Molecular Phylogeny and Population Genetic Structure of the Shallow-water Spiny Lobster *Panulirus homarus* in the South West Indian Ocean Region: Implications for Management

By

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ABSTRACT

The scalloped spiny lobster, *Panulirus homarus* has a subspecies trio that are widely distributed in shallow-water habitats in the South West Indian Ocean. Subspecies are defined by differences in colour and abdominal sculptural pattern. A red variety with the megasculptural carapace pattern, *P. h. rubellus* is distributed along the south east coast of Africa and Madagascar, where they are endemic. Along the African coast *P. h. rubellus* stocks traverse political boundaries, Mozambique and South Africa. This project aimed to facilitate regional fisheries management of shared stocks by employing genetic tools to determine whether stocks (or populations) are indeed shared between countries. Lobster samples were collected from seven localities throughout the east African coast. The mitochondrial cyctochrome *c* oxidase subunit 1 region was sequenced to assess the genetic diversity 1) between different subspecies, *P. h. homarus* and *P. h. rubellus* and 2) between populations of *P. h. rubellus* across its African distribution range. Using DNA barcoding methods, genetic diversity was also found between morphologically distinct subspecies, *Panulirus homarus homarus* and *P. h. rubellus* which differed genetically by *ca.* 2-3% in sequence divergence. Both subspecies were monophyletic relative to the out-group taxa and formed well supported sister clades (BI: 1.00, ML: 93%, P: 100%, NJ: 100%). The distribution of *P. h. rubellus* along the African coast occurs adjacent to different current regimes and therefore varied larval transport modes (*i.e.* Agulhas Current and inshore counter-currents along the Eastern Cape). This may have driven the formation of subpopulations ($\phi_{PT} = 0.104$, $p = 0.010$) which differ by *ca.* 1.7% in sequence difference. The pattern of gene flow of populations of *P. h. rubellus* lends support to the Agulhas Current being a major mode of larval transport as well as corroborates previous abundance and distribution records. Time since population expansion estimates for the *P. h. homarus* and *P. h. rubellus* subspecies as well as for the *P. h. rubellus* subpopulations dated back to the mid-Holocene Epoch in accordance with a warmer, more stable marine environment. Genetically distinct subspecies of *P. homarus* as well as differentiated subpopulations of *P. h. rubellus* calls for a re-visit of the current collective management of *P. homarus* as well as *P. h. rubellus* as a single genetic stock along the south east African coast.

PREFACE

The experimental work described in this dissertation was carried out at the School of Life Sciences, University of KwaZulu-Natal, Westville Campus, Durban. This was carried out under the supervision of Dr A. H.H. Macdonald and Prof. M.H. Schleyer.

These studies represent original work by the author and have not otherwise been submitted in any form for a degree or diploma to any tertiary institution. Where use has been made of the work of others, it is duly noted in the text.

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DECLARATION 1–PLAGARISM

I, Mageshnee Mayshree Reddy declare that:

1. The research in this thesis, except where otherwise indicated, is my original research.

2. This dissertation has not been submitted for any degree or examination at any other University.

3. This thesis does not contain other persons' data, pictures, graphs, or other information unless specifically acknowledged as being sourced from other persons.

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DECLARATION 2–CONFERENCE PRESENTATIONS

1. Reddy, M.M., Macdonald, A.H.H. and Schleyer, M.H. 2011. Are shallow water spiny lobster, *Panulirus homarus* populations genetically structured or panmitic in the South West Indian Ocean region: Implications for management. 7th West Indian Ocean Marine Science Association (WIOMSA) conference, Mombasa, Kenya.

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Signed: ..... **Name:** Miss M.M. Reddy **Date:** 22-01-2013

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LIST OF ABBREVIATIONS

SWIO: South West Indian Ocean
SE: South East
NW: North West
DNA: Deoxyribonucleic acid
Pers. ob: Personal observation
Ma: Million Years Ago
FAO: Food and Agriculture Organisation
R: Rands
SCUBA: Self Contained Underwater Breathing Apparatus
GPS: Global Positioning System
Pers. comm.: Personal communication
SW: South West
Pers. ob.: Personal observation
n: Number of samples
TBE: Tris/borate/EDTA
mA: Milliamps
bp: base pairs
AMOVA: Analysis of Molecular Variance
Indels: Insertions and deletions
F_{st}: Fixation index
PAUP*: Phylogenetic Analysis Using Parsimony
AIC: Akaike Information Criteria
Iss: Index of Substitution
Iss.c.: Critical value of Index of Substitution
IBD: Isolation-By-Distance

CHAPTER 1

Background and Rationale

BACKGROUND

In order to understand genetic patterns and effectively use the genetic outcomes of this study to facilitate management, one must first understand the evolution, distribution, ecology and biology of *Panulirus homarus* and how these aspects may relate to their genetics.

Taxonomy and species description

Historically, *Panulirus homarus* was first described by Linnaeus (1758) and later classified under two different species names, *P. buergeri* de Haan (1851) and *P. dasypus* H. Milne-Edwards (1837). However, under considerable scrutiny it was confirmed that these 'species' were indeed a variety of the same species (Holthuis 1946, Barnard 1950, Gordon 1953, Kubo 1955, George 1963, 1965, 1966, Berry, 1971b). Consequently, Holthuis (1946) proposed synomising these names as a single subspecies complex, *P. homarus*. Gordon (1953) later published the contemporary classification of *P. homarus*; this name change was supported by various authors (George 1963, Kubo 1963, George 1965, 1966) and remains its accepted designation.

The East Coast rock lobster *Panulirus homarus* is a decapod crustacean belonging to the shallow-water scalloped spiny lobster group (George 1966, Smale 1978, Kemp & Britz 2008, Kemp *et al.* 2009). These lobsters have a tough calcified exoskeleton with strong legs and a fan-tail (George 2005a). The characteristic feature of these spiny lobsters, as the name suggests, is spines. Four large spines that are forward-directed and protrude from the outer skeleton of their antennae are often used for defence (George & Main 1967, Berry 1971a), while dense, much shorter spines on their carapace are used to wedge themselves in rocky shelters when threatened (Heydorn 1969, Pollock 1990). In addition, two short forward-directed supra-orbital horns protect their elevated eyes (George & Main 1967, Pitcher 1993, George 2005a).

There are three subspecies of *Panulirus homarus* grouped into two forms, the microsculpta to which *P. h. homarus* Linnaeus (1758) belongs and the megasculpta form which is further broken down into two regional groupings, *P. h. rubellus* Berry (1974a), referred to as the southern megasculpta form, and *P. h. megasculptus* Pesta (1915), referred to as the northern megasculpta form (Berry 1974a). The microsculpta

form is green in colour and the megasculpta form is red in colour (Fig. 1.1). The former descriptions of *P. buergeri* de Haan (1851) and *P. dasypus* H. Milne-Edwards (1837) fit the description of the currently recognised subspecies *P. h. rubellus* and *P. h. homarus*, respectively (Berry 1974a). For a more detailed physical description of *P. homarus* and its subspecies consult Berry (1974a).

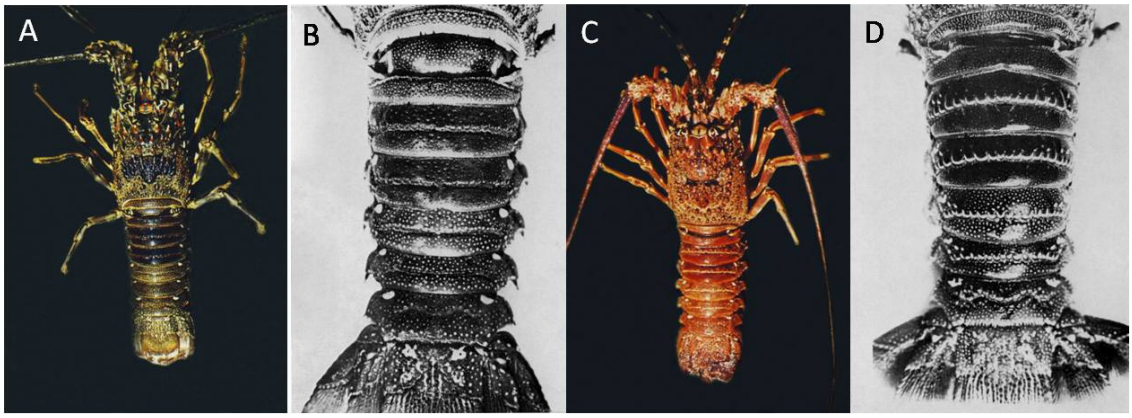

Figure 1.1: Two common variants of *Panulirus homarus* found along the south east coast of Africa, the green form (A) with its associated microsculptured (B) carapace pattern (*Panulirus homarus homarus*) and the red form (C) with its associated megasculptured (D) carapace pattern (*Panulirus homarus rubellus*). Photo modified from Berry (1974a).

Marine lobsters are ecologically and economically valuable crustaceans with the potential for aquaculture (Scholtz & Richter 1995, Chu *et al.* 2001, Cobb 2006, Kemp 2008). Many studies have been conducted on their phylogeny, biology, ecology and population structure and serve as a foundation for the management of these crustaceans (Booth & Phillips 1994, Scholtz & Ricther 1995, Martin & Davis 2001, Porter *et al.* 2005).

Evolutionary history of the palinurids

Decapods appeared in the fossil record around 354-364 Million Years Ago (Ma); the most ancient and well studied records are those of the Reptantia (Porter *et al.* 2005). The Reptantia include all crawling crustaceans such as the crabs, lobsters and crayfish (Scholtz & Ritcher 1995). However, these fossil records are incomplete and seem to be missing information between the late Devonian and Cretaceous period (Porter *et al.* 2005). The oldest fossil record of Reptantia from the late Devonian period is that of *Palaeopalaemon newberryi* and *Imocaris tuberculata* from the Lower Carboniferous period (Shram *et al.* 1978, Schram & Mapes 1984, Porter *et al.* 2005). These records

have been used to calibrate molecular dating studies (Porter *et al.* 2005); however, a suite of molecular markers and several fossils or geological events are recommended to improve the estimation of molecular dating studies (Porter *et al.* 2005).

