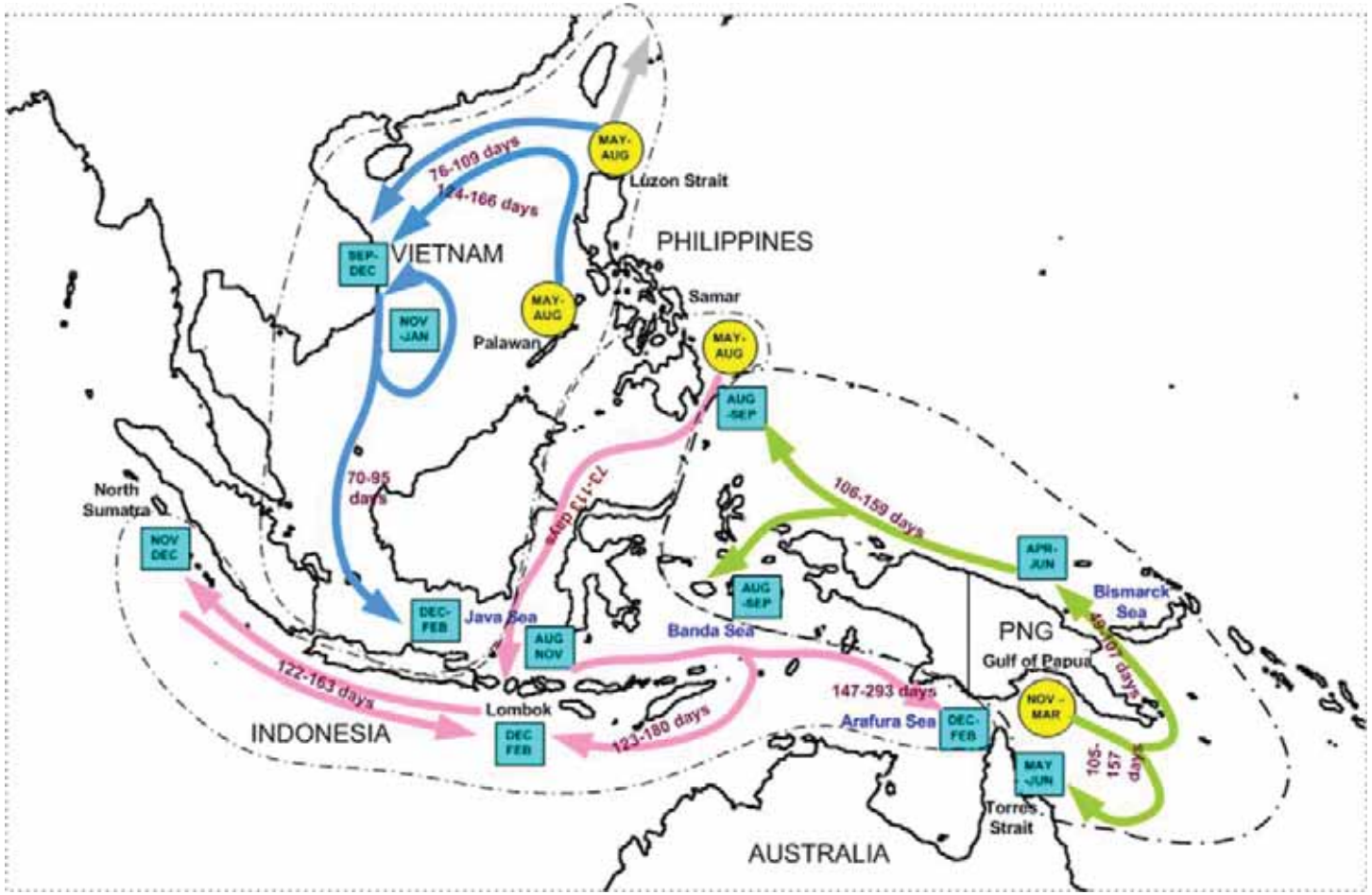

Figure 4.5 Suggested larval dispersal pathways based on the surface water oceanography and the location of spawning grounds, dispersion patterns and connectivity for *Panulirus ornatus* larvae throughout the South-East Asian Archipelago. Round circles indicate spawning grounds where the larvae are released. Square boxes are estimated time that the larvae reach different locations as suggested by the oceanography and confirmed by field data; out of them only two points have no field data, namely arrival times of larvae from Lombok in Torres Strait and the time of transit of the larvae along the north coast of Papua New Guinea. Different arrow colours are different sources that larvae dispersed from.

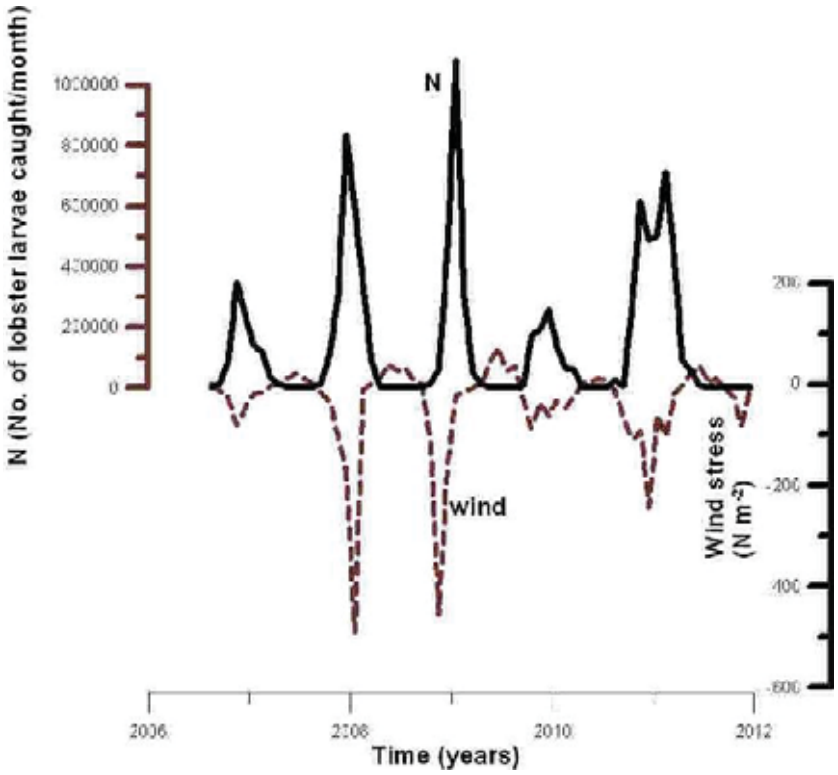

Figure 4.6 Time series plot from 2006 to 2011 of the long shore wind stress over the central Vietnam coastal waters of the South China Sea and of *Panulirus ornatus* larvae/ pueruli monthly catch for Vietnam. A positive wind stress indicates a northward wind and a negative value a southward wind (Hoc Tan Dao, unpublished data).

Table 4.6 Estimate time (*t*) for larvae/pueruli to reach different locations in the South-East Asian Archipelago.

Route	Local Route	Author	Data time	Data depth	*u* (cm/s)		*L*	*t* (days)		
					From	To	(km)	From	To	Average
PNG - Australia - Philippines	Gulf of Papua - Torres Strait	Schiller *et al.* (2008)	Jan-Jul	< 250 m	10	15	1,361	105	157	105 - 157
	Gulf of Papua - Bismack Sea	Schiller *et al.* (2008)	Jan-Jul	< 250 m	15	30	1,381	53	107	49 - 107
		Caravatte *et al.* (2011)	Jan-Jul	< 100 m	15	35	1,381	46	107	
	Bismack Sea - Philippines/Banda Sea	ARGO (2013)	Jun-Sep	< 50 m	20	30	2,744	106	159	106 - 159
Philippines - Vietnam - Indonesia	Luzon Strait - Central Vietnam	Manh & Yanagi (2003)	Oct-Dec	~ 0 m	15	20	1,416	82	109	76 - 109
		Liang *et al.* (2008)	Dec	< 50 m	15	20	1,416	82	109	
		Potemra & Qu (2009)	Dec-Jan-Feb	< 100 m	15	25	1,416	66	109	
	Palawan - Central Vietnam	Liang *et al.* (2008)	Dec	< 50 m	15	20	2,148	124	166	124 - 166
	Central Vietnam - Java Sea	Liang *et al.* (2008)	Dec	< 50 m	25	30	1,818	70	84	70 - 95
		Farshid (unpublished data)	Dec-Jan-Feb	< 10 m	20	30	1,818	70	105	
Philippines - Indonesia - Australia	Eastern Samar - Lombok	Liang *et al.* (2008)	Jul	< 50 m	25	50	2,674	62	124	67 - 119
		Schiller *et al.* (2008)	Jul	< 250 m	25	45	2,674	69	124	
		Potemra & Qu (2009)	Mar-Apr-May	< 100 m	30	40	2,674	77	103	
		Metzger *et al.* (2010)	Mean	< 120 m	25	50	2,674	62	124	
	North Lombok - South Lombok through Banda Sea	Schiller *et al.* (2008)	Jul	< 250 m	20	25	2,663	123	154	123 - 180
		Mayer *et al.* (2010)	Oct	< 700 m	15	25	2,663	123	205	
	South Lombok - North Sumatra	Schiller *et al.* (2008)	Jul	< 250 m	15	20	2,114	122	163	122 - 163
		Potemra & Qu (2009)	Jun-Nov	< 100m	15	20	2,114	122	163	
	Java Sea - Arafura Sea	Forbes & Church (1983)	Jan	< 20 m	10	20	2,536	147	293	147 - 293
		Schiller *et al.* (2008)	Jan	< 250 m	10	20	2,536	147	293	
		Condie (2011)	Jan	< 18 m	10	20	2,536	147	293	

4.4 DISCUSSION

4.4.1 Population structure of *Panulirus ornatus*

In this study, the control region of *P. ornatus* was found to exhibit extremely high genetic variability (h = 0.982 to 1.000 and Pi = 0.079 to 0.113). According to Heyer *et al.,* (2001) and Garcia-Rodriguez & Perez-Enriquez (2008), the control region is the most rapidly evolving region compared to other regions of mtDNA, resulting in high genetic variability, and would require a very large sample size to detect the genetic structure across the geography. Therefore, further studies on *P. ornatus* should focus larger on samples size (in North Sumatra and West Timor), or on other markers with lower mutation rates such as cytochrome oxidase I or 16S rDNA. Both mtDNA control region and microsatellite DNA data in the current study, however, conclusively show that the tropical Ornate Spiny Lobster comprises a single, genetically homogeneous stock across a broad region of the South-East Asian Archipelago, including populations from Vietnam, Indonesia and Australia-PNG. Modelling shows that this lack of genetic population structure in *P. ornatus* is a consequence of both the high dispersal potential of larvae, whereby they remain in the water column for periods of 4.5 to 7 months (Duggan & McKinnon, 2003), coupled with favourable seasonal currents which transport these larvae over long-distances between known adult spawning sites. Moreover, each year a proportion of adult *P. ornatus* lobsters, especially ovigerous females, migrate hundreds of kilometers to mate and spawn in a single location, further aiding dispersal and contributing to broad scale connectivity (Moore & MacFarlane, 1984; MacFarlane & Moore, 1986; Booth & Phillips, 1994; Williams, 2004).

The current study demonstrates genetic panmixia of *P. ornatus* across a large and oceanographically complex region, however, the life-history and long pelagic larval duration of *P. ornatu*s is not unique and similar occurrences of high population inter-connectivity have

been observed at a variety of smaller spatial scales in other *Panulirus* species. For instance, genetic studies on Japanese spiny lobster, *P. japonicus,* failed to reveal any stock heterogeneity within the Japan Sea (Inoue *et al.*, 2007), while low population divergence was observed for *P. gilchristi* in the deep shelf waters along the southern coast of South Africa. In addition, *P. cygnus* along the coast of Western Australia lacks genetic structuring (Thompson *et al.*, 1996). Recently, Chow *et al.* (2011) and Abdullah *et al.,* (2014a) found no genetic structure of pronghorn spiny lobster, *P. penicillatus*, in the Western Pacific region using mtDNA markers, which supports the finding that spiny lobsters might successful disperse throughout the South-East Asian Archipelago. They also suggested that the lack of variation in genetic structure is related to oceanographic flows in the area (e.g., the Kuroshio and Equatorial currents), coupled with a long larval period. Although it may be inferred that the long larval pelagic durations evident in *Panulirus* lobsters universally lead to low population divergence across small to broad spatial scales, this is not always the situation. In *P. argus,* for example, genetic differentiation is present among Bermuda and Florida populations within the Caribbean Sea, as well as those sampled from Venezuela and Brazil (Sarver *et al.*, 1998). Likewise, South African *P. delagoae* and *P. elephas* populations in the Atlantic Ocean and Mediterranean Sea exhibit shallow, but significant, levels of genetic structuring (Gopal *et al.*, 2006; Palero *et al.*, 2008). Thus, whilst some spiny lobster species do not appear to be strongly genetically structured, others have been found to comprise several genetic stocks, most probably as a consequence of the presence of strong regional biogeographic influences, or barriers, to larval dispersal.

The finding that *P. ornatus* is genetically homogeneous across the South-East Asian archipelago was initially surprising given that the populations sampled traverse several known biogeographic barriers to dispersal, including the Torres Strait land bridge between Australia and Papua New Guinea. For Torres Strait, periodic fluctuations in sea level during the

Pleistocene resulted in a land bridge connecting Australia and Papua New Guinea, which effectively formed a physical barrier to gene flow on either side of the Torres Strait (Voris, 2000). This land bridge posed a significant barrier to dispersal, with many marine species examined to date exhibiting genetic differences either side of the Torres Strait. For example, genetic differences have been shown in the tropical reef fish (*Pomacentrus amboinensis* and *Pristipomoides multidens*), barramundi (*Lates calcarifer*) and crustacean species, including penaeid prawns and mud crab (Ovenden *et al.*, 1992; Keenan, 1994; Ward & Elliott, 2001; Ovenden *et al.*, 2002; Ward *et al.*, 2006; Jones C. *et al.*, 2010). In the case of *P. ornatus* the land bridge seems to be a barrier to larval dispersal, but not for the adults, which can swim and walk. Larvae plumes from the Philippines are apparently moved by the ocean surface currents through Indonesia to the western side of Torres Strait (Figure 4.5). Small juveniles of *P. ornatus* (<40mm carapace length) were found in Western Torres Strait in consolidated coral rubble and limestone pavement (Dennis *et al.*, 1997). They remain at this point until mature enough to walk across to the eastern side of the land bridge to spawning grounds in the Gulf of Papua, which was described in studies of Bell *et al.* (1987) and Dennis *et al.* (2001). In conclusion Torres Strait could prevent larval dispersal, but the adult migration might overcome this physical barrier providing connectivity to the western and eastern *P. ornatus* populations.

4.4.2 Implications for management

The panmictic genetic population structure of *P. ornatus* throughout its Indo-Pacific distribution indicates that the species is a single stock requiring a single fishery management plan to ensure sustainable exploitation of this valuable resource. Consequently, a multi-jurisdictional fishery policy should be developed by managers from Australia, Papua New Guinea, the Philippines, Vietnam and Indonesia, because the sustainability of lobster fisheries

in these exploited populations will be problematic if juvenile recruits are derived from degraded, overfished, or otherwise anthropogenically impacted source populations. While the sinks of *P. ornatus* larvae are known, the knowledge of larval sources is still rudimentary, with to date only a few spawning sites confirmed. The present study suggests that an additional spawning ground may also be present in Indonesia based on larval appearance times, but no such site has yet been reported within the literature. Therefore, new studies to identify adult migration and spawning grounds are necessary to ensure that any future multi-jurisdictional lobster fishery management plans capture all spawning sites, which given their importance in "seeding" the wider Indo-Pacific region and maintaining genetic connectivity should be conserved as a priority.

CHAPTER 5. POPULATION GENETIC STRUCTURE OF THE SCALLOPED SPINY LOBSTER *Panulirus homarus*THROUGHOUT THE INDO-PACIFIC REGION

5.1 BACKGROUND

The scalloped spiny lobster *Panulirus homarus* (Linnaeus, 1758) supports an important capture fishery in the Indo-West Pacific. However, commercial catches of this species, like that of other *Panulirus* lobsters, has experienced a significant decline in many countries over recent times. In Oman, for example, the fishery of *P. homarus* represents 33% of the total lobster catch and has declined from 2000t in 1988 to 158t in 2011 (Mehanna *et al.*, 2012). Similarly, Fielding & Mann (1999) indicate that lobster catches in Somalia have also declined significantly in the last few years based on strong reductions of both boat catches (from 125 kg per day to 25-35 kg of lobster per day) and the numbers of lobsters transported to the airport (only one truck load per day in 1998 compared to four truck loads in prior years). A declining trend in catch numbers has also been recorded in south-west India in which *P. homarus* contributed 92% (301 t) of total lobster catches in 1966 and only to 4t in 2002 (Radhakrishnan *et al.*, 2005).

In contrast to the generally declining trend of scalloped spiny lobster landings from wild fisheries, recently there has been increased interest in aquaculture production of spiny lobsters, including *P. homarus*, based on the collection of naturally setting pueruli (Jones C. *et al.*, 2010). Vietnam is currently producing more than 1000t of cage-raised lobsters annually, while the total aquaculture production of spiny lobsters in Indonesia over 10 years from 1999 to 2009 has rapidly increased from 5t to 338t (Phillips, 2013). However, the sustainability of

spiny lobster aquaculture in these countries is of concern, because it is based on a natural resource for which there is currently no, or very limited, harvest management of pueruli, or understanding of the origin of recruits (Williams, 2004).

Mature adult spiny lobsters in the genus *Panulirus* generally migrate along the ocean floor toward deeper-water breeding grounds from shallow nursery areas (George, 2005a; Phillips, 2013; Pitcher, 1993), with migration distances varying from a few to more than 500km (Herrnkind, 1980). This potential in spiny lobsters for adults to migrate long-distances has been suggested as one reason why many Panuliruids show low levels of genetic structure across their distributions (Thompson *et al.*, 1996; Inoue *et al.*, 2007). Although observations on the migratory patterns of *P. homarus* are poor, this species likely exhibits a similar life-history strategy to that of other Panulirids and there have been reports of at least small-scale movements (from 6 to 12 km) of adults (Mohamed & George, 1968) and the offshore movements of egg-bearing females have been observed (Kulmiye, 2004; Kulmiye *et al.*, 2006). Moreover, phyllosoma larvae of *P. homarus* from spawning events have a long planktonic phase, lasting from 4 to 8 months before they settle as pueruli (Booth & Phillips, 1994; Phillips & Matsuda, 2011; Phillips, 2013). This provides the opportunity for larvae to be transported long-distances by ocean currents, therefore increasing the dispersal and gene flow potential among populations of the species. However, across the range of *P. homarus* there are several recognized subspecies based on morphological characters (George & Rao, 1966; Berry, 1974; Pollock, 1993) suggesting that gene flow might be restricted, possibly by oceanographic factors, leading to local adaptation and conceivably genetic divergence (Pollock, 1992, 1993).

Despite the obvious high potential for dispersal and gene flow in Panulirid spiny lobsters, oceanographic factors do appear to be important in shaping the genetic structure of

populations. Several studies have used population genetic analyses to infer metapopulation structure and likely larval dispersal pathways for *Panulirus* species. For example, mtDNA and microsatellite markers revealed genetic differentiation among and within populations of *P. elephas* in the Atlantic and Mediterranean basins (Palero *et al.*, 2008; Palero *et al.*, 2011). Palero *et al.* (2011) attributed differentiation between the Brittany and Scotland/Ireland populations in the Atlantic to be largely due to currents of the Gulf Stream that influence larval dispersal and gene flow patterns. In *P. delagoae,* genetic structure based on the mitochondrial DNA control region was detected between populations in southern Mozambique and eastern South Africa, in which the Mozambique Channel eddies and upper Agulhas Current were suggested to retain the larvae and prevent them from dispersing to South Africa (Gopal *et al.*, 2006). The Agulhas Current in contrast, is regarded to carry the phyllosomas of *P. gilchristi* along the coast of South Africa, leading to widespread larval dispersal and high levels of gene flow in this species (Tolley *et al.*, 2005). Conversely, *P. penicillatus* within the Western/Central Pacific regions lacks genetic structuring (Chow *et al.*, 2006; Abdullah *et al.*, 2014a), implying that the high gene flow of this species is facilitated by large-scale and unimpeded oceanographic-driven dispersal of the pelagic lobster larvae. For *P. homarus* genetic studies are few; however phylogenetic studies by Ptacek *et al.* (2001) and Lavery *et al.* (2014) suggested genetic differentiation by supporting the notion of there being several subspecies. Farhadi *et al.* (2013) also recently used the mitochondrial DNA (mtDNA) control region to reveal significant genetic differentiation of *P. homarus* between Tanzania and several locations in the Arabian Sea, which provided evidence that *P. homarus* as a species indeed exhibits genetic structuring, at least within Africa and the Middle East. There are, however, no population-level genetic studies to date that have covered the broader Indo-Pacific distribution of this species and it is not understood how many genetic units might be present.

To address the issue of broad-scale genetic structuring in *P. homarus* throughout the Indo-West Pacific and to guide fisheries management lobsters were genotyped at both the mtDNA control region and six microsatellite DNA markers. The patterning of genetic structure discovered is then discussed in relation to known oceanographic features between the sampled populations and the validity of the sub-species described by Berry (1974) and Pollock (1993).

5.2 METHODS

5.2.1 Tissue sampling and DNA extraction

This study was carried out in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and the Queensland Animal Care and Protection Act 2001 under Animal Ethics Permit no. A1746, as approved and administrated by the James Cook University Animal Ethics Committee. Animals were collected from commercial aquaculture, or fishery operations, and no specific collection permits were required. Animals are not listed as endangered or threatened.

To determine the genetic structure among *P. homarus* populations across a broad part of the species' geographical distribution, from 2011 to 2013, a total of 229 samples were collected from one location in the west Indian Ocean (Masirah, Oman) and from six localities in the Southeast Asian Archipelago (Taiwan; Da Nang and Binh Thuan in Vietnam, along with West Sumatra, Lombok, and West Timor in Indonesia; Fig 5.1). Specimens from Vietnamese and Indonesian populations were all pueruli caught from wild stock, while those from Taiwan and Oman were adults collected from local fishing markets. All samples (pleopods from adults or abdominal muscle tissue from juvenile lobsters) were preserved immediately in a DMSO-salt preservative solution (Dawson *et al.*, 1998). Genomic DNA (gDNA) from all lobster samples

was extracted from 4mm^2 pleopod clips, or from the abdominal muscle tissue of juveniles using a modified CTAB protocol (Adamkewicz & Harasewych, 1996).

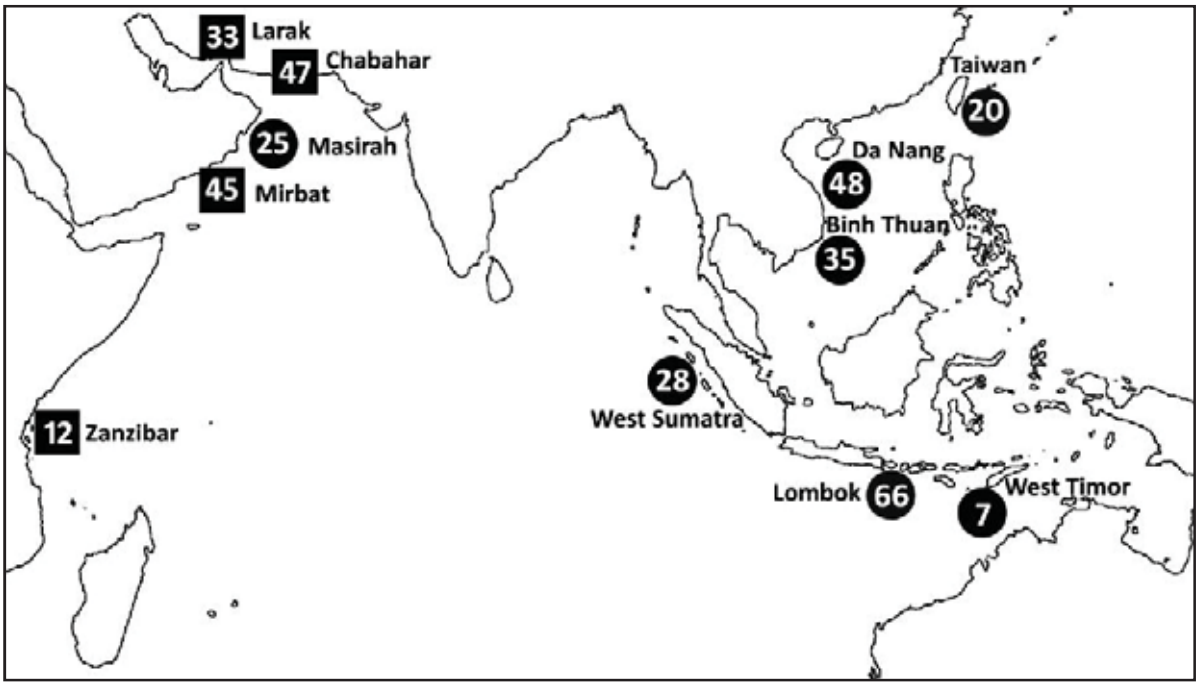

Figure 5.1 Sampling sites and number of *Panulirus homarus* specimens from each location in the present study (circles) and in previous study of Farhadi *et al.* (2013; squares).

5.2.2 mtDNA Control region

DNA extracted from samples was diluted to 10-40ng/µl for use in a polymerase chain reaction (PCR). The control region was amplified in 20µl reaction volumes containing ~5ng DNA, 1× TM buffer (Qiagen), 1.5µM of $MgCl_2$, 0.2µM of dNTPs, 0.1µM of Tag Red (Qiagen) and 0.3µM of forward and reverse primers. PCR was performed on a BioRadC1000 Thermal Cycler (cycling parameters: 3 min at 95 ºC, followed by 35 cycles of 95 ºC for 45s, 50 ºC for 30s, 72ºC for 45s, before a final extension step of 72ºC for 5 min). PCR products were then run on a 1.5% agarose gel for quantity and quality verification, and subsequently cleaned to remove excess primers by precipitation with isopropanol (Sambrook & Russell, 2001). A repeat region in the start of the reverse primed sequence resulted in deterioration of sequences.

Consequently, only DNA sequences from the forward primer was used. To verify nucleotide base calls each sample was sequenced at least twice at the Australian Genome Research Facility (AGRF), Brisbane, Australia. PCR primers previously designed for *P. ornatus* mtCR (PO_F2 5' - ATAAAGGTAATAGCAAGAATC and PO_R1 5' - CAAACCTTTTGTCAGGCATC) were used for the amplification of 800bp of the control region (the same described in chapter 2).

Mitochondrial control region sequences were trimmed to 630bp using Geneious (v. 6.1)to provide the best quality sequences for alignment and subsequent analyses. The resulting fragments corresponded to at least 96% of the same region of the mtDNA control region as that genotyped by Farhadi *et al.* (2013); Genbank accession nos. KC625333–KC625469, in *P. homarus* samples collected from the west Indian Ocean. Therefore, to further increase the number of population samples and the scale of the distribution examined, the 137 *P. homarus* mtDNA control region fragments from Iran (Chabahar and Larak), Oman (Mirbat) and Zanzibar (Tanzania) from Farhadi *et al.,* (2013)were integrated with the 229 new mtDNA genotypes generated in the present study data.