The most ancient ancestors giving rise to the family Palinuridae are the Phemphidea in the Triassic Epoch that later split during the Jurassic Epoch (George & Main 1967), and were suspected to independently evolve into the Stridentes and Silentes lineages 150 Ma (Pollock 1995a). However, the fact that the genera *Palinurus* and *Palinurellus* have an intermediate evolutionary position between the Silentes and Stridentes challenges this view and may represent an early evolutionary offshoot from the Silentes (George 2005a, Groeneveld *et al.* 2007, Tsang *et al.* 2009). Evidence confirms that the basal taxon representing the most ancient Palinuridae species is the Silentes, *Projasus*, *Jasus*, and *Sagmariasus* (George & Main 1967, Pollock 1990, Tsang *et al.* 2009, Chan 2010), while *Palinurellus* is the most primitive intermediate genus that may have lead to the evolution of the Stridentes lineage (Davie 1990, Tsang *et al.* 2009). Subsequent speciation of the Stridentes gave rise to the common Palinuridae known today (George & Main 1967, Pollock 1990). The shallow-water genera of the Palinuridae include *Jasus* and *Panulirus*, while the deep-water taxa include *Puerulus*, *Palinustus*, *Justitia* and *Linuparus* (George & Main 1967).

Phylogenetics

Despite many phylogenetic advances, the phylogeny of marine lobsters remains a contentious issue (Porter *et al.* 2005, Tsang *et al.* 2009). Some aspects of their phylogeny, namely the monophyly of marine lobsters, the acquisition or loss of the stridulating organ over an evolutionary scale, and the direction of evolution between shallow- and deep-water lobsters remains to be clarified.

Monophyly is the term used to describe clades consisting of all the descendants of a common ancestor (Ashlock 1971). Several authors argue that marine lobsters are not monophyletic (Burkenroad 1981, Schram 2001, Dixon *et al.* 2003, Ahyong & O'Meally 2004, Amati *et al.* 2004, Schram & Dixon 2004, Porter *et al.* 2005). However, a comprehensive genetic study by Tsang *et al.* (2008) suggests marine lobsters are indeed monophyletic when some members of the Thalassinidea are excluded (Patek *et al.* 2006,

Tsang *et al.* 2008, Bracken *et al.* 2009, Toon *et al.* 2009, Chan 2010). Some members of the Thalassinidea, namely the squat lobster, are not considered 'true lobsters' (Tsang *et al.* 2008, Chan 2010).

Achelata constitutes an infraorder of marine lobsters consisting of three families, Palinuridae, Scyllaridae and Synaxidae, commonly known as the spiny, slipper and furry or coral lobsters, respectively (Martin & Davis 2001, Palero *et al.* 2009). The divergence of Palinuridae and Scyllaridae is well supported phenetically and genetically, while the Synaxidae was weakly supported as an additional family. Therefore, several authors have recommended classifying the 'family Synaxidae' under the family Palinuridae (Davie 1990, Patek & Oakley 2003, George 2006, Palero *et al.* 2009, Tsang *et al*. 2009) or under the super family Palinuroidea (Pitcher 1993, Tsang *et al*. 2009).

The family Palinuridae consists of 12 genera of spiny lobsters grouped into two lineages, Stridentes and Silentes, depending on the presence of an acoustic stridulating organ (Berry 1971a, Pollock 1990, Tsang *et al.* 2009, Chan 2010).

Table 1.1: Classification of all genera in the family Palinuridae into Stridentes and Silentes

Genus	Stridentes	Silentes	Source
Panulirus	X		White (1847)
Palinurus	X		Weber (1795)
Palinustus	X		A. Milne-Edwards (1880)
Puerulus	X		Ortmann (1897)
Linuparus	X		White (1847)
Justitia	X		Holthuis (1963)
Nupalirus	X		Kubo (1955)
Palinurellus	X		von Martens (1878)
Palibythus	X		Davie (1990)
Jasus		X	Parker (1883)
Projasus		X	George and Grindley (1964)
Sagmariasus (*Jasus*)		X	Holthuis (1991)

***** *Sagmariasus* was formerly classified as *Jasus*

The stridulating organ functions as a defence mechanism as well as for communication. The loud rasping sound emanating from the plectrum (basal appendage of the antennae) being strung against the enlarged ribbed antennular plate on the body of the lobster, affords these lobsters a mechanism to evade predation (Moulton 1953, Palero *et al.* 2009, Tsang *et al*. 2009; also see Patek & Oakley 2003).

The acquisition or loss of the stridulating organ over evolutionary time has been debated and evidence favours the acquisition of the stridulating organ; this is supported by Moultin (1953), Davie (1990), Patek and Oakley (2003), Palero *et al.* (2009) and Tsang *et al.* (2009). Using a set of nuclear molecular markers, Tsang *et al*. (2009) have shown that Silentes constitute the basal lineage to the Stridentes, suggesting a more primitive Silentes lineage. Moreover, Tsang *et al.* (2009) were able to demonstrate that the Stridentes are indeed monophyletic and, thus, the stridulating organ is a synamorphic feature that appeared only once within their evolution. The stridulating organ is a complex one and it is doubtful that this organ formed independently (Davie 1990, Patek *et al.* 2006, Tsang *et al.* 2009). Thus, the monophyly and primitive (basal lineage) nature of the Silentes provide supporting evidence for the acquisition of the stridulating organ over an evolutionary scale. An additional feature setting the Stridentes apart from the Silentes is their different sperm characteristics. The sperm of Stridentes have a characteristic tar spot appearance while that of the Silentes have a gelatinous texture and appearance (George 2005b, c); this feature also hints at their distinct and divergent evolution.

There is also controversy surrounding the evolution of spiny lobsters and the direction of habitat invasion (from shallow water to deeper water or *vice versa*). Various authors suggest that shallow-water spiny lobsters originated or evolved from deep-water populations (George & Main 1967, Baisre 1994, Pollock 1995a, George 2005c, Groeneveld *et al.* 2007), while others argue that spiny lobsters from shallow waters later proliferated into deeper waters (Feldmann & Tshudy 1989, Davie 1990, Feldmann & Schweitzer 2006, Tsang *et al.* 2009, Tsoi *et al.* 2011). George and Main (1967) and George (2005a) provide examples of physiological adaptations that may have facilitated spiny lobsters proliferating in shallow-water environments from deeper-waters. These include enhanced eyesight, evident in the elevated eye position, enlarged and protected supra-ortibals, stout strong legs used to cling to rock surfaces, a tough exoskeleton and carapace spines used to lock into shelters during unfavourable, turbid conditions (Berry 1971a, George 2005a, Tsang *et al.* 2009). Nevertheless, such inferences are in opposition to molecular data that place *Panulirus*, a shallow-water lobster, basal to deeper water genera (Tsang *et al*. 2009).

The few scenarios mentioned above indicate the need for phylogenetic clarification within spiny lobsters. Perhaps clarification of marine lobster phylogenetics requires an understanding of the reason differences arise, *i.e*. past genetic isolation.

Phylogeography

George and Main (1967) suggest that the present day distribution of Palinurid species occurred when the once continuous equatorial seas became fragmented due to tectonic plate movements that may have isolated populations.

The scalloped spiny lobster, *Panulirus homarus*, and its subspecies provide an example of speciation as a result of past oceanographic regimes. The Agulhas Current and associated inshore gyres are suspected to have contributed to the isolation of *P. h. homarus* and *P. h. rubellus* along the south east African coast (Berry 1974b, Pollock 1992, George 2005c). In addition, *P. h. megasculptus* probably became isolated from *P. h. homarus* and *P. h. rubellus* due to monsoon and upwelling conditions apparent in the Arabian Sea (Pollock 1992). This oceanographic system has been a consistent feature over geological time and indeed has strengthened since the Miocene Epoch (George 2005c).

In the *Panulirus homarus* subspecies complex*, P. h. homarus* is by far the most widely distributed subspecies of *P. homarus*, occurring along the Indian and West Pacific Oceans and is thought to have emerged in the Pliocene (3.5 Ma). It has been suspected that *P. h. homarus* may hybridize with both *P. h. rubellus* and *P. h. megasculptus* in regions of their overlapping distribution (Berry 1974a, George 2005b, c). The cross-fertilisation between *P. homarus* subspecies and of *P. longipes* and its subspecies are the only two documented cases of cross-fertilisation between closely related taxa inhabiting areas of overlapping distribution in the genus *Panulirus* (Berry 1974a, Pollock 1993, George 2005b). Based on experimental cross-fertilisation studies, it has been suggested that *P. h. homarus* and *P. h. rubellus* hybridize but, given the inability to rear these larvae, the existence and reproductive viability of such offspring remains in question (Berry 1974a).

Distribution

Within the family Palinuridae, the most diverse genus, *Panulirus*, has 19 extant species, seven of which are resident in the Indian Ocean. Five species, *P. homarus* Linnaeus (1758), *P. pencillatus* Olivier (1791), *P. longipes* A. Milne-Edwards (1868), *P. ornatus* Fabricus (1798) and *P. versicolor* Latreille(1804) are found along the SE coast of Africa (Berry 1971a, George 1973). However, these species are not limited to the Indian Ocean and occur in parts of the Atlantic and Pacific Oceans (Berry 1971a). As an example, *P. homarus* is widely distributed in the Indo-Pacific and occurs in parts of the Pacific Ocean (Berry 1971a, George 1973, George 2005c).

George and Rao (1965) recognised that *Panulirus homarus* populations from different regions were morphologically distinct and these authors suggested that subspecies would be found across this large distribution range. These differences exist in populations on the east Aden coast, Sri Lanka and the SW Indian coasts (George & Rao 1965, Berry 1974a, b).

Since the formal recognition of three subspecies of *Panulirus homarus*, their distributional range was broken down into three major regions in which each of these subspecies is found. *Panulirus homarus homarus* is commonly found in the Indo-West Pacific (Holthuis 1991), *P. h. rubellus* (Berry 1974a, b) along the SE African and Madagascan coasts (Berry 1974a, Holthuis 1991) and *P. h. megasculptus* along the northern Arabian Sea and Somalian coast (Fielding & Mann 1999, Kulmiye *et al.* 2006). The classification of these subspecies was based solely on differences in morphological characteristics that are still applicable today (Pesta 1915, Berry 1974a, Holthuis 1991) but lacks any genetic evidence supporting this delineation.