Sequence data were aligned using Geneious v.6.1 with default alignment parameters and checked manually for misalignments. The statistical packages MEGA6 (Tamura *et al.*, 2013), DNAsp v.5.1 (Rozas *et al.*, 2003) and ARLEQUIN v.3.5 (Excoffier *et al.*, 2005) were used to perform calculations of nucleotide diversity, haplotype diversity, neutrality tests (Tajima's *D*; Tajima, 1989), as well as partitioning of genetic structure Φ_{ST} (using genetic distance). For calculation of the statistical significance of the Φ_{ST} values obtained, a significance test with 10,000 permutations was carried out with ARLEQUIN v. 3.5 (Excoffier *et al.*, 2005). Φ_{ST} and pairwise Φ_{ST} comparisons between populations were estimated using the T92 model (Tamura 1992), with a gamma correction ($\alpha = 0.633$) as determined by Model Selection in MEGA6.

The median-joining network (Bandelt *et al.*, 1999) for the haplotypes generated was constructed using NETWORK v. 4.6.1.0 (http:\\www.fluxus-engineering.com).

5.2.3 Microsatellites

Six highly polymorphic microsatellite markers developed originally for *P. ornatus* (Orn_01, Orn_02, Orn_11, Orn_12, Orn_16 and Orn_20 (Dao *et al.*, 2012) were used for population genetic analyses based on the nuclear genome of 216 specimens belonging to seven sample localities. This analysis could not include the individuals from the study by Farhadi *et al.,* (2013), as their work focused only on genotyping individuals based on the mtDNA control region.

DNA was diluted to 10–40ng/µl for use as template in a polymerase chain reaction (PCR). Microsatellites were individually amplified in 10µl reaction volumes containing ~20ng DNA, 1× Type-it Multiplex PCR Master Mix (Qiagen), 0.04µM of fluorescent labelled forward primer (TET, FAM or HEX), and 0.2µM of reverse primer. PCR was performed on a BioRadC1000 Thermal Cycler (cycling parameters: 5 min at 95ºC, followed by 28 cycles of 95ºC for 30s, 58ºC for 90s and 72ºC for 30s, before a final extension step of 60ºC for 30 min). The PCR products were then checked for consistent amplification by visualization on a 1.5% agarose gel. After this step, the PCR products were pooled according to size, fluorescent label as well as product quantity, and the pooled products subsequently purified using Sephadex G-50 resin before loading on a Megabace 1000 Capillary Sequencer for size separation of alleles (Amersham Biosciences). Alleles were scored on the basis of fragment size using the Fragment Profiler 1.2 package (Amersham Biosciences).

Summary statistics such as the number of alleles, as well as observed and expected heterozygosity were calculated for microsatellites in GENALEX v.6.1 (Peakall & Smouse,

2006), which was also used to test for deviations from Hardy-Weinberg Equilibrium (HWE). GENEPOP on the web (http://genepop.curtin.edu.au/) was used to test for linkage disequilibrium among microsatellite loci. Corrections for multiple comparisons (HWE and linkage disequilibrium) were adjusted using the False Discovery Rate (FDR) method (Benjamini & Hochberg, 1995). Polymorphic Information Content (PIC) was also calculated for each locus with CERVUS v.3.0 (Kalinowski *et al.*, 2007) and the presence of null alleles and scoring errors were checked using MICROCHECKER v.2.2.3 (van Oosterhout *et al.*, 2004).

The level of genetic structure of *P. homarus* based on microsatellite markers was analysed using an Analysis of Molecular Variance (AMOVA) with 10,000 permutations, as well as by calculating pairwise F_{ST} comparisons between populations, both of which were carried out with ARLEQUIN v.3.5 (Excoffier *et al.*, 2005). Further to these analyses, the Bayesian clustering algorithm implemented in STRUCTURE v.2.2.3 (Pritchard *et al.*, 2000) was used to determine spatial genetic discontinuities by inferring the highest probable number of genetic clusters present within the dataset with prior knowledge of the individual's origin. Individuals are placed in K predetermined sub-groups based on their likelihood of belonging to that sub-group, which is calculated using allele frequencies of multiple loci. The K value was chosen in advance and ranged from 1 to 10, and the populations were assumed to be admixed (an individual could belong to any population) in origin. Burn-in and run length were set to 100 000 MCMC (Markov chain Monte Carlo) repetitions and each run was iterated 10 times. This approach implements a model-based clustering method for inferring population structure and assigning individuals to the most probable genetic sub-group or population. The package Structure Harvester (http://taylor0.biology.ucla.edu/structureHarvester/) was used to determine the optimum number of clusters used in the analysis and CLUMPP (http://www.stanford.edu/group/rosenberglab/clumpp.html) was also used to create

averages across the replicate runs, after which the outputs were put through DISTRUCT (http://www.stanford.edu/group/rosenberglab/distruct.html) to graph average q values.

5.3 RESULTS

5.3.1 mtDNA control region

Including the data from Farhadi *et al.* (2013) mitochondrial DNA control region sequences were obtained from a total of 344 *P. homarus* individuals (Genbank accession no. for present study KM186313-KM186519). From these sequences, 612 sites were non-variable and 480 were polymorphic without insertions or deletions. In total, 307 haplotypes were identified, with 89% of the individuals sampled possessing a unique haplotype (Table 5.1).

Table 5.1Genetic indices for the mtDNA control region characterized in *Panulirus homarus* from 11 sample sites/collections. *N* = sample size; *H* = number of haplotypes; *hd* = haplotype diversity; and*Pi* = nucleotide diversity.

Locality	***N***	***H***	***hd***	***Pi***	**Tajima's *D* *(p-value)***
Taiwan	17	16	0.993±0.023	0.031±0.004	-1.219 (0.098)
Da Nang, Vietnam	49	49	1.000±0.004	0.040±0.003	-1.103 (0.123)
Binh Thuan, Vietnam	41	41	1.000±0.005	0.037±0.003	-1.344 (0.067)
West Timor, Indonesia	5	5	1.000±0.126	0.047±0.009	
Lombok, Indonesia	62	59	0.998±0.003	0.036±0.002	**-1.460 (0.037)**
West Sumatra, Indonesia	24	24	1.000±0.012	0.034±0.002	-1.130 (0.150)
Zanzibar, Tanzania	12	11	0.985±0.040	0.038±0.005	-1.338 (0.081)
Masirah, Oman	9	9	1.000±0.052	0.029±0.004	-0.593 (0.283)
Mirbat, Oman	45	41	0.996±0.006	0.004±0.006	**-2.253 (0.000)**
Chabahar, Iran	47	39	0.992±0.006	0.006±0.007	**-1.886 (0.008)**
Larak, Iran	33	30	0.994±0.009	0.007±0.007	**-1.640 (0.038)**
Total	344	307	0.999±0.0003	0.047±0.006	-1.397 (0.089)

Haplotype diversity for the 11 populations was extremely high, ranging from 0.985 (Zanzibar) to 1.000 (Da Nang, Binh Thuan, West Timor, West Sumatra and Masirah; Table 5.1). The nucleotide diversity of these populations ranged from 0.004 (Mirbat) to 0.047 (West Timor).

Among the 22 shared haplotypes discovered, one was represented at three sampling sites, 14 were found among two sites, while the other seven were shared only among individuals at the same site (Appendix 1).

Global AMOVA testing based on mtDNA control region sequences revealed significant population structure (Φ_{ST} = 5.31%; P=0.00; Table 5.2). In general, significant genetic differentiation was detected between the three populations from the Arabian Sea (Mirbat, Oman; Larak and Chabahar, Iran) and all other populations, for which the pairwise Φ_{ST} among populations were high, ranging from 6.1% to 18.6%, P<0.05, Table 5.3). No clear significant mtDNA control region structure was found between populations within the southeast Asian Archipelago region, as well as between the southeast Asian Archipelago and Zanzibar (Tanzania), or Masirah (Oman) in the west Indian Ocean.

Table 5.2 Summary table of analysis of molecular variance (AMOVA) describing the partitioning of genetic variation for*Panulirus homarus* populations based on both mtDNA control region sequences (Φ_{ST}; 11 populations) and six microsatellite loci (F_{ST}; 7 populations).

	Source of variation (%)			
	Among population	**Within population**	Φ_{ST}/ F_{ST}	**Probability ± SD**
mtDNA-control region	5.31	94.69	0.053	0.000 ±0.000
Microsatellites	6.02	93.97	0.060	0.000 ±0.000

Table 5.3Genetic differentiation between *Panulirus homarus* from collection locations using pairwise Φ_{ST} for mtDNA-control region data (upper diagonal) and pairwise $\boldsymbol{F_{ST}}$ for microsatellite loci (lower diagonal). Significant values after False Discovery Rate correction are in bold. Note: there are no microsatellite data for Mirabat, Larak and Chabahar.

	Indo-West Pacific Ocean					**Indian Ocean**				
		Vietnam		Indonesia		Tanzania	Oman		Iran	
	Taiwan	Da Nang	Binh Thuan	Lombok	Sumatra	Zanzibar	Masirah	Mirbat	Larak	Chabahar
Taiwan		**0.016**	**0.026**	0.005	**0.027**	**0.021**	**0.033**	**0.186**	**0.142**	**0.123**
DaNang	0.005		-0.010	0.003	-0.003	0.007	0.011	**0.092**	**0.082**	**0.067**
BinhThuan	0.001	**0.010**		0.006	-0.011	0.006	0.014	**0.083**	**0.073**	**0.060**
Lombok	**0.043**	**0.044**	**0.052**		0.006	0.010	**0.024**	**0.127**	**0.113**	**0.102**
Sumatra	**0.051**	**0.041**	**0.058**	**-0.001**		0.014	0.007	**0.088**	**0.071**	**0.064**
Zanzibar							0.014	**0.121**	**0.085**	**0.061**
Masirah	**0.181**	**0.175**	**0.198**	**0.085**	**0.075**			**0.109**	**0.073**	**0.061**
Mirbat									-0.006	-0.001
Larak										-0.004
Chabahar										

As further evidence for significant genetic structuring between the three Arabian Sea populations (Mirbat, Oman; Larak and Chabahar, Iran) and all others, the haplotype network tree revealed that the haplotypes clustered into geographical regions, or location-based groups, with the majority of haplotypes being single or unique units (Figure 5.2). Except for one haplotype from Larak, all haplotypes from the Mirbat-Oman samples (red dots), together with the Larak and Chabahar - Iran individuals (pink dots) clustered together only on the left side of the haplotype network tree, while the haplotypes of other populations were scattered throughout the network.

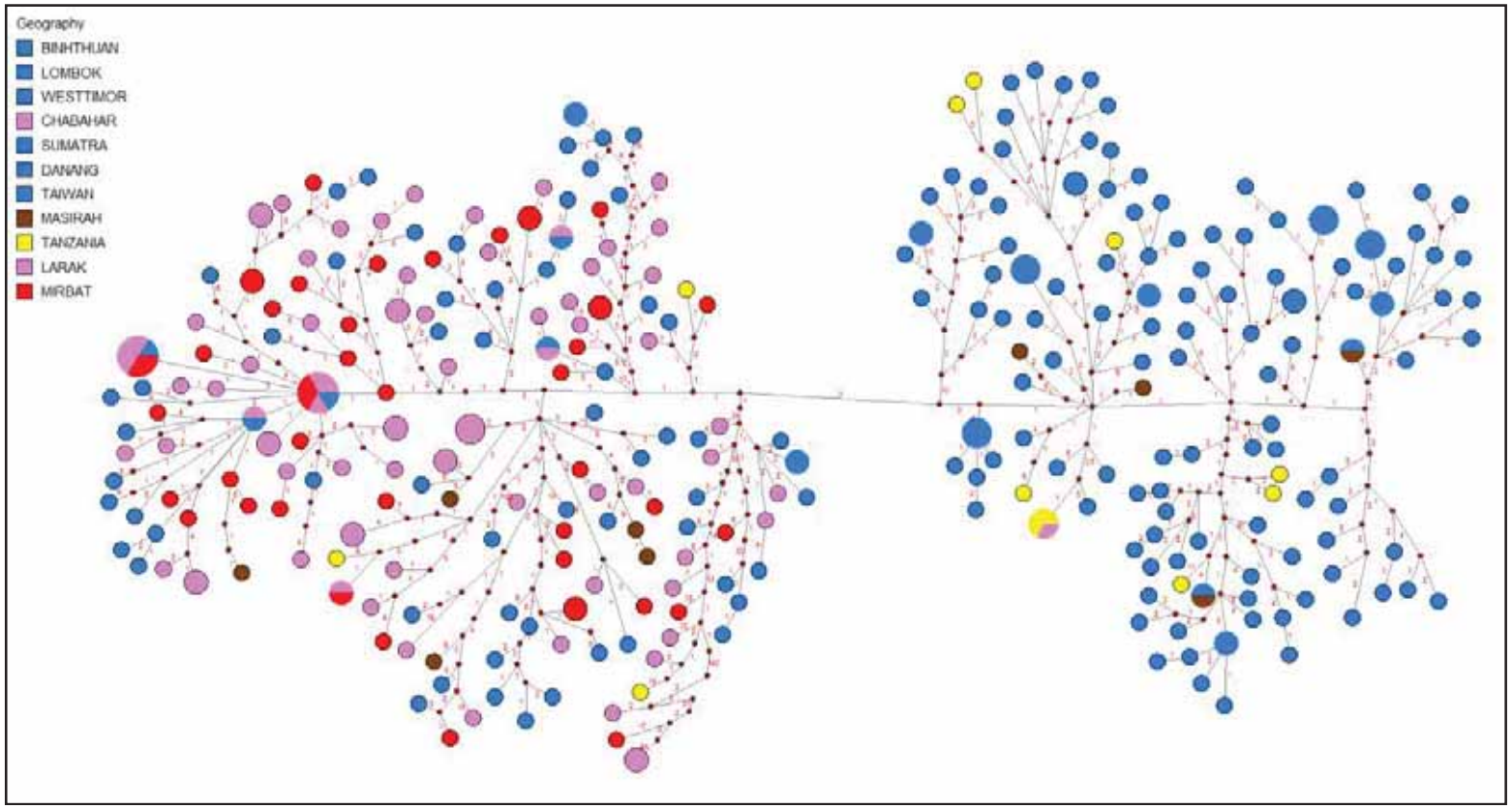

Figure 5.2 Haplotype network of *Panulirus homarus* mtDNA control region sequences from eleven collection locations from Indo-Pacific Ocean. Each circle represents a haplotype, whose diameter is proportional to the number of individuals with that haplotype. The numbers on the connecting lines are the number of mutations between haplotypes. Small red dots are missing haplotypes. The blue colour indicates Southeast Asian Archipelago's samples, while the brown colour and other colours present samples from different locations from West Indian Ocean in this study and previous study of Farhadi *et al.* (2013).