Ecology

The scalloped spiny lobster, *Panulirus homarus*, is commonly found in shallow-water rocky habitats within 200 m of the shore (Berry 1971a, Tsang *et al*. 2009). *Panulirus homarus* is limited to depths of 1-90 m (Holthuis 1991, Kulmiye *et al.* 2006), but is commonly found at 5 m (Berry 1971a, Kulmiye & Mavuti 2005). Its vertical distribution mirrors that of its primary food source, the brown mussel, *Perna perna* (Berry 1971a, Kemp & Britz 2008). In addition, they are also known to be an

opportunistic scavenger feeding on molluscs, limpets, other small or dead crustaceans, echinoderms, algae and seagrasses (Pitcher 1993). *Panulirus homarus* hunts nocturnally and spends most of the day in rock crevices to avoid predation; other evasion tactics include camouflaged carapace colouration, sharp spines, tail flipping and the rasping sound of stridulation (Berry 1971a, Smale 1978, Pitcher 1993). In shelters, *P. homarus* is known to live in close association with moray eels (Berry 1971a). This association is particularly beneficial to these lobsters, as eels are known to prey on octopi that prey on these lobsters. In essence, moray eels and *P. homarus* share a mutualistic relationship as the moray eels provide protection to the lobsters from octopi and in turn, the lobsters attract octopi on which the moray eels feed (Berry 1971a). Other marine predators of *P. homarus* include sharks, rays and rock-cod (Pollock & Melville-Smith 1993).

Sub-adult spiny lobsters generally migrate toward deeper water breeding grounds from shallow nursery areas (Pitcher 1993, George 2005a). Adult *Panulirus homarus* are not known to undertake long migrations like many other Palinuridae species and, therefore, their dispersal abilities may be restricted (Mohamed & George 1967, Pitcher 1993, George 2005a). Mohamed and George (1967) found that, even though *P. homarus* populations along the Indian coast were locally restricted, they had a general northward and shoreward movement. Their shoreward movement may be explained by their search for suitable rocky habitats (Mohamed & George 1967, George 2005a) and their northward movement possibly to breeding grounds (George 2005a). Adult migration may contribute to homogeny among geographically isolated populations. Migration toward suitable breeding grounds is an adaptive evolutionary strategy also seen in *Palinurus gilchristi* and *P. delagoae* (Groeneveld 2002, Groeneveld & Branch 2002, George 2005a).

Apart from biotic limitations such as predation and searching for suitable breeding grounds, abiotic limitations such as habitat availability and temperature also influence the local distribution and migration of *Panulirus homarus* (Kemp 2008). Lobster shelters are primarily compromised by sand inundation (Berry 1971a). When sand inundation prevails, lobsters crowd in shelters and, when sand scouring occurs they are sparsely distributed, although still gregarious. As such, sand scouring and inundation have a large influence on local movement patterns (Berry 1971a).

Another abiotic factor that limits their distribution on a larger geographical scale is temperature. Temperature is the single most important abiotic factor responsible for biological and genetic breaks along areas of the coastline (Williams *et al.* 2002, Atkinson & Clark 2005, Teske *et al*. 2011). These 'breaks' are often associated with the thermal tolerance range of species. Palumbi (2003) highlights the prevalence of marine species breaks along coastlines globally. In South Africa, these breaks are classified into bioregions that are delineated by differences in sea surface temperature (Atkinson & Clark 2005). The temperature tolerance of *Panulirus homarus rubellus*, studied in captivity, ranged from 24-30°C (Smale 1978, Kemp & Britz 2008). Below 24°C, the inter-moult period and moult period of the lobster are reduced, thus affecting its growth, but its survival is not compromised. It is not surprising then, that the highest abundance of *P. h. rubellus* along SE Africa occurs along the subtropical KwaZulu-Natal coast and decreases along the cooler, warm temperate Eastern Cape coast (Atkinson & Clark 2005, Kemp & Britz 2008). Thus, it is probable that temperature limits the southern distribution of *P. homarus* along the SE African coast (Kemp 2008, Kemp & Britz 2008) and may even limit its northern distribution.

Biology

The link between temperature and geographical distribution exists because temperature influences growth in *Panulirus homarus* as well as in other lobsters (Smale 1978, Hartnoll 1982, Kemp *et al*. 2009). For example, the warm water tropical *P. h. homarus* grows at a faster rate than the sub-tropical *P. h. rubellus* (Kemp & Britz 2008). The discrepancy in size of *P. homarus* subspecies from different localities can be easily explained by the difference in environmental conditions and hence growth. In an experimental study, Kemp and Brtiz (2008) found that food consumption of *P. h. rubellus* was proportional to an increase in temperature and in turn increased its metabolism, leading to increased growth (Booth & Phillips 1994, Kemp 2008).

Growth

Although growth in lobsters does not appear to follow the general growth pattern (increase in mass over time) seen in most members of the animal kingdom, internal tissue growth is constant (Pitcher 1993). Growth is commonly measured in terms of

moulting and successive inter-moult periods of the exoskeleton (Mauchline 1977, Pitcher 1993). Moulting is a process whereby lobsters shed their old exoskeleton as they increase in carapace length or mass and form a new one able to accommodate their increase in size (Berry 1971b, Chittleborough 1975). The average inter-moult increment decreases and the interval increases with increasing carapace length until sexual maturity is attained and, thereafter, a constant rate of inter-moult successions occur (Berry 1971b, Pitcher 1993). Sexually mature females partition more of their energy toward egg bearing than growth (Pitcher 1993). Therefore, sexually mature males are often larger than sexually mature females (Berry 1971b, Pitcher 1993). On average, sexually mature *Panulirus homarus* moult and spawn between 4-5 times a year (Berry 1971b, Pollock & Melville-Smith 1993).

However, growth in lobsters, their moult frequency and, therefore, their size at sexual maturity is influenced by food availability, shelter/density and temperature (Berry 1971b, Morgan 1980, Pitcher 1993, Pollock 1995b, Kemp 2008); factors that vary with location. Not surprisingly, differences in size at sexually maturity in *Panulirus homarus* have been noted at different localities (Table 1.2.).

Size at sexual maturity

Table 1.2: Comparison of size at sexual maturity of *Panulirus homarus* from different localities

Location	Size at first sexual maturity	Source
South coast of Sri Lanka	38-47 mm CL	Jayakody (1989)
West coast of Sri Lanka	55-59 mm CL	De Bruin (1962)
East Aden	60-70 mm CL	George (1963)
South Africa, Transkeian waters	50 mm CL	Heydorn (1969)
South Africa, Natal waters	54 mm and greater CL	Berry (1971a)

*Adapted from Jayakody (1989)

Warmer waters are associated with a high rate of moulting, which is strongly correlated with a higher growth rate (Chittleborough 1974, 1975, Hartnoll 1982, Pollock 1995b, Kemp 2008). The onset of sexual maturity is age- and not size-dependent and, therefore, sexual maturity may be attained at a larger size in areas with warmer waters (Plaut 1993, Pollock 1995b, Kulmiye *et al.* 2006). *Panulirus homarus homarus* off the Sri Lankan coast, attains sexual maturity at 59.3 mm carapace length (CL) (Jayakody

1989); while Kulmiye *et al.* (2006) found, using the berry method, the same subspecies off the Kenyan coast attains sexual maturity at 63.4 mm CL. In Sri Lanka *P. h. homarus* reach sexual maturity at a smaller size than those in Kenya, owing to differences in temperature and, thus, growth (Plaut 1993, Kulmiye *et al.* 2006). Pollock (1995b) emphasizes that, although, the correlation between temperature and size at sexual maturity may hold true for some species of spiny lobsters, it may not for others.

Previous authors have also shown differences in size at sexual maturity between subspecies of *Panulirus homarus*; *P. h. rubellus* reach sexual maturity at 54 mm CL off the east coast of South Africa (Berry 1971b) and *P. h. megasculptus* at 58 mm CL off the Somalian coast (Fielding & Mann 1999). Moreover, *P. h. megasculptus* has also shown considerable variation in size at sexual maturity, not only in different areas but also at different landings in the same area (George 1963, Johnson & Al-Abdulsalaam 1991, Mohan 1997, Fielding & Mann 1999). Intraspecific differences in size at sexual maturity have also been shown in another palinurid lobster, *Jasus tristani* (Pollock 1995b). Variations within subspecies are due to extrinsic factors outlined by Kulmiye *et al.* (2006): water temperature, food availability and population densities, while those between subspecies are often a combination of extrinsic and intrinsic genetic factors. On average, *P. h. rubellus* and *P. h. homarus* along the SE coast of Africa reach sexual maturity within three years of larval settlement (Berry 1971b, George 2005a).

Reproduction

Sexually mature *Panulirus homarus* breed all year round, with a marked peak in breeding during the austral summer months *viz*. between November and March (Berry 1971b, Kemp 2008) and minimal to no breeding during the winter months (Berry 1971b, Pollock & Melville-Smith 1993). A moult prior to egg bearing is a physiological adaptation that assists in mating and has little effect on growth (Berry 1970, Pitcher 1993). Hence, sexually mature females mate a short while after moulting when the exoskeleton is in a hardened state. This is often used to identify breeding females (Berry 1970).

Sexually mature *Panulirus homarus* females are distinguished by the presence of ovigerous setae on their pleopods or the presence of eggs on their sternum; mature

females bearing eggs are said to be in berry (Berry 1970, Jayakody 1989, Pitcher 1993). Larger females have been shown to be more fecund and may produce several broods per breeding season compared to smaller females (Berry 1971b, Pollock 1990, Pitcher 1993). Typically, mature *Panulirus homarus rubellus* are known to produce one to four broods annually, while *P. h. homarus* produces two to four broods (Berry 1971b, George 2005a). In males, there is no primary discerning feature indicating sexual maturity although the second and third pairs of walking legs extend at the onset of sexual maturity and may act as a secondary feature (Berry 1970, Pitcher 1993). This growth allows males to access females when they retreat into their shelters during mating, and aids in overturning the female whilst mating (Berry 1970). It is, therefore, less probable that males reproduce with a female of larger size (Berry 1970), but this may not always hold true (Chittleborough 1974).

Panulirus homarus is known to display polygamous mating behaviour (Berry 1970). However, there is a hierarchy in mating whereby males display acts of aggression and, as a result, the dominant males feature high in the mating rank (Berry 1970). Male reproductive dominance corresponds to size; therefore, larger males are often dominant. Larger males also produce more sperm and are able to mate many more times than smaller males (Berry 1970, Pitcher 1993, George 2005a). The production of sperm occurs in the gonads and is released from the gonophores (Berry 1970). The male requires frontal contact with the female (sternum to sternum) to deposit a spermatophore on her sternum (Berry 1970, Kemp 2008).

The spermatophore hardens on the female's sternum and the protective layer is actively scraped away exposing spermatozoa. The timing of this event is variable among species and may occur within 24 hours or up to 42 days later (Berry 1970); in *Panulirus japonicus* sperm-scraping occurs within ten minutes and in *P. cygnus* within 69 days (MacDiarmid & Kittaka 2000, George 2005a). Once the protective outer layer is successfully scraped away, oviposition begins (Berry 1970). The interval between mating and oviposition is variable but often occurs a week after mating in *P. homarus* (Berry 1970). Ova are released sequentially in such a way as to ensure that they pass over the spermatophoric mass (Berry 1970). After oviposition, eggs are incubated in a

brood chamber for 29-59 days in captive *P. h. rubellus* and approximately 30 days in captive *P. h. homarus*, after which larvae are formed (Berry 1971b, George 2005a).

Larvae

Spiny lobsters have a unique lifestyle in that they include a phyllosoma larval stage in their development (Gurney 1942, Booth & Phillips 1994, Sekiguchi & Inoue 2002, Dixon *et al.* 2003). Phyllosoma larvae are planktonic, transparent, dorso-ventrally flattened and may reach a length of 50 mm in some species (Pitcher 1993, Booth & Phillips 1994). They have well-defined mouthparts and pereiopods used to capture food (Booth & Phillips 1994) and feather-like exopodites to swim (Pitcher 1993). Their main diet consists of gelatinous plankton and medusae (Pollock 1995a).

Phyllosoma larvae are poor horizontal swimmers and rely on ocean currents for transport (Booth & Phillips 1994, George 2005a). As such, phyllosoma larvae are often carried offshore by ocean currents where they complete their phyllosomal development (Booth & Phillips 1994, George 2005a). In an attempt to avoid predation, phyllosoma larvae migrate vertically at night and, in the day, are carried by ocean currents into deeper water (Phillips & Sastry 1980, Booth & Phillips 1994). The presence of phyllosoma larvae in off-shore waters has been confirmed by their capture with plankton nets on research cruises that operate beyond the Exclusive Economic Zone (Chow *et al*. 2012). The phyllosoma larval stage of *Panulirus homarus rubellus* and *P. h. homarus* lasts for 4-6 months, followed by a series of moults and metamorphosis through 9 and 10 stages respectively (Berry 1974b, Booth & Phillips 1994). Detailed descriptions of these stages are provided by Berry (1974b).

On completion of the phyllosoma larval stage, larvae return to coastal areas where they complete their puerulus development. Pueruli are referred to as the ‘competent’ stage in larval development (Pollock 1990). In this stage, pueruli resemble miniature, transparent spiny lobsters that are able to swim forward, backward and flicker their tail in the characteristic defence response observed in adult spiny lobsters (Pitcher 1993, Pollock & Melville-Smith 1993). Pueruli are characterised by the presence of natatory pleopods in semi-planktonic and semi-benthic juveniles that are between 6-12 mm (CL) in size (Berry 1974b, Booth & Phillips 1994). They are nocturnally active but

presumably do not feed, as the mouth appendages are incomplete; they thus rely on energy reserves acquired during the phyllosoma stage (Booth & Phillips 1994, Briones-Fourzán *et al*. 2008). Pueruli spend most of the day demersally and the night at the ocean surface in the plankton (Booth & Phillips 1994). It has been suggested that the smaller size of pueruli in *Panulirus homarus* compared to other palinurid lobsters is an evolutionary advantage that allows these larvae to transit undetected through tropical and subtropical regions, where conditions are favourable but predation is high (Pollock 1995a).

Sensors on their antennae allow pueruli to perceive the noise of breaking waves near suitable, coastal 'home' environments, to which they swim; their movement to coastal areas is aided by ocean currents and fronts (Phillips & McWilliam 1986, Booth & Phillips 1994, Briones-Fourzán *et al*. 2008). The metamorphosis from phyllosoma larvae to pueruli occurs at the edge of the continental shelf where oceanic processes, changes in salinity, contact with the benthos, or other physical and chemical cues associated with suitable 'home' environments act as driving forces toward this metamorphosis (Pollock 1990, Booth & Phillips 1994). McWilliam and Phillips (1997) attribute the exhaustion of energy reserves stored in the phyllosoma stage as the main driver toward metamorphosis rather than environmental cues. However, a combination of both nutritional exhaustion and environmental cues is more likely the reason for metamorphosis (Pollock 1995a). The end of the puerulus stage marks the beginning of the post-larval stage.

During the post-larval stage, the pleopods are no longer natatory and adult morphology develops (Berry 1974b). The recruitment of juveniles to suitable rocky habitats begins the bottom-dwelling life of spiny lobsters (Booth & Phillips 1994) and the development of carapace pigmentation (Booth & Phillips 1994).

Larval recruitment provides a good index of subsequent adult recruitment into lobster fisheries (Pitcher 1993, Briones-Fourzán *et al*. 2008). However, larval recruitment and settlement are influenced by oceanographic variation and are therefore highly variable, necessitating long-term data (Briones-Fourzán *et al*. 2008) that can be used to implement sound management plans. The planktonic larval stage in spiny lobsters and

their transport, thus, have major implications for larval recruitment, population structure and their proliferation as adults.

POPULATION GENETICS

The long-lived larval stage, wide dispersal capacity and the ability to settle on various suitable 'home' 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, Pollock 1995a, Thorpe *et al*. 2000, Cowen *et al*. 2006).

Larval dispersal

Spiny lobsters, as with many invertebrates with low mobility or sedentary adult life phases, rely heavily on the dispersal capacity of their planktonic larvae for population expansion, maximisation of fecundity, minimising overcrowding and inbreeding, settlement in favourable habitats and the exploitation of new food sources (Booth & Phillips 1994, Thorpe *et al.* 2000, Groeneveld *et al.* 2007).

The geographical distribution of spiny lobsters is largely determined by the transport of phyllosoma larvae by various transport modes and subsequent post-settlement onto suitable substrata (Mohamed & George 1967, Berry 1971b, Booth & Phillips 1994). The various modes of larval transport include oceanic currents, fronts, gyres, local eddies and wind drift (Berry 1971a, Pollock 1995b, von der Heyden *et al.* 2007a). Thorpe *et al.* (2000) include additional mechanisms, apart from conventional larval dispersal modes, that may contribute to larval dispersal such as rafting on natural substrata (wood, pumice, turtles, algae) or anthropogenic material such as plastics, buoys and other floating objects. Recently, some studies have also highlighted the role of ships and ballast water in transporting organisms or larvae that invade and proliferate in a new territory (Thorpe *et al.* 2000 & references therein).

Compared to some members of the Scyllaridae, Palinuridae generally have a long-lived planktonic, phyllosoma larval stage. Indeed, spiny lobsters have the longest planktonic larval stage compared to other crustaceans (Pollock 1995a). The planktonic larval duration in Palinuridae persists for 2-24 months, depending on the species (Sarver *et al.*

2000, Palero *et al.* 2008). As an example, *Panulirus homarus* has a planktonic phyllosoma stage that is more moderate in duration (4-6 months) compared to some species of *Jasus* (up to 24 months; Pollock 1990, Booth 1994). The long-lived larval stage combined with a variety of potential modes of transport results in the relatively wide dispersal of lobster larvae (Pollock & Melville-Smith 1993, Silberman *et al*. 1994, Pollock 1995a, b, von der Heyden *et al*. 2007a).

Larval dispersal is associated with one of two models, the island (Nei *et al*. 1977) or stepping stone model (Nei & Maryuyama 1975), but in oceans these models, and many other landscape genetic assumptions/theories, may not always hold true owing to the complexity of the marine environment (Palumbi 2003, Selkoe *et al.* 2010).

In oceans, the potential for larval mixing is high and, consequently, makes gene flow between distant geographic populations possible (Pollock 1995b, Palumbi 2003, da Silva *et al*. 2011). Although there are several models of gene flow that vary in some aspects, all ultimately assume homogeneity in populations unless subjected to some sort of genetic isolation (Thorpe *et al.* 2000). Given this, there must be some degree of isolation for subspecies or genetically structured populations to exist (Pollock 1995b, Sarver *et al*. 1998, 2000, Gopal *et al*. 2006). Therefore, genetic divergence implies limiting factor(s) on gene flow (Palumbi 2003). These factors include vicariate barriers, ocean currents, larval retention, larval behaviour, larval duration or a combination of these factors that may result in genetic isolation or, at the very least, some extent of genetic structure (Pollock 1993, 1995b).

Typically, in the absence of vicariant barriers, species with a longer planktonic larval duration have the potential to settle over large distances (Pollock 1990, Silberman *et al*. 1994, Briones-Fourzán *et al*. 2008, Butler *et al.* 2011) and may comprise less structured or panmictic populations compared to those with a shorter planktonic larval duration (Silberman *et al*. 1994, Palero *et al.* 2008). A long planktonic larval duration allows for high rates of gene flow between populations that may result in panmixia (Tolley *et al.* 2005, Palero *et al.* 2008, Babbucci *et al.* 2010). In addition, high levels of adult migration between populations may also contribute to panmixia (Silva & Russo 2000, Tolley *et al.* 2005). Conversely, larvae with a shorter planktonic duration will presumably disperse and settle close to their point of origin, thus limiting gene flow

and, in turn, forming more genetically structured populations (Silva & Russo 2000, Babbucci *et al.* 2010). Genetic structure exists because of an accumulation of mutations and genetic drift, ultimately leading to speciation if sustained over a geological scale (Silva & Russo 2000).

In several species of the genus *Jasus*, planktonic larval duration can persist for up to 24 months (Phillips & McWilliam 1986, Booth 1994, Palero *et al.* 2008). The implication of this is a less structured population, seen among populations of *Jasus edwardsii* on the coasts of southern Australia and New Zealand (Ovenden *et al*. 1992, Silberman *et al*. 1994, Palero *et al.* 2008). On the other hand, *Sagmariasus* (*Jasus) verreauxi* populations between southern Australia and New Zealand are more genetically structured due to the Tasman Sea acting as a barrier to larval dispersal (Brasher *et al*. 1992, Palero *et al.* 2008). The aforementioned scenario demonstrates the role of barriers (vicariate, physical or physiological) on larval survival and population genetic structure.

Nevertheless, the dispersal capacity of even long-lived planktonic larvae does not necessarily translate into a wide distributional range for some species (Brasher *et al*. 1992, Pollock 1995b, Thorpe *et al*. 2000, Palumbi 2003; also see Bohonak 1999) and highly structured populations can be formed (Knowlton & Keller 1986, Todd *et al*. 1998, Thorpe *et al*. 2000, Gopal *et al*. 2006, von der Heyden *et al*. 2007a). This may be due to a number of reasons including barriers (vicariate, physical or physiological), the influence of ocean currents and intrinsic genetic factors that may influence larval settlement (Palero *et al.* 2008).