5.3.2 Microsatellite markers

Six highly polymorphic microsatellite markers were successfully amplified and the PCR products of 229 *P. homarus* samples genotyped. A total of 119 alleles were observed, ranging from 9 (marker Orn_04) to 34 (marker Orn_11) alleles per locus (Table 5.4). Significant departures from HWE were observed for some loci; however, null alleles were detected only for loci Orn_04 and Orn_12 at Masirah and Orn_20 at Da Nang. Therefore, the dataset was reanalysed with and without these markers in appropriate populations to test if they were significantly influencing the results obtained. No differences were found in genetic structure indices when these markers were included, so the complete dataset of markers were analysed and are presented here. No linkage disequilibrium was detected among the six loci genotyped.

Significant genetic structure was detected among the seven sites *P. homarus* was sampled from (AMOVA, $F_{ST} = 0.060$, $P < 0.001$). Due to the low number of individuals collected from West Timor (7 lobsters) the higher F_{ST} values involving this population are likely a result of random sampling effects and small sample size. About 6.0% of the observed genetic variation occurred among localities (Table 5.2), and pairwise F_{ST} revealed significant genetic structure between three groups of sample sites. The first group included only Omani samples (Masirah), the second represented the Vietnamese (Binh Thuan and Da Nang) and Taiwanese sampling sites, while the Indonesian sampling sites from Sumatra and Lombok fell in the third group. Around 17.5% to 19.8% of the genetic differentiation detected originated among samples from Oman and the second group of sites, while lower levels of separation (from 7.5% to 8.5%) were evident among Masirah (Oman) and Indonesian samples (Table 5.3).

The partitioning of genetic variability seen in pairwise F_{ST} estimates was consistent with the results obtained using Bayesian assignment tests. Results applied to Structure Harvester (http://taylor0.biology.ucla.edu/structureHarvester/) indicated K = 2 genetic clusters as the

most probable number of clusters within the dataset. Structure analysis confirmed the likelihood of regional genetic structuring between the Arabian Sea and Indo-West Pacific *P. homarus* populations. All samples from Masirah - Oman were assigned with a degree of genomic coancestry of > 70% to the first (white) genetic cluster (Figure 5.3), while the majority of the samples collected from Vietnam and Taiwan were assigned with high coancestry to the second (grey) genetic cluster. Indonesian samples were allocated a mixture of assignments between the two clusters. Also of interest, was the observation that some individuals from West Sumatra, Lombok or Da Nang also had similar genetic coancestry proportions to those from Masirah - Oman.

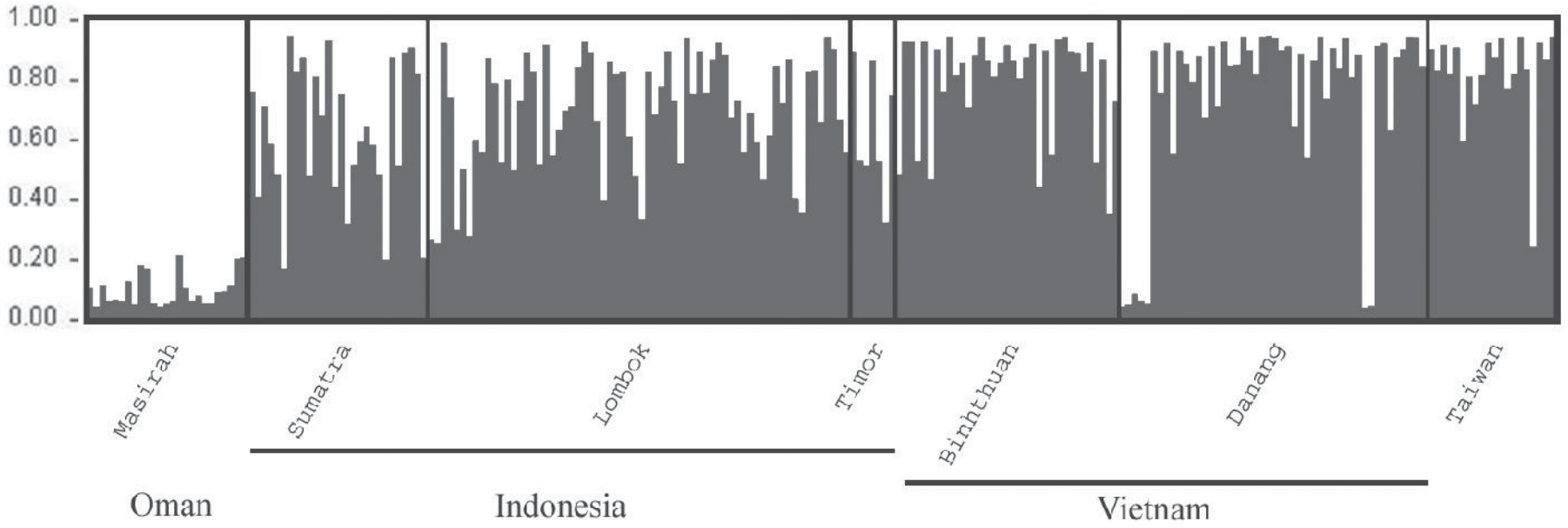

Figure 5.3 *Panulirus homarus* Bayesian individual assignment analysis for K = 2 for individuals genotyped at six microsatellites across seven sampling sites. Colours (white or grey) represent the probability (y-axis) of individuals being assigned to each genetic cluster, whilst numbers (x-axis) represent animals sampled from Oman (Masirah), Indonesia (West Sumatra, Lombok, West Timor), Vietnam (Binh Thuan and Da Nang) and Taiwan. Sampling locations were used as priors.

Table 5.4Genetic indices for six microsatellites characterized in *Panulirus homarus* at seven sample sites/collections. *N*= sample size, N_A= number of alleles, H_O= observed heterozygosity, H_E= expected heterozygosity, P_{HWE}= Hardy-Weinberg equilibrium significance value at P<0.05 after FDR correction, *ns*= non-significant, *bold text*= significant at P< 0.05, *= presence of null allele, and*PIC*= polymorphic information content.

Locality		**Microsatellites**					
		Orn_04	**Orn_05**	**Orn_11**	**Orn_12**	**Orn_16**	**Orn_20**
Masirah	***N***	24	25	25	24	25	24
	Na	7	14	22	16	14	3
	Ho	0.46	0.88	0.92	0.54	0.80	0.08
	P_{HWE}	**0.00***	0.84	0.74	**0.00***	0.55	1.00
Binh Thuan	***N***	35	35	29	35	35	35
	Na	5	11	12	15	9	6
	Ho	0.63	0.63	1.00	0.86	0.69	0.17
		0.01	**0.01**	0.74	0.21	0.59	1.00
Da Nang	***N***	48	47	48	48	48	48
	Na	6	13	21	19	13	11
	Ho	0.42	0.60	0.90	0.83	0.63	0.27
	P_{HWE}	0.91	**0.00**	**0.00**	1.00	0.05*	**0.00***
Taiwan	***N***	20	20	20	20	20	20
	Na	4	5	12	12	8	3
	Ho	0.55	0.70	0.85	0.90	0.80	0.15
	P_{HWE}	0.45	0.81	0.82	0.02	0.36	0.03
West Sumatra	***N***	27	28	28	28	28	24
	Na	4	7	13	16	10	3
	Ho	0.63	0.54	0.86	1.00	0.71	0.46
		0.95	1.00	0.68	0.33	0.33	0.66
LomBok	***N***	65	65	66	66	65	64
	Na	6	10	16	18	9	5
	Ho	0.63	0.65	0.86	0.82	0.71	0.48
	P_{HWE}	0.70	0.81	0.26	0.25*	**0.00**	**0.00**
West Timor	***N***	6	6	7	7	7	7
	Na	3	4	8	10	6	2
	Ho	0.50	0.83	0.86	1.00	0.57	0.29
	P_{HWE}	0.88	0.80	0.59	0.55	0.87	0.43
Mean	***N***	32	32	32	33	33	32
	Na	5	9	15	15	10	5
	Ho	0.54	0.69	0.89	0.85	0.70	0.27
	PIC	0.55	0.67	0.88	0.93	0.68	0.54
Total	***N***	225	226	223	228	228	222
	Na	9	22	34	25	18	11
	Allele size range (bp)	362-378	166-216	158-235	256-370	151-191	305-349

5.4 CONCLUSION AND DISCUSSION

Both mtDNA control region and microsatellites reveal the presence of significant structure among *P. homarus* populations across the species' geographic distribution. High pairwise Φ_{ST} values (6.0 - 18.6%) were evident between the Southeast Asian Archipelago samples (Taiwan; Da Nang and Binh Thuan in Vietnam, along with West Sumatra, Lombok, and West Timor in Indonesia) and the Arabian Sea samples from the Indian Ocean (Mirbat in Oman; Larak and Chabahar in Iran). However, the mtDNA control region marker sutilised in this study, failed to detect genetic differentiation amongst the Southeast Asian Archipelago localities (Φ_{ST} = 0.004; P >0.05), or between the Southeast Asian Archipelago and other Indian Ocean populations (Masirah, Oman and Zanzibar, Tanzania). In contrast to this limitation in the mtDNA control region data, six polymorphic microsatellites could reveal genetic structure among populations(overall F_{ST} = 0.060, P < 0.05), with moderate differentiation between the Southeast Asian Archipelago and Masirah populations (pairwise F_{ST} = 7.5% - 19.8%). The different genetic partitioning patterns discovered among the Southeast Asian Archipelago populations and the Masirah (Oman) population were also evident in the Bayesian individual assignment analysis (Figure 5.3). All samples from Masirah exhibited high co-ancestry with the first genetic cluster, however, a few individuals collected from West Sumatra, Lombok and Da Nang also exhibited high co-ancestry to several Omani samples. Despite this, the majority of samples were assigned with high co-ancestry to the second genetic cluster.

These results from the molecular data may be explained when looking at the morphotypes of *P. homarus* individuals across the sampling distribution. The *P. homarus* samples collected in the Indian Ocean (Masirah in Oman) as part of the present study, as well as Indian Ocean samples from the previous study (Farhadi *et al.*, 2013), belong to the megascupta form (Appendix 2), which was described by Berry (1974) as *P. homarus megasculptus*. In the

Southeast Asian Archipelago, however, most lobsters exhibit the microsculpta form which is described as *P. homarus homarus*, although a small proportion of individuals possessing the megasculpta morphotype have also been recognized from the region (Pham, 2000). The genetic data herein therefore suggests that there may be one-way dispersal of *P. homarus megasculptus* from the western Indian Ocean into the Southeast Asian Archipelago. In addition, the genetic differentiation patterns among the Southeast Asian Archipelago and Masirah populations were only detected using microsatellite markers, which would not have been detected by mtDNA control region markers as they are maternally inherited. Given this, it is possible that only female lobsters disperse towards the Southeast Asian Archipelago from Indian Ocean source populations, giving rise to a genetic pollution effect in the Southeast Asian Archipelago (pairwise F_{ST} = 4.1% - 5.8% among populations).

The genetic breaks in population structure between the Arabian Sea populations (Mirbat, Larak and Chabahar) and other populations shown in the present study may be a consequence of ocean movement patterns which restrict gene flow among localities. Pollock (1993) explained the occurrence of *P. h. megasculptus* using oceanographic features. In this assessment, it was posited that weaker oceanic circulation during the previous glaciation event in the Arabian Basin, together with a shallower Laccadive-Chagos ridge to the southwest of the Indian continent could have increased retention of larvae of a northern *P. homarus* population within the Arabian Sea. Therefore, the restricted circulation patterns would have reduced the degree of larval interchange amongst widespread populations of the same species and natural selection would promote gradual genetic divergence towards *P. homarus megasculptus* within partially isolated stocks (see Pollock (1993); Figure 5.4). Previous study of Abdullah *et al.,* (2014b) found strong regional structuring between notherwestern and other Indian Ocean populations of *P. penicillatus* due to the ocean currents' pattern.

Within Indian Ocean populations, there was surprisingly no significant genetic differentiation detected between lobsters sampled from the Arabian Sea (Masirah, Oman) and eastern Africa (Zanzibar; pairwise Φ_{ST} = 1.4%,P > 0.05). It is also surprising that clear differentiation between animals collected from Masirah and the other Arabian Sea sampling locations was found (the pairwise Φ_{ST} ranged from 6.1 to 10.9%, P < 0.05; Table 5.3). The Masirah animals were collected at a local fish market, where lobsters captured from both nearby fishing grounds as well as more distant adjacent areas are likely to have accumulated. Given this, it is possible that the individuals sampled at Masirah originated from a different location, which could help to explain the genetic differences between these samples and other Arabian Sea collections from Mirbat, Larak and Chabahar. If, however, the Masirah animals were caught in local fishing grounds, this would indicate that the *P. homarus* population in the Arabian Sea might contain two different genetic stocks, consisting of the local stock (e.g. samples originating from Mirbat, Oman and Larak or Chabahar, Iran), and the migrant stock from the adjacent areas of the western Indian Ocean.