Typically, if different populations are geographically separated, it is likely these populations may diverge and, in time, not only become genetically isolated, but may also undergo speciation, genetic drift and natural selection (Pollock 1995b, Silva & Russo 2000, Thorpe *et al.* 2000). Local-scale oceanographic systems, however, often reduce genetic isolation by allowing some level of migrant exchange; even a low rate of genetic exchange may influence the divergence of populations (Silva & Russo 2000, Thorpe *et al.* 2000). Characteristically, genetic differentiation manifests in those species separated by large distances (Thorpe *et al.* 2000 & references therein); however, local scale differentiation has also been detected in some species (Palumbi 2003).

Thus, the culmination of ocean current patterns and the life history of lobsters are key in explaining current day lobster population genetic structure (Babbucci *et al.* 2010). Incidentally, such concepts are the basis on which the application of seascape genetics was developed.

Seascape genetics

Recent terrestrial population genetic studies employ landscape genetics as a major tool to explain complex population patterns; in more recent times these methods have been adapted to the concept and application of 'seascape genetics' (Galindo *et al*. 2006, Selkoe *et al.* 2010). Landscape genetics, however, has made many more advances than its marine counterpart, as the latter is challenged by complexity in the marine environment (Galindo *et al*. 2006, 2010). Thus, seascape genetics is still in its infancy and requires many paradigm shifts from existing landscape genetic theories (Selkoe *et al.* 2010).

The many genetic studies on freshwater populations and hypotheses, unfortunately, cannot be applied as easily to marine organisms as the marine environment is more complex and is not confined by definitive boundaries (Thorpe *et al.* 2000, Galindo *et al*. 2010). Most freshwater organisms are restricted by geographical barriers, while marine organisms are often confined only by ecological barriers such as temperature and ocean currents (Thorpe *et al.* 2000, Selkoe *et al.* 2010).

Seascape genetics broadly aims to link oceanography, biology, ecology and genetics (Galindo *et al*. 2010, Selkoe *et al.* 2010). Selkoe *et al.* (2010) found that environmental and biological data often help resolve the chaotic genetic patterns apparent in marine systems. In seascape genetics, population genetic structure is not merely attributed to straightforward isolation-by-distance (IBD) models; an array of environmental and biological factors that better explain genetic patterns are also incorporated (von der Heyden 2009, Selkoe *et al.* 2010).

High-resolution ocean circulation models used in 'seascape genetics' have helped unravel some of the complexity in marine environments (Selkoe *et al.* 2010). Thus, oceanographic data, superimposed by environmental factors and biological data have

successfully helped explain contemporary population genetic patterns (Foll & Gaggiotti 2006, von der Heyden 2009, Selkoe *et al.* 2010).

Principles and hypotheses derived from seascape genetics may, however, apply to some species, but not to others (Selkoe *et al.* 2010). A suite of environmental drivers may result in high gene flow and connectivity in some species and low gene flow and connectivity in others (Foll & Gaggiotti 2006, Selkoe *et al.* 2010). Thus, an in-depth understanding of the unique ecology, biology, evolution, phylogeography of a species and its environment is instrumental when applying seascape genetics. Since the inception of seascape genetics, the popular assumption of landscape homogeneity has been abandoned (Foll & Gaggiotti 2006). Studies have now shown that factoring in landscape variables (landscape heterogeneity) produces results that are more reliable and that better explain population genetic patterns than conventional methods such as isolation-by-distance (Foll & Gaggiotti 2006, Galindo *et al*. 2006, Storfer *et al.* 2010).

MOLECULAR MARKERS

Currently, several molecular markers are routinely used in genetic studies such as allozyme electrophoresis, mitochondrial DNA (mtDNA) restriction analysis, microsatellite DNA, minisatellite DNA, Random Amplified Polymorphic DNA (RAPD), Single Stranded Conformational Polymorphism (SSCP) and DNA sequencing (Thorpe *et al.* 2000), all having their respective advantages and disadvantages. Each marker provides different aspects of genetic information. Thus, it is best to use a suite of molecular markers in order to obtain a better resolution of genetic relationships.

There are three protein analysis methods widely used in genetic studies, total protein analysis, serological methods and locus-specific allozyme markers (Sweijd *et al.* 2000). Serological markers are frequently used in species identification. However, allozyme electrophoresis is a more popular molecular technique, probably due to its cost-effective nature and its usefulness with large sample sizes (Thorpe *et al.* 2000). Allozymes have long been considered neutral markers. However, studies have shown that these markers may incur more selection than DNA markers (García-Rodríquez & Perez-Enriquez 2006). Furthermore, Brooker *et al.* (2000) discourages the use of allozymes due to their low variability and, thus, possibly unreliable results that may mask fine-scale population

level genetic relationships that are concerned with migration and gene flow. In particular, protein variability is low in decapod crustaceans (Brooker *et al.* 2000) and allozymes may exhibit strong correlations to environmental and biological factors as outlined by García-Rodríguez & Perez-Enriquez (2006). Allozymes should thus be used with caution in decapod crustacean population genetic studies regarding gene flow and migration (Brooker *et al*. 2000, García-Rodríguez & Perez-Enriquez 2006). However, this marker has been successfully used to resolve species boundaries and systematics of species or subspecies.

Even though allozyme markers are widely used in genetic studies, for the most part, many protein methods have been superseded by DNA techniques. These are favoured as DNA is more stable, minute quantities of tissue are required, preservation of tissue samples is simple, and DNA can be extracted even from fossils (Silva & Russo 2000). However, large population sizes are required for population genetic studies, and, thus DNA techniques are often expensive, especially when sequencing is needed (Silva & Russo 2000).

Nevertheless, Silva and Russo (2000) showed that most population genetic studies employ mtDNA markers, followed by the popular use of nuclear markers. MtDNA markers are (usually) maternally inherited markers that are suitable for the assessment of population genetic structure and for phylogeography studies (Yamauchi *et al*. 2002, Tolley *et al*. 2005). Such markers have been popular in evolutionary studies as the mitochondrial gene is (usually) maternally inherited and does not undergo genetic recombination (García-Rodríguez *et al*. 2008). The common functional and transcribed coding genes used in the mtDNA genome are the large subunit 16S ribosomal RNA and cytochrome *c* oxidase subunit 1 (COI); these are slower evolving regions in the gene and are therefore routinely employed in phylogenetic studies. On the other hand, the control region has a rapid evolutionary rate and is often used in population genetic studies (García-Rodríguez & Perez-Enriquez 2006).

Some of the advantages of using mtDNA are its haploid nature, smaller size and relatively rapid rate of evolution compared to nuclear DNA, its lack of intermolecular genetic recombination and its sensitivity to genetic drift (Katsares *et al*. 2003). On the other hand, some of the caveats associated with the use of mtDNA include the

amplification of a single locus as well as the possible amplification of mtDNA pseudogenes (Buhay 2009). Pseudogenes (mtDNA) may reveal population genetic structure or inferences of gene flow between populations that are in fact panmictic (Williams *et al*. 2002, Schultz 2009). Moreover, high substitution saturation rates and high A/T content in these markers often result in high levels of homoplasy. This may impede the resolution of deeper nodes in phylogenetic studies (Tsang *et al*. 2008). Thus, the use of slower evolving mtDNA regions or RNA nuclear markers is common alongside mtDNA markers to address the above concerns (Chu *et al*. 2001).

Nuclear genes have lower mutation rates compared to mtDNA due to a larger effective population size in the former; thus mutations become fixed in mtDNA at a faster rate (Silva & Russo 2000). Consequently, these nuclear markers are widely applied in phylogenetic studies and allow inferences concerning events distant in time that may be used to explain current distribution and behavioural patterns (García-Rodríguez *et al*. 2008). Nuclear markers are less informative at a population level unless a selectively neutral intron (non-coding region) with a higher mutation rate is targeted (Silva & Russo 2000).

In population genetics, microsatellites are markers of choice and are routinely used in combination with mtDNA and other molecular markers (Silva & Russo 2000, Selkoe & Toonen 2006).

There are currently several molecular markers available, as discussed earlier, and appropriate markers should be chosen carefully to answer specific research questions and avoid bias in the results of a study. Yang (1998) recommends the use of both fast and slow evolving molecular markers to obtain clear phylogenetic relationships. The number of genes or molecular markers used in phylogenetic studies is just as important, if not more so, than the sample size (Rokas & Carroll 2005, Palero *et al.* 2009). Babbucci *et al*. 2010, Palero *et al.* (2009) and Tsang *et al.* (2009) demonstrate that a comprehensive phylogenetic picture is painted by using both a larger sample size as well as many molecular markers.

Two major factors relevant in choosing molecular markers are the costs and the relatedness or scale of resolution required for the purpose of the study (Sweijd *et al.*

2000). Some studies may require a finer resolution to discriminate between subspecies while others may require an appropriate resolution to differentiate between species or genera.

Application of molecular markers in spiny lobsters

The discovery rate of new marine lobster species remains high and in the last decade (2000-2010) alone at least 29 species have been discovered (Chan 2010), with Groeneveld *et al.* (2006) and Sekiguchi and George (2005) each naming a new species in the genera *Palinurus* and *Panulirus* respectively. In recent times, the identification of novel, cryptic and undescribed species has largely been aided by molecular markers.

Taxonomy and systematics

Historically, taxonomy and systematics were based on morphological differences (Schultz 2009). However, some identifying morphological characters that may have evolved convergently (homoplasy), analogously or have been lost over an evolutionary scale may pose a problem in this type of classification (Schultz 2009). Although extensively used in the past, taxonomy based on morphological characters has faced criticism (Sarver *et al.* 1998). Nevertheless, this type of taxonomy has been successfully used in the past and is still used at present. Moreover, some taxonomic classifications are corroborated genetically (Tsang *et al.* 2009).

In decapods, cladistic classification has proven difficult owing to their vast diversity in morphological features and their wide range of shared, derived characters (Sarver *et al.* 1998, Dixon *et al.* 2003, Porter *et al.* 2005, Tsang *et al.* 2008). Some of the common morphological features used for classification in the past were tail length (Linnaeus 1758, Boas 1880), gill type (Huxley 1878), number of chelae (Beurlen & Glaessner 1930) or locomotion (Boas 1880). Earlier taxonomic studies classified species within the genus *Panulirus* based on the morphological appearance of the second and third maxillipeds (George & Main 1967, Picther 1993).