The current study provides evidence for genetic structuring of *P. homarus* among populations across the species' geographic distribution, even though the species has a high dispersal potential, which is mediated via adult spawning migrations and a long pelagic larval duration (5.5 to 8 months; Phillips, 2013). This suggests the need for several management units over the species distribution and given that *P. homarus* is under intense fishing pressure and there are declining annual catch rates (Radhakrishnan *et al.*, 2005; Mehanna *et al.*, 2012; Phillips, 2013), it is necessary to have better management controls for stocks of this species.

Finally, although the data from the mtDNA study of (Farhadi *et al.*, 2013) could be integrated with mtDNA data from the current study there is currently no microsatellite marker data for *P. homarus* from the western Indian Ocean from locations such as Tanzania and Iran. As a result

of this, the levels of genetic differentiation within the nuclear genome between Zanzibar (Tanzania) and Masirah (Oman) or Zanzibar (Tanzanian) and Indo-West Pacific samples could not be evaluated. Therefore, it is suggested in future to sample these locations and to use additional microsatellite markers for future research into the population structure of *P. homarus* throughout the species' range. It would also be advantageous to combine these investigations with studies on the ecology of the species, with special attention being paid to the locations of breeding grounds and spawning times, which could be applied to a bio-physical model to understand the oceanographic dispersal pathway of *P. homarus* larvae across their entire geographic distribution. These studies will provide essential information about the present, as well as future status of *P. homarus* stock for more effective management of the fishery associated with this species.

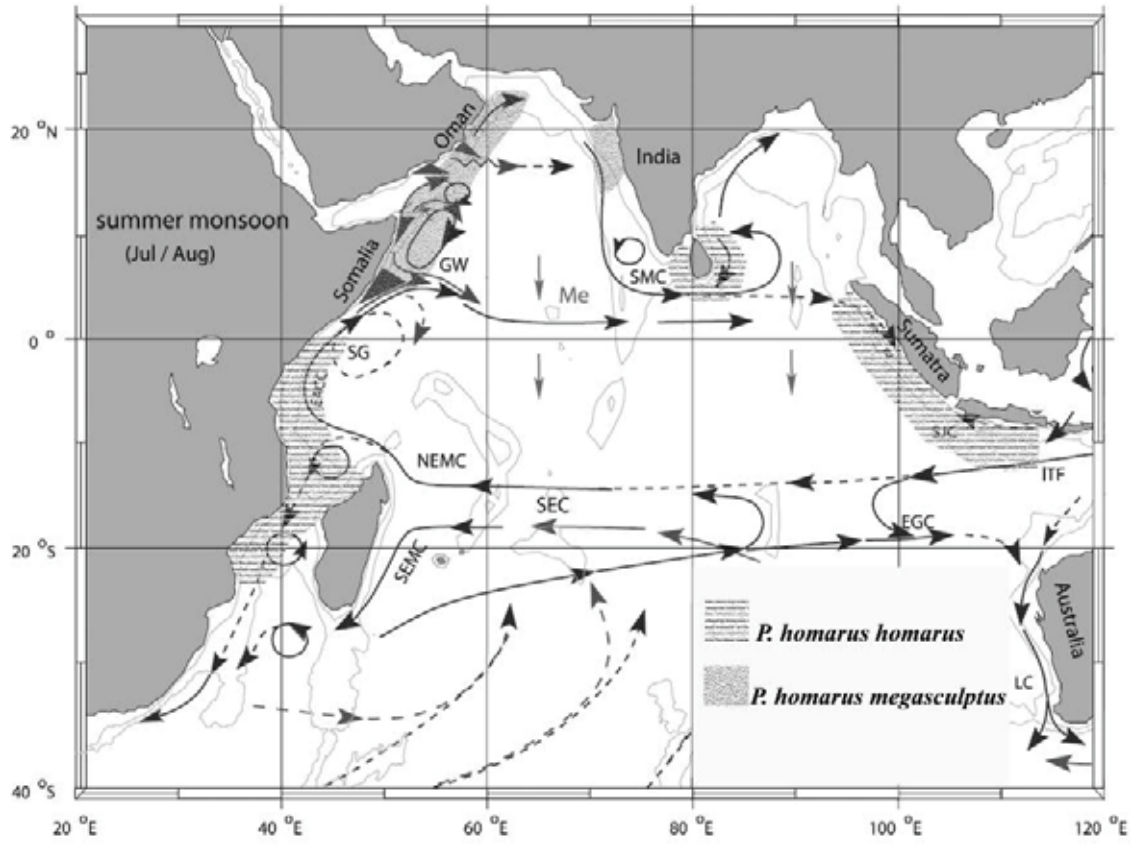

Figure 5.4 Distribution of two*Panulirus homarus* subspecies (*P. h. homarus* and *P. h. megasculptus*) in the Indian Ocean in relation to modern (interglacial) surface circulation patterns during southwestern monsoons (July/August). Current branches indicated are the South Equatorial Current (SEC), South Equatorial Countercurrent (SECC), Northeast and Southeast Madagascar Current (NEMC and SEMC), East African Coastal Current (EACC), Somali Current (SC), Southern Gyre (SG), Great Whirl (GW), Southwest and Northeast Monsoon Currents (SMC and NMC), South Java Current (SJC), East Gyral Current (EGC), and the Leeuwin Current (LC).Vectors (Me) show directions of meridional Ekman transports. Modified from the studies of Pollock (1993) and Schott & McCreary Jr (2001)

CHAPTER 6: *IN SILICO* DEVELOPMENT AND EVALUATION OF A DNA PARENTAGE MARKER SUITE FOR *Panulirus ornatus* TO ASSIST FUTURE BREEDING PROGRAMS

6.1 BACKGROUND

Grow-out of the tropical spiny lobster *P. ornatus* for aquaculture is well developed in Vietnam and is also undergoing rapid expansion in Indonesia. Seed supply for lobster farming is currently based on the collection of naturally settling pueruli, which in Vietnam at least, shows large variability in total annual catch rates (Jones C. *et al.*, 2010; Long & D. Hoc, 2009). Reliance on wild seed creates unstable conditions for the aquaculture industry, and as a consequence, hatchery technology to produce captive bred *Panulirus ornatus* juveniles has been actively pursued with the first successful production of captive pueruli achieved in 2006 by an Australian company, Lobster Harvest Pty Ltd. Hatchery production technology is now on the threshold of commercialization and it is likely that future aquaculture of spiny lobsters will be derived at least in some part from captive bred progeny (Jones & Shanks, 2009; Jones C. *et al.*, 2010; Phillips & Matsuda, 2011).

Once hatchery production becomes routine, the industry is likely to want to move rapidly towards the establishment of breeding programs and the domestication of superior stocks. Forward planning and the development of tools to assist future breeding programs are therefore prudent. Tropical spiny lobsters are particularly fecund, with *P. ornatus* breeding females producing an excess of 500,000 eggs per spawning (Passfield, 1988). From a commercial hatchery perspective, this means that just a few breeding females can produce

more than enough eggs to stock entire farms. However, there is great risk in using a small number of broodstock to supply seed, particularly in relation to a breeding program, as this may quickly lead to inbreeding and associated phenotypic consequences of inbreeding depression.

Hatchery-bred populations may have greatly reduced genetic diversity compared to wild populations (Sbordoni *et al.*, 1986; Beardmore *et al.*, 1997;Xu *et al.*, 2001), which if left unmanaged can result in a reduction in fitness of traits, such as growth rate, or viability through the inbreeding of related genotypes (Falconer & MacKay, 1981; Wolfus *et al.*, 1997). In order to minimize the threat of inbreeding in a breeding program based on family selection, reliable pedigree information is essential (Jerry *et al.*, 2004; Jerry *et al.*, 2006). Parentage inference using DNA markers such as microsatellites has become commonplace to establish pedigrees (Estoup *et al.*, 1998; Garcia de Leon *et al.*, 1998; Wolfus *et al.*, 1997; Xu *et al.*, 2001; Hara & Sekino, 2003; Jerry *et al.*, 2004; Jerry *et al.*, 2006; Dong *et al.*, 2006; Castro *et al.*, 2007; Wang *et al.*, 2012). Therefore, if breeding programs for *P. ornatus* are to be developed, a valuable tool to assist such programs will be the identification of a suite of DNA microsatellite markers that can be applied to determine parentage of individuals. Having a powerful DNA parentage marker suite will allow the industry to establish reliable family pedigree data and to accurately make mate choices that will avoid inbreeding.

Molecular DNA parentage analysis is based on the simple concept that diploid parents pass on only one of the possible two alleles at each locus in their gametes to offspring. Offspring therefore carry one allele from the father and the other derived from the mother (Herbinger *et al.*, 1995). With the development of DNA markers (e.g., microsatellites) and sophisticated computer software such as CERVUS (Kalinowski *et al.*, 2007), COLONY (Jones & Wang, 2010), FAP (Taggart, 2007)and PAPA (Duchesne *et al.*, 2002) to analyse relationships among

individuals, molecular parentage analysis has made the reconstruction of pedigrees is possible in many taxonomic groups, including aquaculture species (Danzmann, 1997; Estoup *et al.*, 1998; Herbinger *et al.*, 1995; Wilson & Ferguson, 2002; Jackson *et al.*, 2003; Jerry *et al.*, 2004; Sekino *et al.*, 2004; Jerry *et al.*, 2006; Castro *et al.*, 2007; Wang *et al.*, 2012). In Chapter 3, 30 microsatellites were isolated for genetic analysis in the Ornate Spiny Lobster *P. ornatus*and 10 of these were used to investigate the population genetic structure of two different *Panulirus* species (in Chapter 4 & 5; Dao *et al.,*2013). Despite their power to elucidate broad-scale population genetic structure, there is no current information on the potential of these same microsatellite DNA parentage markers to assign parentage for future commercial *Panulirus* breeding programs.

CERVUS v.3.0 (Marshall *et al.*, 1998) is a computer simulation program that has been used extensively to assign offspring of various species to their parents using genetic markers. Whilst CERVUS v.3.0 is primarily used to assign parentage, it also can be used to simulate theoretical broodstock genotypes and thus is helpful in the initial design of DNA parentage marker suites. In the absence of having large numbers of hatchery-bred spiny ornate lobster progeny families that can be produced and genotyped due to current industry breeding limitations of the species, simulation approaches are useful in assessing the probable performance of a DNA marker and in establishing the number of loci required to assign parentage based on population allelic frequencies. In this chapter, previously acquired genotypes from the Ornate Spiny Lobster (Chapter 4) were used as *in silico* broodstock under various mating strategies in order to establish the best combination of microsatellite markers which would be useful for a DNA parentage marker suite for the species.

6.2 METHODS

6.2.1 Computer simulation of parentage assignment

From six populations in Vietnam, Indonesia, and Australia, 120 individuals of *P. ornatus* with no missing genotypes (in Chapter 4) were chosen and used as the theoretical broodstock for simulations. Genotypes at 10 microsatellite loci previously (Orn_01, Orn_02, Orn_11, Orn_12, Orn_16, Orn_17, Orn_18, Orn_20, Orn_21, and Orn_25) generated in the *P. ornatus* population genetic study (Table 3.1; in Chapter 3 and Dao *et al.* (2013)) were used in all simulations. In each simulation, potential male and female parents were randomly chosen (using Excel function - RAND) from the pool of these 120 individuals and their genotypes mated to create family genotypes of progeny *in silico* (assuming no mutation or transmission error between parents and their progeny) using HYBRIDLAB v.1.0 (Nielsen *et al.*, 2006). Fifty progeny per parental cross were generated.

In order to estimate the number of loci that would be required to confidently assign parentage in a theoretical breeding population of *P. ornatus*, three mating scenarios with three replicates for each scenario were simulated. These three scenarios were:

Scenario 1. This simulation evaluated the power of markers under the assumption that all progeny were grouped into only full-sib families (*ie* no half-sib families produced). Here 10, 20, 30, 40 and 50 candidate dams (maternal parents) were mated to one of an equal number of candidate sires (paternal parents; Figure 6.1). Fifty progeny from each family were simulated and pooled to replicate a pond grow-out scenario where multiple families would be co-reared.

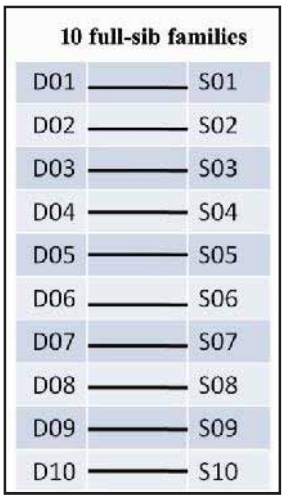

Figure 6.1 Mating design in the first simulation illustrating the mating design where 10 full-sib families were produced. A similar design was used for the production of 20, 30, 40 and 50 full-sib families. D = Dam; S = Sire.

Scenario 2.

This scenario compared the power of microsatellites to assign a pool of progeny from both full-sib and half-sib families or only half-sib families, including two simulations:

One simulation evaluated the power of microsatellites under the mating scenario where some females and males selected randomly from the candidate parent group could mate with more than a single broodstock individual (*i.e.* progeny could be both half- and full-sibs; Figure 6.2). Under this simulation, one female could randomly mate with more than one male and vice versa, but not all broodstock were allowed multiple matings. Although the exact mating behaviour of *P. ornatus* is not known, this simulation was based on the known occurrence of occasional multiple matings in many lobster species (Alves & Paiva, 1976; Nelson & Hedgecock, 1977; Ferguson, 2002; Gosselin *et al.*, 2005). A total of 10 random matings (50 progeny per mating) were simulated where the majority of progeny from this simulation belonged to half-sib families (multiple paternal or multiple maternal), while the rest of the progeny were derived from only full-sib families (e.g. S10-D07 in replicate 1). This

simulation identified the number of loci required for a pool of progeny derived from both full-sib and half-sib families to correctly assign parentage.

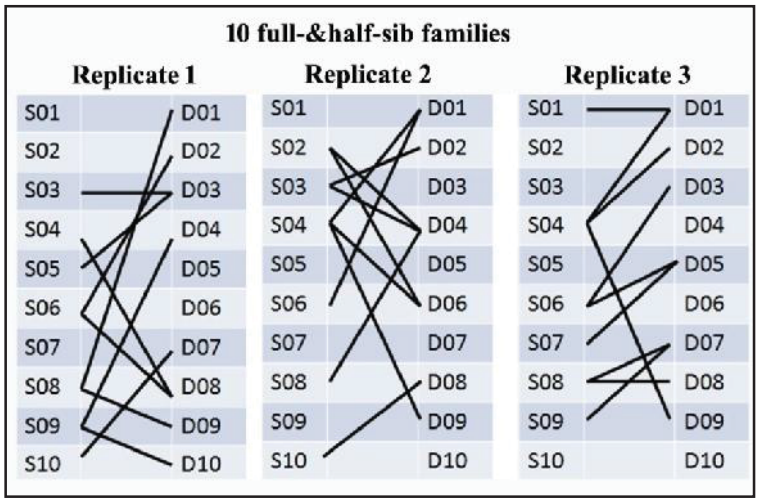

Figure 6.2 Mating design in the second simulation involving the production of 10 full-&half-sib families (three replicates). D = Dam; S = Sire.

The other simulation identified the power of microsatellites to assign parentage was evaluated under the scenario where all progeny were derived from only half-sib families obtained from multiple matings of female broodstock. Here 10 candidate dams and 30 candidate sires were randomly chosen from the pool of 120 individuals and each female mated to three males to produce a total of 30 half-sib families (50 progeny from each family). This scenario was chosen to represent the case where a single female may be put into an individual mating tank with multiple males (Figure 6.3).

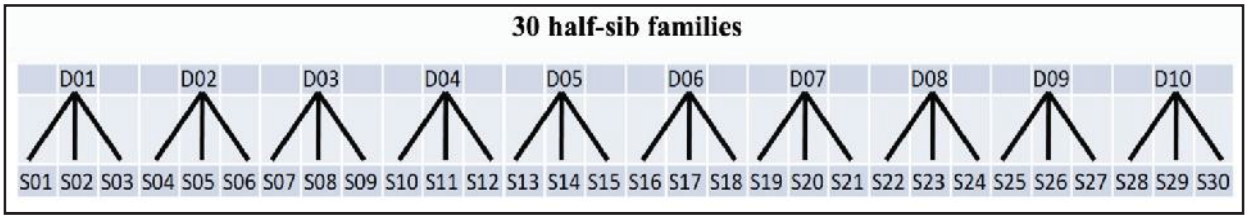

Figure 6.3 Mating design in the third simulation to produce 30 half-sib families where each female is mated to three males (10 candidate dams and 30 candidate sires). D = Dam; S = Sire.

CERVUS v. 3.0 (Marshall *et al.*, 1998; with the parent's sex known) was used to estimate how accurately progeny could be assigned to their putative parent. Simulation parameters were performed for 10,000 replication cycles, with the proportion of parents and corresponding loci genotyped set to 1.0 and a 1% error rate in likelihood calculations (Jerry *et al.*, 2006). Simulation and parentage assignment tests were started using the locus with the highest polymorphic information content (PIC), followed by sequentially adding other loci to the marker suite depending on their PIC (from the next highest PIC). In simulations, therefore, the order of the 10 loci that were used to predict assignment success of the Ornate Spiny Lobster were Orn_12, Orn_11, Orn_21, Orn_16, Orn_01, Orn_18, Orn_25, Orn_02, Orn_20 and Orn_17. The minimum allowable confidence level at which a candidate parent's assignment was accepted as being correctly assigned was 95%.

The results of these simulations were presented as an average of three replicates with standard error (SE). Data was analysed statistically using one-way analysis of variance (ANOVA) with a 95% level of confidence.

6.3 RESULTS

6.3.1 Genetic diversity of microsatellites

Allelic diversity in the genotyped broodstock at the 10 microsatellite loci varied, with values ranging from three alleles present in locus Orn_02 to 21 alleles in locus Orn_11 (Table 6.1). This high allelic diversity correspondingly manifested in a high overall heterozygosity of broodstock and high PIC of markers. The two most informative microsatellite loci were Orn_12 and Orn_11, which possessed 20 and 21 alleles respectively, and that had PIC higher than 0.9.

Table 6.1 Number of alleles, observed heterozygosity (H_O), expected heterozygosity (H_E), polymorphic information content (PIC) of 10 microsatellite loci where broodstock were genotyped and markers evaluated for their ability to correctly assign *P. ornatus* progeny to their known parents. These values were estimated from real genotyped data of 120 *Panulirus ornatus* individuals in Chapter 4.

Locus	No. of alleles	H_O	H_E	PIC
Orn_12	20	0.990 ± 0.07	0.946 ± 0.001	0.931 ± 0.002
Orn_11	21	0.885 ± 0.020	0.925 ± 0.002	0.908 ± 0.003
Orn_21	7	0.786 ± 0.013	0.833 ± 0.001	0.800 ± 0.003
Orn_16	9	0.806 ± 0.010	0.747 ± 0.004	0.707 ± 0.004
Orn_01	8	0.651 ± 0.014	0.699 ± 0.011	0.653 ± 0.014
Orn_18	9	0.711 ± 0.010	0.690 ± 0.006	0.659 ± 0.007
Orn_25	7	0.710 ± 0.019	0.690 ± 0.008	0.628 ± 0.013
Orn_02	3	0.605 ± 0.016	0.618 ± 0.006	0.539 ± 0.006
Orn_20	12	0.462 ± 0.010	0.480 ± 0.009	0.463 ± 0.010
Orn_17	8	0.454 ± 0.017	0.501 ± 0.019	0.466 ± 0.019
Mean/ total	10.5	0.706 ± 0.014	0.713 ± 0.007	0.675 ± 0.019

6.3.2 Parentage assignment

Scenario 1 - Assignment success (%) of Panulirus ornatus progeny derived from 10, 20, 30, 40 and 50 full-sib families

Simulations demonstrate that a microsatellite marker suite of the six most powerful loci (with over 95% correct assignment rate) was sufficient to correctly assign lobster progeny to their correct family grouping, even when up to 50 full-sib families were mixed (Figure 6.4). However, it was observed that correct assignment to the correct families with only 5 loci and family sizes affected the correct parental assignment (Figure 6.5). There was a decreasing trend in correct assignment rate to their families when increasing the family number in the simulation from 10 to 50 full-sib families. The correct assignment to their families required only 3 loci in 10 full-sib families, while it was necessary to utilise at least 4 (in 20 and 30 full-

sib families) or 5 loci (in 40 and 50 full-sib families) to achieve over 95% of correct assignment to their families (Figure 6.5). In addition, 100% assignment was not reached if the number of full-sib families was higher than 30 (98% for 40 full-sib families, 97% for 30 full-sib families).

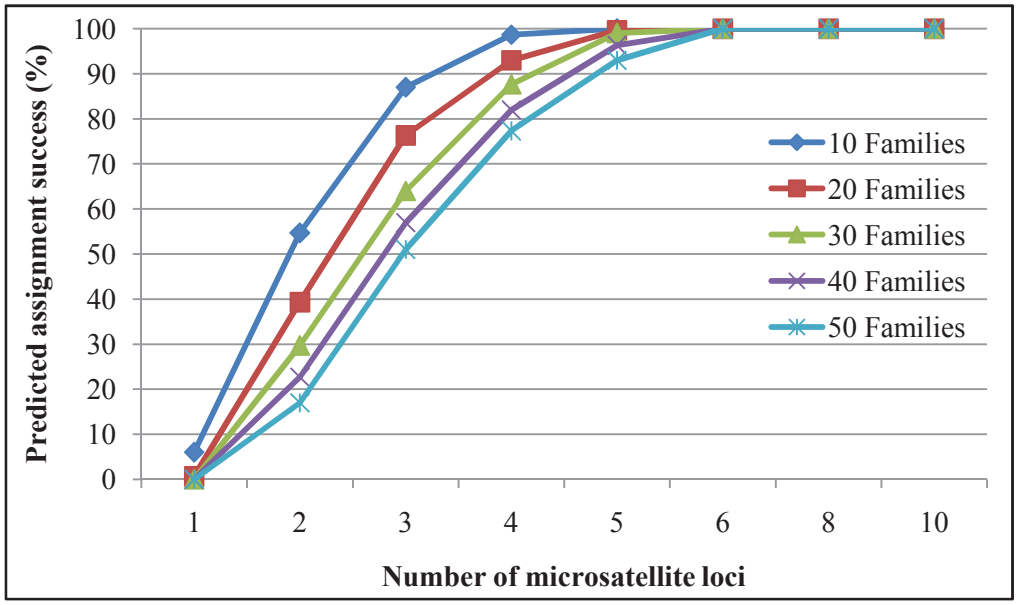

Figure 6.4 Predicted assignment success (%) of *Panulirus ornatus* progeny to their correct families based on simulations involving a total population pool of 10, 20, 30, 40, and 50 families using up to 10 microsatellite markers.

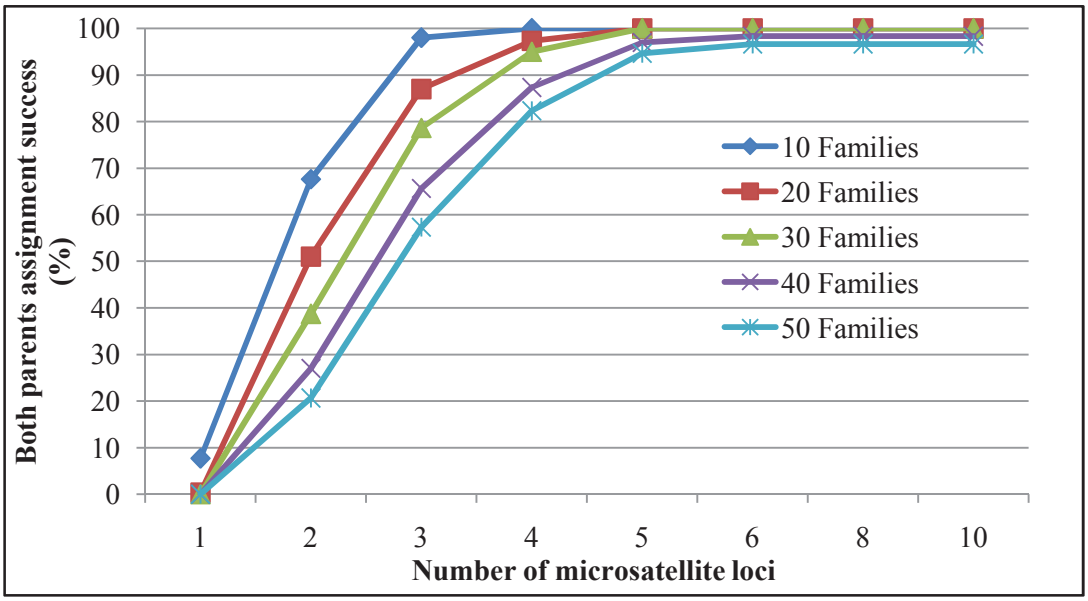

Figure 6.5Assignment rate (%) of *Panulirus ornatus* progeny derived from 10, 20, 30, 40 and 50 families to their correct families.

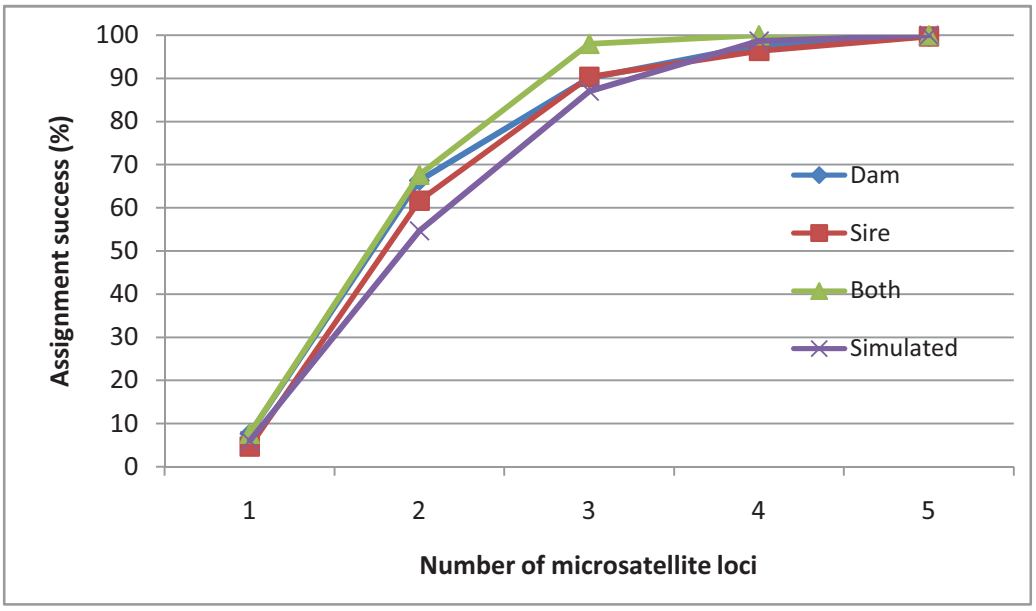

Figure 6.6 Correct assignment rate (%) of *Panulirus ornatus* progeny derived from 10 full-sib families to their correct dam, sire, or families.