These simplistic methods were later superseded by a more comprehensive approach that used a range of morphological characters as well as differences in biological traits, such as larval and spermatozoan characteristics (Dixon *et al.* 2003). Some authors, involved

in more recent phylogenetic studies have argued that diagnostic features commonly used in past cladistic studies were biased as the two most common features used in decapod phylogeny 1) the development of the tail and 2) reduction in the first abdominal pleuron were plesiomorphic features (Scholtz & Richter 1995, Tsang *et al.* 2008). However, the first cladistic study (Scholtz & Richter 1995) on the phylogenetic relationship of Reptantia (most common decapods crabs, crayfish and lobsters), compared well with the phylogenetic placement of some Reptantia taxa in later studies (Schram & Ahyong 2002, Dixon *et al.* 2003, Tsang *et al.* 2008, Chan 2010).

A molecular approach often complements traditional classification methods, particularly when dealing with cases that are more perplexing. A combination of traditional and contemporary approaches may provide sound substantiation for current day taxonomic nomenclature (Schultz 2009). Therefore, a genetic approach accompanied by a morphological or cladistic approach can help resolve the many difficulties associated with classifying species (Ahyong & O'Meally 2004). Moreover, genetic methods are less time-consuming than traditional methods (Schultz 2009) and are particularly important when working with cryptic species, *i.e.* species that are similar morphologically but genetically different, or similar genetically but are morphologically different (Knowlton 1993, Sweijd *et al*. 2000). Molecular identification has also been helpful in identifying early stage phyllosoma larvae or pueruli in species that cannot be distinguished morphologically (Chow *et al*. 2006), e.g. *Panulirus homarus* and *P. ornatus* (Booth & Phillips 1994).

DNA barcoding and the wide application of COI

DNA barcoding (Herbert *et al*. 2003 & 2004) is being used in a worldwide campaign dedicated to fingerprinting all extant species of the world in an attempt to catalogue the world's biodiversity (Hajibabaei *et al.* 2007, da Silva *et al.* 2011). The DNA barcoding gene is a fragment of cytochrome oxidase *c* subunit I (COI). The COI region is genetically informative at various taxonomic levels but has commonly been used at the species and population level (Herbert *et al*. 2003 & 2004, da Silva *et al.* 2011).

Molecular data presented by da Silva *et al.* (2011) showed a higher molecular divergence at higher taxonomic levels. This pattern loosely corroborates past taxonomic

studies based on degree of differences in morphological traits (da Silva *et al.* 2011). As such, the COI marker serves as an ideal tool for the identification of organisms down to species level, known as DNA taxonomy (Hajibabaei *et al.* 2007). Morphological features used in the taxonomic classification of species may show plasticity in response to environmental changes, thus misidentification may occur and this reinforces the need for the supplemental use of DNA identification (da Silva *et al.* 2011). However, the aim of this campaign is not to displace traditional taxonomy but rather to validate taxonomic classifications and species evolutionary groupings based on biological, ecological and biogeographic information (Hajibabaei *et al.* 2007, da Silva *et al.* 2011).

Even though COI provides a snapshoot look into recent evolutionary relationships between and within taxa (da Silva *et al.* 2011), supplementary molecular markers or regions better suited for phylogenetic inferences should be used to complement this marker in phylogenetic studies, providing a comprehensive look at evolutionary relationships (da Silva *et al.* 2011). In population genetic studies, COI has also been widely used for assessing migration patterns and genetic drift (Hajibabaei *et al.* 2007).

Using mtDNA, COI and 16S ribosomal RNA markers, Sarver *et al.* (1998) successfully demonstrated that Caribbean and Brazilian populations of *Panulirus argus* that differ in colour were genetically different and could be considered subspecies. They proposed two provisional subspecies and George (2005b, c) has tentatively accepted these names. However, Chan (2010) argues that the name change was provisional and, according to the International Code of Zoological Nomenclature (1999), names proposed conditionally after 1961 were not acceptable.

Similarly, *Panulirus homarus* subspecies have been distinguished by colour and differences in abdominal sculptural pattern (Berry 1974a). However, genetic evidence validating the subspecies of *P. homarus* is not yet available but, if acquired, will provide a sound basis for this subspecies classification. Such genetic classification of *P. homarus* will prove particularly useful in understanding the molecular phylogeny and population genetic patterns in these lobsters.

Population genetic studies

Panmixia is commonly observed in spiny lobster populations around the world an observation made possible by the use of various molecular markers. García-Rodríguez and Perez-Enriquez's (2006) genetic results suggest that the Californian spiny lobster, *Panulirus interruptus*, exists as a panmictic population owing to the California Current that promotes high gene flow in this region. Panmixia was also found in the spiny lobster population of *Palinurus gilchristi* in southern Africa where they are endemic (Tolley *et al*. 2005).

In Hawaii, *Panulirus marginatus* also exhibited panmictic populations, revealed by allozymes (Shaklee & Samollow 1984, García-Rodríguez & Perez-Enriquez 2006). However, the choice of this molecular marker dictates that additional markers are needed to verify panmixia in this population (Brooker *et al.* 2000, García-Rodríguez & Perez-Enriquez 2006). There was also no genetic differentiation found among Caribbean populations of *P. argus* from Bermuda and Florida, using both Random Fragment Length Polymorphism (RFLPs) and allozymes (García-Rodríguez & Perez-Enriquez 2006). However, there were significant differences in the same species among Venezuela and Brazil using mtDNA (Sarver *et al.* 1998, García-Rodríguez & Perez-Enriquez 2006).

The literature bears abundant information on the biology, ecology, evolution and phylogenetics of *Panulirus homarus* but a paucity of genetic information. Such information on population genetic structure and genetic diversity are particularly useful, not only from a scientific perspective but also from a management point of view, in deciding whether stocks can better be managed on a regional or national scale, particularly in the case of shared stocks.

MANAGEMENT

The identification of genetically distinct populations is pivotal in determining management and conservational taxonomic units (Waples 1998, Ward 2000). Therefore, the benefits of genetic studies are far reaching and go beyond unravelling the biology, ecology and evolution of species; they can be further applied to the conservation and management of species (Waples 1998).

The lobster fishery

The alarming increase in food and other prices, currently at a pinnacle, has drastically affected some three billion people around the world that rely on seafood as a primary source of protein (FAO 2010). These price increases have set in motion a higher demand on both vertebrate and invertebrate fisheries. The enormous pressure on invertebrate fisheries has lead to many fisheries becoming fully commercially exploited and, in some cases, over-exploited (Thorpe *et al*. 2000, Babbucci *et al*. 2010, FAO 2010, Palero *et al*. 2011). Traditional management has clearly failed these fisheries and little use has been made of newer methods such as the application of genetics in fisheries. Despite a few efforts, genetic information and the application of genetic techniques are largely lacking in the management of these fisheries (Thorpe *et al.* 2000, Ward 2000, Diniz *et al*. 2005).

Lobsters are tremendously valuable commodities to many countries, with lobster tails being the primary unit of sale, fetching high retail prices globally (Sweijd *et al.* 2000, Chan 2010). The high demand and market potential of this resource has lead to an intensified exploitation of wild stocks (Sweijd *et al.* 2000, Chan 2010, FAO 2010). According to the FAO (2010), 80 000 tons of spiny lobsters are harvested annually from around the world, contributing substantially to the global seafood market. As such, spiny lobsters have been the subject of numerous biological, ecological, phylogeographic, molecular phylogeny and aquaculture studies (Palero *et al.* 2009 & refer to references in this Chapter). Although presently abundant in large numbers (Fielding *et al*. 1994, Fielding 1996, 1997, Fielding & Mann 1999), *Panulirus homarus* along the east African coast may face the bleak future of overfishing. Due to severe fishing pressure, the European spiny lobster, *Panulirus elephas*, showed signs of a dwindling population for several years before any action was taken to mitigate the problem (Babbucci *et al*. 2010). Negligence was involved as historically high catch rates resulted in a false sense of security (Babbucci *et al*. 2010). It is therefore likely that many other lobster species will be susceptible to a similar fate.

As the demand for stocks come under scrutiny, so too does the issue of shared stocks among neighbouring countries (Caddy & Cochrane 2001, von der Heyden *et al*. 2007b). Many neighbouring countries have come to heated disputes as to whom wild stocks

belong (FAO 2010). However, marine organisms are not confined to clearly delineated political boundaries and therefore the genetic identification of distinct stocks is fast becoming instrumental in the conservation and management of shared stocks (Knowlton 1993, 2000, von der Heyden *et al*. 2007b). Their genetic identification will therefore, not only be helpful from an ecological or biological standpoint but also from a socio-economic and political viewpoint.

The Panulirus homarus fishery

The shallow-water scalloped spiny lobster, *Panulirus homarus rubellus*, supports various types of fisheries along its distribution (the south east coasts of Africa and Madagascar), ranging from a small-scale commercial fishery along its southern most distribution-the Eastern Cape coast, South Africa-, a recreational fishery along the KwaZulu-Natal coast (east coast of South Africa) to artisanal fisheries along southern Mozambique and south east Madagascar (Fielding *et al*. 1994, Kemp 2008, Steyn *et al*. 2008, Steyn and Schleyer 2011). Little is known about the artisanal fisheries, local distribution patterns, abundance or genetic structure of *P. h. rubellus* along southern Mozambique and Madagascar or the implementation of any management schemes in these countries. The target subspecies along much of Mozambique, Tanzania, Kenya, Somalia and India is *P. h. homarus* and this subspecies also supports various types of fisheries across its wide distribution range.

Management in South Africa

Along the South African coast a high abundance of *Panulirus homarus rubellus* and a low abundance of *Panulirus homarus homarus* occur. No distinction is made between the two subspecies by fisheries and, therefore, current management focuses on the collective management of *P. homarus* as a species. The East Coast rock lobster fishery largely supports poor coastal communities in the Eastern Cape Province (Fielding *et al*. 1994, Steyn *et al*. 2008), where approximately 2000 fishermen possess lobster fishing permits (Steyn *et al*. 2008). The small-scale commercial fishery in the Eastern Cape fetches more than R 500 000 annually (Kemp 2008, Steyn *et al*. 2008), while the large recreational fishery on the KwaZulu-Natal coast harvests up to 150 tons of *P. h. rubellus* annually (Fielding *et al*. 1994, Kemp 2008). Management of *P. homarus* is still

largely conventional, relying solely on permit regulations and policing (Kemp 2008, Steyn and Schleyer 2011). This involves a minimum legal size of 65 mm CL, a closed season from the 1st of November to the end of February the following year, gear restrictions (no use of boats or artificial breathing apparatus), a ban on collecting females in berry or soft-shelled lobsters and a bag limit of eight lobsters per day per permit holder (Fielding *et al*. 1994, Steyn *et al.* 2008).

In the Eastern Cape, the sale of undersized lobsters remains a huge problem, partly due to the ready market amongst holiday makers that are willing to buy these undersized catches, be it through ignorance or disregard for the fishery (Fielding *et al*. 1994, Steyn *et al.* 2008). However, the number of holiday makers and, therefore, the demand on the fishery is highly seasonal. In an effort to provide a constant income for these fishers, all year round buying stations have been established in and around the Eastern Cape. These buying stations have commercial licenses and buy most of the catches from fishers with permits (Steyn *et al.* 2008). This allows a stable income for the lobster fishermen as well as a better policing of regulations by the Department of Environmental Affairs (DEA), Branch Oceans and Coasts that routinely police these buying stations (van der Berg, R.S. 2011, pers. comm.[1]). Steyn *et al.* (2008) suggested that a stricter restriction on fishing gear, capping the permit numbers, community educational programmes and a better implemented co-management strategy were nevertheless needed. Apart from the aforementioned control measures, the role of genetic identification of possibly distinct stocks has the potential to make a significant impact on the way this fishery is managed.

The application of genetics to management and conservation

The identification of discrete populations, particularly for exploited or commercially important species, is imperative in developing sound conservation and management strategies for such species (Crivello *et al.* 2005, Babbucci *et al.* 2010). Several DNA-based identification techniques have been successfully used to investigate the population genetic structure of marine organisms (Thorpe *et al.* 2000, Ward 2000). The identification of genetically distinct stocks is particularly important when a species

[1] Ruan S. van der Berg, commercial lobster buyer at Live Fish Tanks Wild Coast, Ruan@livefishtankswc.co.za.

range traverses political or management boundaries (von der Heyden *et al*. 2007b). Such stock identification can impact the way these fisheries are managed (Waples 1998, Ward 2000).

The identification of distinct stocks is a powerful tool and has proven invaluable to commercially exploited fisheries (Ward 2000). The export of marine resources often involves manipulation of these organisms into sometimes unrecognisable entities (Sweijd *et al.* 2000), such as the popular export of lobster tails, removal of head and fin appendages, and filleting of fish (Sweijd *et al.* 2000, Ward 2000). This may encourage the replacement of high end-products with lower valued congener species or the mislabelling of products. Since these products cannot be identified morphologically at a species level, there is a greater need for rigorous DNA identification techniques to police and discourage these illegal acts (Sweijd *et al.* 2000).

In South Africa, the over-exploited abalone fishery has been closed indefinitely. However, canned South African abalone products were falsely labelled and sold as an Australian brand (Sweijd *et al.* 2000). DNA identification techniques were used to discriminate between abalone from South Africa and those from Australia and proved that the product did in fact originate from South Africa. This gave the abalone industry a powerful tool that was used to limit the illegal trade of these already over-exploited animals (Sweijd *et al.* 2000). More recent genetic studies that use forensic techniques as a tool in fisheries include von der Heyden *et al*. (2010) and Cawthorn *et al*. (2012).

Stock over-exploitation is a common problem that is apparent amongst many species around the world, including the palinurid lobsters. In an effort to forecast over-exploitation as a potential problem in the future, an apt knowledge of the population genetic structure of palinurid lobsters is required before a sound management plan can be implemented.

RATIONALE

The scalloped spiny lobster, *Panulirus homarus*, is a subspecies complex widely distributed in shallow-water, rocky habitats of the South West Indian Ocean (SWIO). *Panulirus homarus* supports artisanal fisheries in Mozambique, Tanzania, Kenya and Madagascar, and small-scale commercial as well as recreational fisheries in South

Africa. This subspecies complex comprises of three subspecies, separable by differences in colour and abdominal sculpture with each being found in different geographic regions. *Panulirus homarus rubellus* (SE Madagascar and Africa), *P. h. homarus* (eastern Africa to Japan, Indonesia and Australia) and *P. h. megasculptus* from the NW Indian Ocean, including Somalia and the Arabian Sea (Berry 1974a, Holthuis 1991, Al-Marzouqi *et al*. 2007).

Panulirus homarus homarus and *Panulirus homarus rubellus* differ in colour and abdominal sculptural pattern. Subspecies of *P. argus* which are separable based on colour differences have been shown to differ genetically as well (Sarver *et al.* 1998, 2000). Therefore, it is likely that morphologically delineated *P. homarus* subspecies are also genetically distinct.

Along the SE coast of Africa *Panulirus homarus rubellus* is distributed along a stretch of coastline adjacent to different current regimes (*i.e.* Agulhas Current, Mozambique Channel eddies, East-Africa Coastal Current, inshore counter-currents along the Eastern Cape). This, together with the occurrence of *P. h. rubellus* across different bioregions suggests population genetic structure may exist across its African range. Genetic structure between spiny lobster populations in the SWIO region was recently shown for another spiny lobster species, *Palinurus delagoae*, off South Africa and Mozambique (Gopal *et al.* 2006).

One of the objectives of the South West Indian Ocean Fisheries Project (SWIOFP) is to facilitate regional fisheries management strategies of shared stocks. Thus, it is important to determine whether stocks (or populations) are shared between countries (*i.e*. regional stocks), or whether they are genetically distinct local stocks, better managed on a national basis. This project employed genetic tools to determine whether *Panulirus homarus* along the east African coast should be managed regionally or at a finer scale.

***Objective*:** This project aimed to determine the spatial distribution pattern of genetic variation in *Panulirus homarus* from seven localities along southern Mozambique and South Africa. The outcomes will provide novel genetic information on this species.

***Specific aims*:**

The aims of this study were to:

a) Investigate whether the colour and sculptural differences observed in the two variants (*Panulirus homarus homarus* & *P. h. rubellus*) constitute taxonomically distinct subspecies or species, as well as provided inferences on reproductive isolation and environmental selective pressures, such as habitat and diet.

b) Optimize and apply mitochondrial DNA (mtDNA) primers for *Panulirus homarus rubellus* to describe the genetic stock structure present in populations along localities in southern Mozambique and South Africa.

c) Provide a genetic basis for management decisions on the regional and national management strategies for the different stocks.

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CHAPTER 2

Genetic discrimination between two morphologically distinct subspecies of *Panulirus homarus*

ABSTRACT

In this Chapter, the classification of two subspecies of *Panulirus homarus* based on morphological and colour differences are challenged genetically using DNA barcoding. As per the standard DNA barcoding method, a fragment of the COI gene was sequenced and 10 representatives each from the microsculpta form (*P. h. homarus*) and megascultpa form (*P. h. rubellus*) used for the present study. An assortment of intra- and inter-population and phylogenetic analyses was carried out to test whether or not subspecies were genetically different. Results indicate that both subspecies are indeed genetically different from one another as evident from the AMOVA, the haplotype network, genetic distance comparisons, as well as character and distance phylogenetic tree based analyses. Both, *P. h. homarus* and *P. h. rubellus* were monophyletic with respect to the out-group taxa and each subspecies formed well supported sister clades (BI: 1.00, ML: 93%, P: 100%, NJ: 100%). Mismatch distribution plots, Fu's *Fs* and Harpending's Raggedness Index indicate both subspecies have undergone a historic population expansion, likely a range expansion estimated to have occurred during the mid-Holocene Epoch. An assortment of genetic analyses and nucleotide divergence (3%) showed marked genetic differences between *P. h. homarus* and *P. h. rubellus* and were comparable to species differences. However, according to the Biological Species Concept, these subspecies cannot be deemed to be species due to possible interbreeding and, therefore, some level of gene flow between subspecies that may prevent complete genetic isolation, provided hybrid offspring are reproductively viable. Historic range expansion may account for the present day marginal overlap in distribution of *P. h. homarus* and *P. h. rubellus* in southern Mozambique. However, different modes of larval transport and possibly larval homing were suggested as major factors geographically separating these subspecies and, thus, shaping their genetic structure. In terms of management, these subspecies have been found to be genetically distinct and should therefore be managed separately.

Key words: Panulirus homarus, DNA Barcoding, Molecular Phylogeny

INTRODUCTION

Since the Oligocene and early Miocene Epochs, sea level has risen by 500 m (Pollock 1990). Subsequently, during the Pleistocene Epoch, paleotectonic plate movements, changes in climate and oceanic current regimes created new, shallow marine habitats (George & Main 1967, George 2005a). Accordingly, the shallow water genus *Panulirus* appeared in the fossil records in the early Pleistocene or at least during the late Pliocene about 2-3 Ma (George & Main 1967, Pollock 1990, 1995a).

Members of the genus *Panulirus* split into four major groups (Group I-IV) after glacial activity during the Pleistocene Epoch, each believed to have become isolated and to have undergone speciation in different regions of the global ocean basins (George & Main 1967). Two mechanisms associated with the speciation of *Panulirus* were 1) the selection of phyllosoma larvae able to recognise suitable home environments and 2) barriers to gene flow due to changes in and possible strengthening of paleo-circulation systems (Pollock 1990). Changes in climate and current circulation patterns as a result of the collision and subsequent uplift of the Himalayas and New Guinea mountains may have shaped speciation in group III and IV species (including *P. homarus*) in the Indo-West Pacific (Patek *et al*. 2006). Indeed, oceanographic circulation patterns impeding gene flow were instrumental in speciation of the current *Panulirus* species resident in various ocean basins as seen today (Pollock 1995a).

Panulirus homarus and its subspecies provide an example of speciation associated with paleo-circulation patterns. The *Panulirus homarus* subspecies complex was formerly described as two species, one red in colour, *Panulirus buergeri* De Haan (1851), the other green, *Panulirus dasypus* H. Milne-Edwards (1837). However, after much deliberation these species were designated subspecies with the addition of a third subspecies Berry (1974a). Currently, this subspecies complex is grouped into two forms based on abdominal sculptural pattern and colour; the microsculpta form to which *Panulirus homarus homarus* Linnaeus (1758) belongs and the megasculpta form to which *Panulirus homarus rubellus* Berry (1974a) referred to as the southern megasculpta form and *Panulirus homarus megasculptus* Pesta (1915) referred to as the northern megasculpta form, belong (Berry 1974a). The former has well developed squamae along the transverse abdominal grooves and is green in colour, while the latter

has poorly developed squamae along the transverse abdominal grooves and is red in colour (Berry 1974a). Both forms have pale yellow to white spots along the entire length of the body, often concentrated on the posterior margins (Berry 1974a, pers. obs.). These spots may only be partially developed in some individuals or absent (pers. obs.).