At three loci in the simulation of 10 full-sib families, the successful assignment to their families was 98%, much higher than that to dam only or to sire only (90%; Figure 6.6). There was no difference between the results when a marker suite of more than three loci was used to correctly assign progeny to their families (Table 6.2).

Scenario 2 - Assignment success (%) of Panulirus ornatus progeny derived from 10 full-&half-sib families and 30 half-sib families

In both simulations of 10 full-&half-sib families and 30 half-sib families, four loci were powerful enough to correctly assign parents to their families (Figure 6.7 & 6.8). The assignment success (%) of progeny to dam and to sire were equal across all loci sets in 10 full-&half-sib families simulation (Figure 6.7), and these values were lower than those to their families. However, in the simulation of 30 half-sib families, with a marker suite of less than 6 loci, the correct assignment rate (%) of progeny to sire was much lower than other assignment success (%) of progeny to dam or to their families. As an example, the assignment success of

progeny to sire alone was 80% at four loci, whereas this increased to 96% when assigning progeny to dam alone, or assignment to their families together (Figure 6.8, Table 6.2).

In the comparison between the three types of simulations, only three markers with the highest PIC are needed to achieve over 95% correct assignment parentage rate in the simulation of 10 full-sib families, while at least four of the highest PIC loci are required for the simulation of 10 full-&half-sib and 30 half-sib families (Figure 6.9).

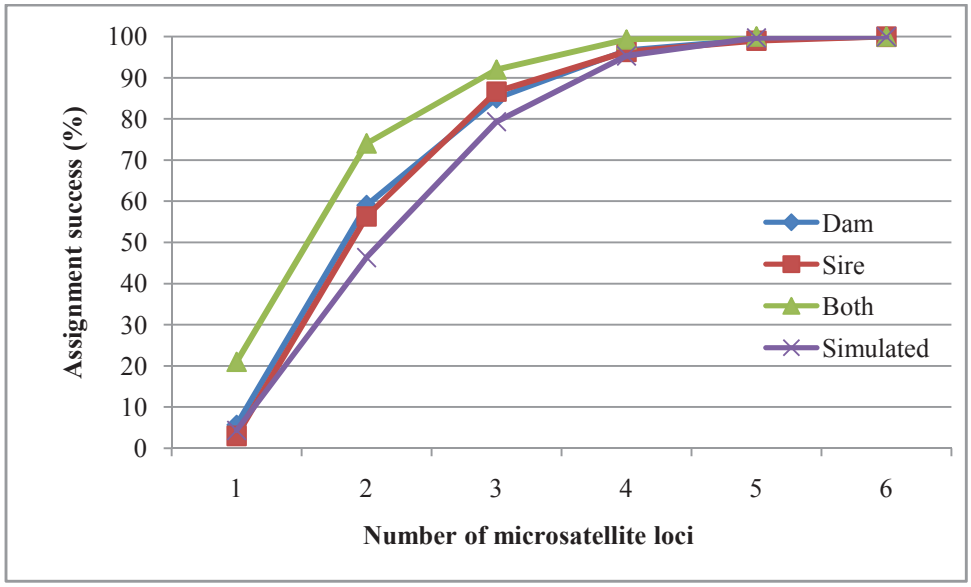

Figure 6.7 Correct assignment rate (%) of *Panulirus ornatus* progeny derived from 10 full-&half-sib families to their correct dam, sire, or families.

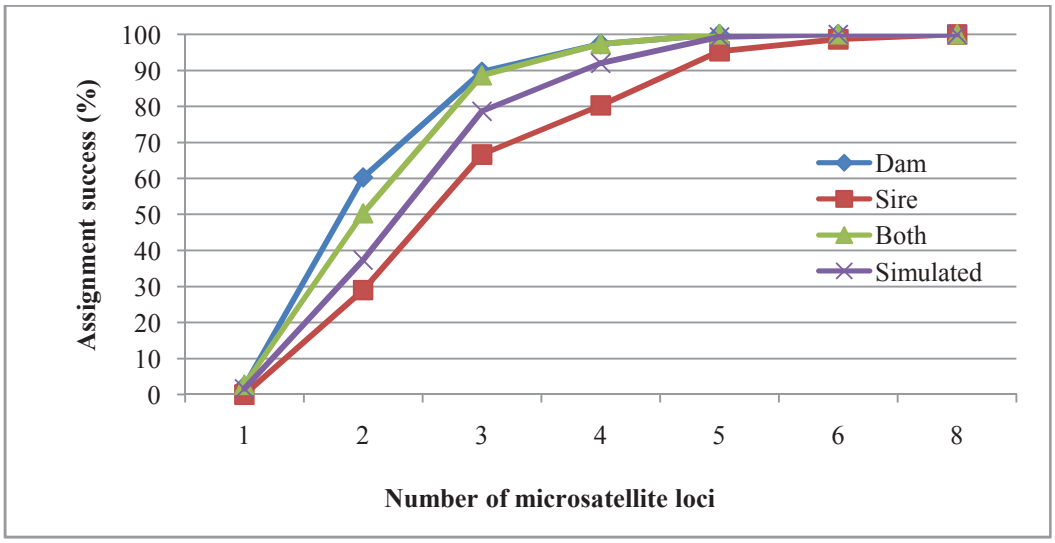

Figure 6.8 Correct assignment rate (%) of *Panulirus ornatus* progeny derived from 30 half-sib families to their correct dam, sire, or families.

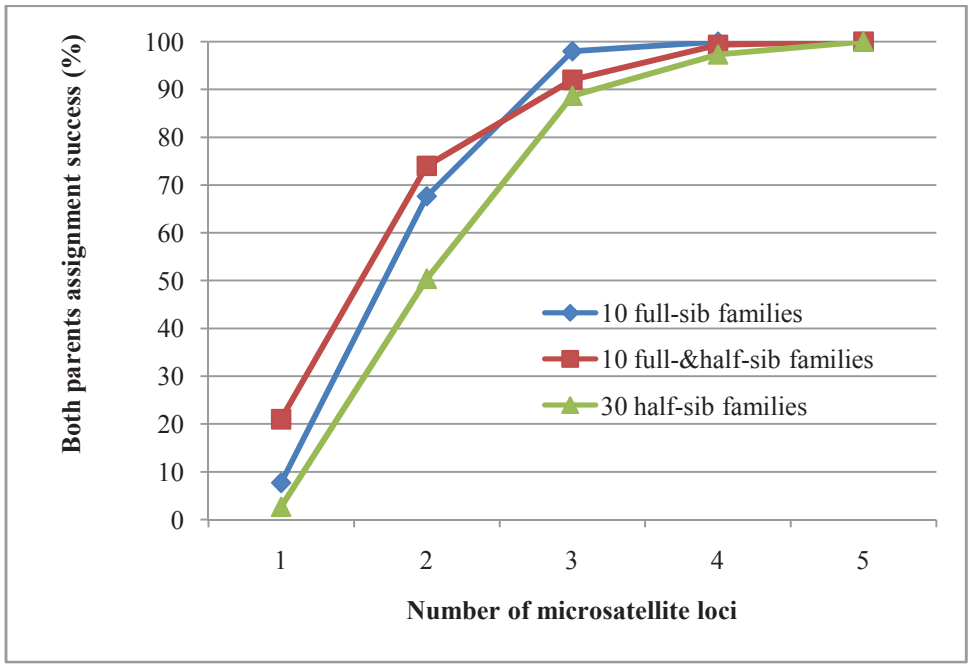

Figure 6.9 Assignment rate (%) of *Panulirus ornatus* progeny derived from 10 full-sib, 10 half-sib and 30 half-sib families to their correct families.

6.4 DISCUSSION

The results indicated that a marker suite of the five loci with the highest PIC could be used to correctly assign parentage in the Ornate Spiny Lobster *P. ornatus*. In the simulation of full-sib families, the marker suite of five loci reliably assigned both parents in pools of progeny with up to 30 families with 100% accuracy, and only reduced to 97% and 95% accuracy where the parentage of progeny had to be discriminated from pools of 40 families or more. For the 10 full-sib families simulation, only 3-4 microsatellite markers were needed to assign parentage, while the results of using 5 loci produced the highest assignment success rates to their families (100%) in 10 full-&half-sib and 30 half-sib families simulated. In addition, the assignment success rates of dams were higher than those of sires in the simulation of 30 half-sib families, but this value was similar in 10 full-sib or 10 full-&half-sib family simulations.

The number of microsatellite markers and number of alleles per loci both contributed to increased assignment success. In all simulations the more loci that were used led to higher assignment success, however, after the most six polymorphic markers were used in the marker suite, assignment success rapidly asympoted. These results were consistent with many previous studies on the effects of the numbers of loci used to determine parentage (Bernatchez & Duchesne, 2000; Jerry *et al.*, 2004; Vandeputte *et al.*, 2004; Dong *et al.*, 2006; Jerry *et al.*, 2006; Wang *et al.*, 2012). All these studies indicated that allocation success was highly dependent on the number of loci used, with the efficiency of parentage assignment obviously being higher when using more microsatellites. In addition, microsatellite markers with many alleles have more discriminatory power to assign parentage. For example, the use of eight markers with an average of 7.8 alleles per locus gave the theoretical capability of assigning 95% or more offspring to the correct parents for common carp (*Cyprinus carpio* L) in a population of 10 dams x 24 sires (Vandeputte *et al.*, 2004). Estoup *et al.,* (1998) found that the efficiency of parentage assignment for a population of rainbow trout with 34 males and 34 females when also using eight loci (8.4 alleles per loci) was 95%. A simulation to assign parentage for the swimming crab *Portunus trituberculatus* demonstrated that a marker suite of six loci containing an average of 16.7 alleles per microsatellite could reliably assign parentage in up to 50 families (Wang *et al.*, 2012). Therefore, with an average of 13 alleles per microsatellite, the six most powerful *P. ornatus* loci evaluated here could correctly assign offspring to their families in up to 50 full-sib families. Furthermore, the loss of alleles per locus in offspring may lead to higher assignment success when using microsatellite markers, which have more alleles, as the number of alleles per locus in offspring was found to be lower when compared to their parents (Hara & Sekino, 2003). Castro *et al.* (2004) also found that the low accuracy of microsatellite loci in parentage assignment was affected by the presence of null alleles.

Differences in the numbers of candidate dams and sires in the broodstock population also affected the assignment success rate. Full-& half-sib families or half-sib families required more microsatellite markers to assign parentage than full-sib families. In this computer simulation, a pool of progeny comprising of 10 full-sib families needed only 3–4 loci to correctly assign over 95% offspring to their true parents, while simulations involving 10 full-& half-sib and/or 30 half-sib families required six loci. This is likely due to the difference of the relationship between a parent and offspring in full-sibs and half-sib families, 50% and 25%, respectively (Gjedrem & Baranski, 2010). It is also possible that the fewer potential candidates in the population could reduce the probability of individuals sharing the same alleles (Jerry *et al.*, 2004; Gjedrem & Baranski, 2010). Adding more families to the broodstock population resulted in a decrease in correct assignments, even when the numbers of loci were increased. A similar effect was seen in simulations for *P. monodon* by Jerry *et al.* (2006), who showed that seven loci could predict progeny assignment success in up to 20 families, but in a larger number of families assignment success rate decreased.

Whilst simulations are useful in determining the most powerful markers to use in a DNA parentage marker suite, it is important to consider the limitations of using computer simulations, as simulation approaches often have been shown to be limited in deciding how many loci are actually required when evaluated under real-world conditions (Jerry *et al.*, 2004; Dong *et al.*, 2006). Differences have been found between simulations and real data sets due to the presence of null alleles at loci that were not accounted for in the simulations, and/or substantial allelic dropout caused by poor quality DNA (Jerry *et al.*, 2004). Also in aquaculture, the level of relatedness between broodstock are often higher than that simulated based on random mating, which might lower the overall power of microsatellite markers (Norris *et al.*, 2000). Simulation studies are commonly used to estimate the assignment abilities of microsatellite markers to indicate how many loci are actually required and to

evaluate how these loci would perform under real situations. For example, Dong *et al.* (2006) found that using five loci could correctly assign 97% of the progeny for the Chinese shrimp (*Fenneropenaeus chinensis*) to their true parents in simulations using CERVUS v.2.0.; while only 90.7 % of 215 offspring were assigned to their parental pairs exclusively in real mixed family groupings (6 candidate mothers and 5 candidate fathers) produced in aquaculture. A similar result was found for Kuruma shrimp *Penaeus (Marsupenaeus) japonicas*, where assignment success of progeny to their ‘‘true’’ mother was lower than predicted by simulations when using microsatellite markers to assign parentage (Jerry *et al.,* 2004). According to Jerry *et al.* (2006), the assignment success is high if lower numbers of progeny are required to be assigned in real-world assignment tests with the marker suite, compared to CERVUS simulations which use much higher numbers of assigned progeny. The authors indicated that in computer simulations, 7 microsatellites could be used to identify progeny from up to 20 communally stocked families for *P. monodon*, while with 5 loci, over 95% of real progeny could be correctly assigned. Finally, with 7 loci, 100% assignment of real progeny to their families could be achieved. Given this, experimental evaluation is needed to examine the results of this study due to the differences between the simulations and real data.

In conclusion, the current study provides the first estimates of the number of microsatellite markers required (a suite of up to 10 loci) for assigning parentage confidently to a theoretical breeding population of the Ornate Spiny Lobster, *P. ornatus*. From the original 30 microsatellites that have been published, the 10 most highly polymorphic markers were used to evaluate the efficiency of determining pedigree. The results of these simulation approaches suggest that a marker suite of six loci with the highest PIC could be used to correctly assign parentage in this species. This marker suite might be also cost-effective in reality as the low number of loci . While the simulations performed have provided important knowledge on the minimum number of loci required to correctly assign parentage, experimental evaluation is

still needed to confirm the overall power of the marker suite in assignments. This can only occur once mass hatchery production of the ornate spiny lobster is realised.

CHAPTER 7: GENERAL DISCUSSION

7.1 SIGNIFICANCE

For many countries in the Southeast Asian Archipelago, such as Vietnam and Indonesia, the aquaculture of spiny lobsters is based on wild stock supplies. The exploitation of wild pueruli has raised concerns about the sustainability of harvesting large numbers of naturally recruiting juveniles. However, prior to the current work there was very little scientific data available on levels of recruitment and connectivity of populations that could be used in the creation of informed management policies. There was, therefore, a critical need to gather reliable data on the fishing pressure (species, quantity, locality, season of catch, etc), that tropical lobsters are subjected to, along with recruitment patterns of these species for both aquaculture and fishery management. By conducting surveys on levels of lobster seed collection, the effects of wind stress and season on catch rates, and the population genetics of the two commercially important spiny lobster species, *P. ornatus* and *P. homarus*, work presented in this thesis makes a significant contribution to our understanding of factors to be considered in the sustainable management of these species. Chapter 2 analyses long term data (six years) of recruitment of *Panulirus ornatus* and *P. homarus* along the central coasts of Vietnam and showed large fluctuations in annual catch in both species. This chapter also examined the correlation between the change of wind stress (the shear stress exerted by the wind on the surface of oceans) and the variation of *P. ornatus* and *P. homarus* pueruli catch along the Vietnamese coast during that time. High correlation was detected when comparing the total catch of *P. ornatus* puerulus in Vietnam coastal regions and the wind stress caused by winds prevailing over the ocean surface. As a result of this, wind stress could be used as a good tool to predict seasonal recruitment patterns of *P. ornatus*, particularly in Vietnam. The recruitment pattern of *P. homarus* in Vietnam, in contrast, might be controlled by different

environmental factors as not as strong a relationship between wind stress and recruitment was observed.

To understand the source of possible recruitment and connectivity of populations of these two spiny lobsters, molecular tools were used to examine levels of genetic diversity both on the connectivity within and among populations. What is currently known of the life-strategy and ecology of *P. ornatus* would negate the likelihood of genetically discrete populations occurring in the Southeast Asian Archipelago region. Its long oceanic larval development and wide larval transport during this development would imply a genetically well mixed population. Genetic techniques need to be sensitive enough to be able to infer the rate of exchange, i.e. whether gene flow is rare (essentially self-recruiting), or frequent enough to be confident that if a population is fished down too hard, it could be replenished fairly rapidly from another one. Genetic markers (mtDNA control regions and microsatellites) were developed to reveal the population genetic structure of 216 *P. ornatus* individuals from throughout the Southeast Asian Archipelago. The results from both mtDNA control region and microsatellites suggested a single genetic population of *P. ornatus* throughout the region. Based on the biological and oceanographic data, a dispersal pathway of *P. ornatus* larvae was developed in this thesis, which suggested that three generations are all that is required for complete genetic connectivity among populations in Southeast Asia, Australia and Papua New Guinea. The dispersal pathway also suggested that most of the recruitsof *P. ornatus* in Vietnam might be transported from spawning grounds in the Philippines, while Indonesia likely receives recruitment from many different sources from the Philippines and Australia.

Similar genetic studies were applied on 229 individuals of *P. homarus* from Vietnam, Indonesia, Australia and northeast Africa (Oman). The results of this study were combined with that of a previous genetic study on *P. homarus* populations in Africa using mtDNA by

Farhadi *et al.*(2013), which detected genetic variation between northeast and eastern African populations. Work presented in this thesis using microsatellites detected significant genetic differentiation among populations of *P. homarus* throughout the Southeast Asian Archipelago, and between populations from Africa (Oman) and Southeast Asian. Bayesian individual assignment analysis also shows that the Masirah, Oman samples were more homogeneous to a single genetic cluster, which was not revealed by the mtDNA, suggesting the possibility of a one-way migration pattern for females.

Once hatchery production becomes routine, the industry is likely to want to move rapidly towards the establishment of breeding programs and the domestication of superior stocks. Forward planning and the development of tools to assist future breeding programs are therefore prudent. In this thesis, previously acquired genotypes from the Ornate Spiny Lobster (Chapter 4) were used as *in silico* broodstock under various mating strategies in order to establish the best combination of microsatellite markers which would be useful for a DNA parentage marker suite for the species. The results indicated that a marker suite of the six loci with the highest PIC could be used to correctly assign parentage in the Ornate Spiny Lobster *P. ornatus*.

7.2 IMPLICATIONS FOR MANAGEMENT

The panmictic genetic population structure of *P. ornatus* throughout its Indo-Pacific distribution indicates that the species is a single stock requiring a single fishery management plan to ensure sustainable exploitation of this valuable resource. Consequently, a multi-jurisdictional fishery policy should be developed by managers from Australia, Papua New Guinea, the Philippines, Vietnam and Indonesia because the sustainability of lobster fisheries in these exploited populations will be problematic if juvenile recruits are derived from degraded, overfished, or otherwise anthropogenically impacted source populations. While the

sinks of *P. ornatus* larvae are known, the knowledge of larval sources is still rudimentary, with to date only a few spawning sites confirmed. The present study suggests that an additional spawning ground may be present in Indonesia based on larval appearance times, but no such site has yet been reported within the literature. Therefore, new studies to identify adult migration and spawning grounds are necessary to ensure that any future multi-jurisdictional lobster fishery management plans capture all spawning sites, which given their importance in "seeding" the wider Indo-Pacific region and maintaining genetic connectivity, should be conserved as a priority. In terms of *P. homarus*, the genetic isolation of samples from Masirah (Oman) suggests the management must be done at the level of self-recruiting populations. Microsatellites also revealed low levels of population genetic structuring throughout the South China Sea. However, due to lack of knowledge about the ecology of this species, especially the location of spawning grounds, the connectivity among populations within species distribution could not be explained. Therefore, it is essential to identify and protect the spawning biomass producing larvae.

It would also be useful to calculate the number of spawning lobster stock needed to sustain the seed collection in Vietnam, Indonesia or Malaysia, and the number of seed needed for overall natural stock replenishment. To ensure sustainability of recruitment of spiny lobster, various efforts like this have been applied in other countries for other species. For example, a proportion (25%) of the estimated *Jasus edwardsii* seed catch has been identified as being able to be sustainably caught when they are 12 months old, or a trade-off of adult quota catch has been applied (New Zealand). However, in Vietnam and other countries neighbouring the South China Sea, management practices such as seasonal closures, size limitations, catch quota, gear restrictions, ban on the taking of berried females, are unlikely to be adopted and/or effectively enforced. It has been suggested to prohibit the taking of lobster seed of less than a certain size (e.g. less than 7mm carapace length). However, given the expected very high

mortality of settling and recently settled puerulus, this is likely to be counter-productive. A better policy would be to encourage the taking of very small seed (where natural mortality is high), but restrict the taking of larger juveniles (e.g. over a carapace size of say, 20mm). The logic for this is that natural mortality is expected to be inversely related to size and lobsters successfully surviving 20mm carapace length would have a good chance of recruiting to the adult population.

7.3 WIDER APPLICATION OF GENETICS FOR AQUACULTURE

Hatchery production technology is now on the threshold of commercialization and it is likely that future aquaculture of spiny lobsters will be derived, at least in some part, from captive bred progeny (Jones & Shanks, 2009; Jones C. *et al.*, 2010; Phillips & Matsuda, 2011). Once hatchery production becomes routine, the industry is likely to want to move rapidly towards the establishment of breeding programs and the domestication of superior stocks. Forward planning and the development of genetic tools to assist future breeding programs are therefore prudent. In this study it was shown that the microsatellite DNA genetic resources developed will have application to the determination of parentage of *P. ornatus* in future breeding programs, thereby becoming a valuable tool for managers to control inbreeding and to link families with superior culture attributes to their pedigree.

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Appendix 1. Spatial distribution of control region haplotypes among *Panulirus homarus* from one locality in Arabia Sea and six localities in the Indo-West Pacific.

Locality	Haplotype No.																																																		
										1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	3	3	3	3	3	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4	5	5	5
	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2
BinhThuan	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	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
DaNang	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
Lombok	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
Sumatra	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Taiwan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Timor	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tanzania	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Masirah	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chabahar	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Larak	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mirbat	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

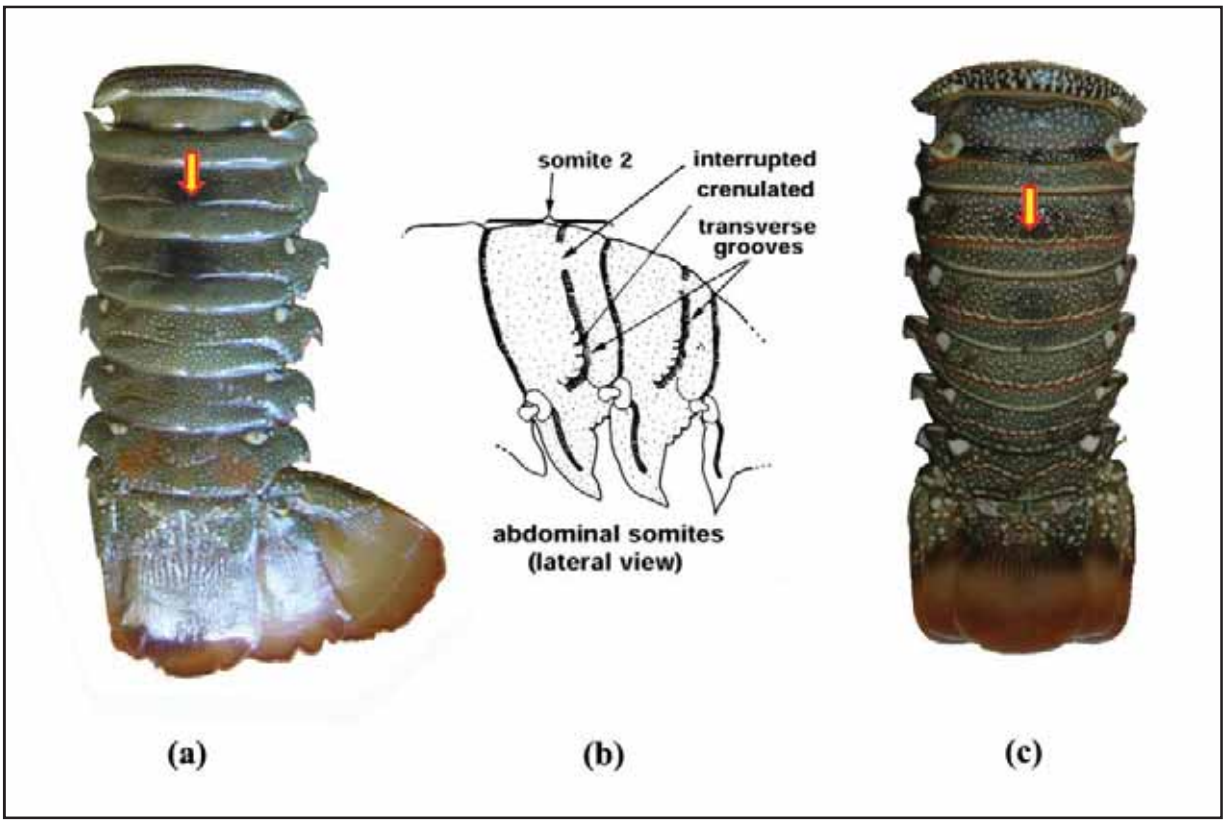
Locality	Haplotype No.																																																		
																																																	1	1	1	1	1
	5	5	5	5	5	5	5	6	6	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7	8	8	8	8	8	8	8	8	8	8	9	9	9	9	9	9	9	9	9	9	0	0	0	0	0
	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4
BinhThuan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DaNang	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	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lombok	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Sumatra	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Taiwan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Timor	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tanzania	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Masirah	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chabahar	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Larak	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mirbat	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Locality	Haplotype No.																																																		
	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	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	1	1
	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	3	3	3	3	3	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5
	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6
BinhThuan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DaNang	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lombok	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	0	0	0	0	0	0	0	0	0	0	0
Sumatra	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
Taiwan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Timor	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tanzania	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Masirah	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0
Chabahar	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Larak	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mirbat	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Locality	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208
BinhThuan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DaNang	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lombok	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sumatra	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Taiwan	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	2	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Timor	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tanzania	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	2	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Masirah	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0
Chabahar	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	3	2	1
Larak	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mirbat	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Locality	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260
BinhThuan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DaNang	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lombok	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sumatra	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Taiwan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Timor	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tanzania	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Masirah	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chabahar	1	1	1	1	2	1	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	2	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Larak	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	1	1	1	1	1	2	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1
Mirbat	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Locality	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307
BinhThuan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DaNang	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lombok	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sumatra	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Taiwan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Timor	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tanzania	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Masirah	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chabahar	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Larak	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mirbat	0	0	0	0	0	0	0	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	1	1	1	1	1	2	1	1	1	1	1	1



Appendix 2. Two morphotypes of *Panulirus homarus*: the microsculpta form (a) photo of sample collected from fishing market in Vietnam (b) lateral view figure with interrupted crenulated transverse grooves (Holthuis, 1991); and the megascuplta form (c) photo of sample collected from Masirah Island, Oman in the present study.