Panulirus homarus homarus is the most widely distributed of these subspecies and its distribution marginally overlaps that of *P. h. megasculptus* in the north and *P. h. rubellus* in the south (Berry 1974a, Pollock 1993). *Panulirus homarus homarus* is commonly found in the Indo-West Pacific (east Africa to Japan, India, Indonesia, East Indies, Australia and New Caledonia) (Holthuis 1991, Kulmiye *et al.* 2006, Kemp & Britz 2008), *P. h. rubellus* along the SE African coast, (particularly around southern Mozambique and along the east coast of South Africa), and along the south east Madagascan coast (Berry 1974a, Holthuis 1991, Kulmiye *et al.* 2006), and *P. h. megasculptus* along the northern Arabian Sea and Somalian coast (Fielding & Mann 1999, Kulmiye *et al.* 2006).

Although there is subtle overlap in the latitudinal distribution of *Panulirus homarus* subspecies, the predominance of one species often marks the exclusion of another (George & Main 1967, George 2005a). However, in regions where a marginal overlap in distribution occurs, *P. homarus* subspecies exist sympatrically and may even interbreed (Berry 1974a, George 2005a, b). Indeed, the cross-fertilisation between *P. homarus* subspecies and among subspecies of *P. longipes* are the only two documented cases of cross-fertilisation between closely related taxa in the genus *Panulirus* (George 2005b). Genetic and ecological divergences have been considered evolutionary strategies that limit competitive exclusion between closely related taxa, allowing for sympatric habitation (von der Heyden *et al.* 2007). Therefore, Knowlton (1993, 2000) suggests that sibling species often display minor differences in morphology and colour, and may also have different ecological and habitat preferences when they occur in sympatry.

Colour is an important diagnostic feature used for the identification and characterisation of lobster (sub) species (Tsoi *et al.* 2011). However, colour differences in lobsters may be genetically (permanent) or environmentally (temporary/reversible) induced. The

latter may be induced by re-allocation of pigment containing cells (Knowlton 1993, Tsoi *et al.* 2011), anthropogenic chemicals that enter the ocean and temporarilly stain lobsters (Wowor 1999, Tsoi *et al.* 2011) or preservation methods (Berry & George, 1972, Tsoi *et al.* 2011). In addition, differences in habitat preference have also been known to influence the colour of lobsters (Wowor 1999). Two populations of the deep-sea clawed lobster, *Linuparus sordidus* from Hong Kong and Taiwan are distinguished by colour differences (Wowor 1999). However, a difference in colour between these populations did not translate into genetic differences and has been attributed to environmental variability (Chan & Saint Laurent 1999, Tsoi *et al.* 2011). On the contrary, two populations of *Panulirus argus* from Brazil and the Caribbean/Western Atlantic, also distinguished by colour differences, were found to be genetically different (Sarver *et al.* 1998), as were two subspecies of *P. longipes longipes* and *P. l. fermoristriga* (Chan & Chu 1996).

Traditional taxonomy and systematics were largely based on differences in morphological traits (Schultz 2009) such as differences in abdominal pattern and colour in the two forms of *Panulirus homarus* (Berry 1974a). However, such morphological traits may be constrained by environmental selective pressure and therefore may not necessarily reflect genetic differences (Patek *et al.* 2006).Thus, in recent times, many studies use a combination of traditional and contemporary methods for taxonomy and systematics (Chan & Chu 1996, Macpherson & Machordom 2001, da Silva *et al.* 2011). A popular contemporary method is the use of mtDNA (Costa *et al.* 2007); DNA barcoding of animals has been used in particular by an increasing number of authors (Herbert *et al*. 2003, Hajibabaei *et al.* 2007, da Silva *et al.* 2011).

The universal DNA barcoding gene is a protein coding region of the mitochondrial genome, a fragment of the cytochrome oxidase *c* subunit I (COI) (da Silva *et al.* 2011). The COI region is conserved in the mitochondrial genome and is, therefore, routinely applied in phylogenetic and phylogeography studies (Avise *et al*. 1987, Ball & Avise 1992). Although genetically informative at various taxonomic levels, this marker yields better resolution for identification at the species and population level (da Silva *et al.* 2011).

Here we investigate whether morphological differences between the megasculpta (*Panulirus homarus rubellus*) and microsculpta (*P. h. homarus*) forms of *P. homarus* are corroborated by their genetics using DNA barcoding methods. The megasculpta form consists of two subspecies. However, in the present study a single representative was used, *P. h. rubellus*.

MATERIALS AND METHODS

***Study area*:** The study area comprised of seven localities along the south east African coast (Fig. 2.1). Ten representatives each from the microsculpta form (*Panulirus homarus homarus*) and the megasculpta form (*P. h. rubellus*) spanning the geographic range of the study area (where possible) were used for phylogenetic analyses.

***Sample collection*:** Lobsters were collected by SCUBA diving or snorkeling, or acquired from lobster catchers and buyers. The carapace length was measured using calipers, lobsters were sexed, sorted into subspecies and a picture of each taken as a voucher for identification. Subspecies identification followed the physical description by Berry (1974a) in which individuals with a microsculptured carapace pattern and green colour represented *Panulirus homarus homarus* and individuals with a megasculptured carapace and red colour represented *P. h. rubellus*. A leg segment was excised from each lobster, preserved in 70% ethanol, transported on ice and stored at 4ºC for later DNA isolation. If lobsters were collected by SCUBA diving or snorkelling, they were returned to the ocean.

Geographic co-ordinates were recorded at each locality using a Global Positioning System (GPS). At localities in Mozambique, a GPS was not available and co-ordinates were obtained from Google Maps (www.maps.google.com). Co-ordinates were converted into decimal units and are presented in table 3.1.

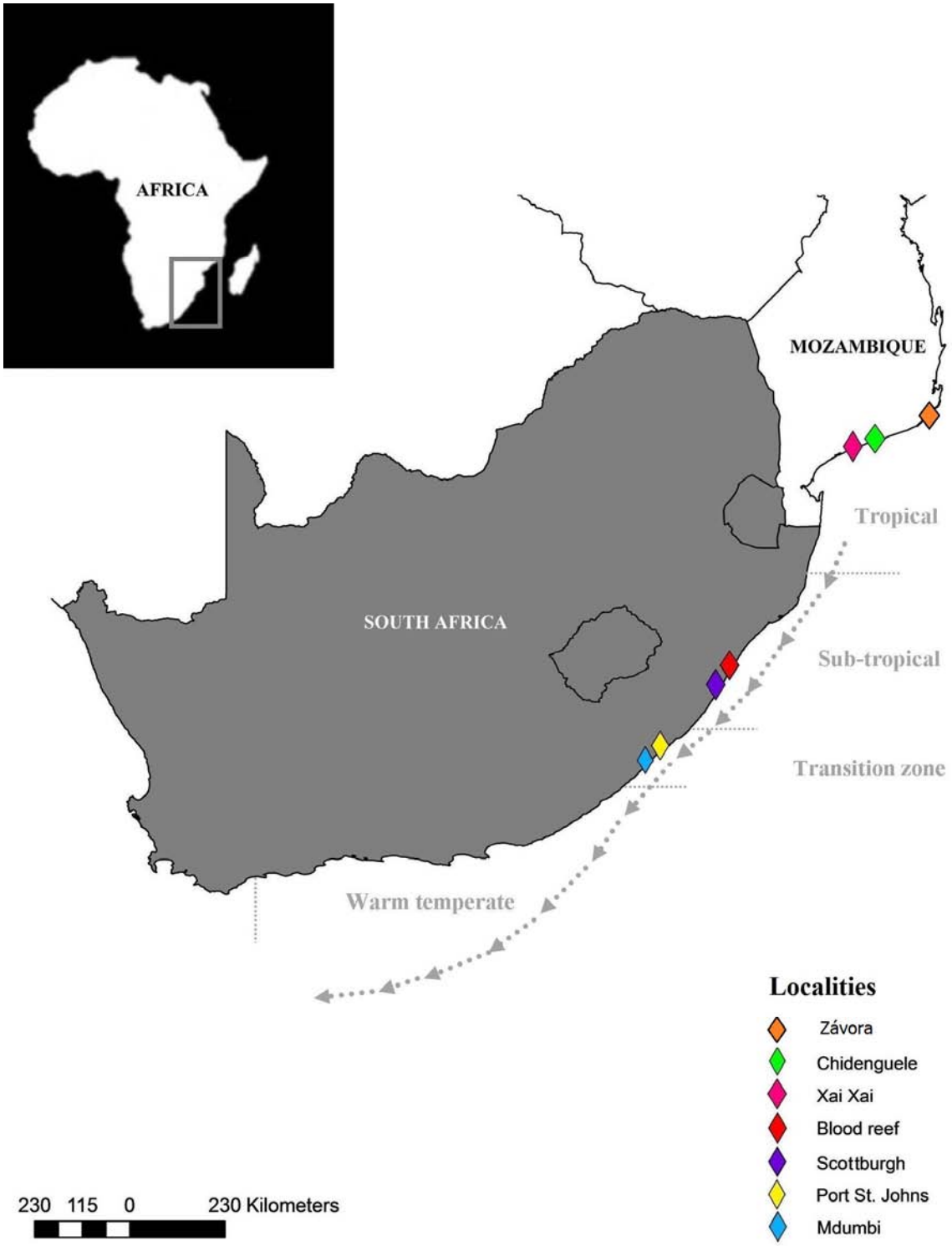

Figure 2.1: Study area showing localities at which *Panulirus homarus* (*Panulirus homarus homarus* and *Panulirus homarus rubellus*) was collected in southern Mozambique and South Africa. The relevant bioregions (Griffiths *et al*. 2008) and direction of flow of the Agulhas Current are included.

***DNA isolation*:** Genomic DNA was isolated from approximately 50 mg pereiopod tissue. The standard protocol for the ZYMO ZR96 tissue and insect DNA kit™ (Inqaba Biotechnical Industry (Pty) Ltd) was modified, by adding 20 µl of proteinase K (Fermentas, Inqaba Biotechnical Industry (Pty) Ltd) to finely chopped pereiopod tissue followed by an overnight incubation at 56°C.

After isolation, two aliquots of *ca*. 30 ng µl $^{-1}$ DNA each in 100 µl of elution buffer was attained; one of which was stored at 4°C and used as a working stock and the second was frozen as a back-up.

***PCR amplification*:** The cytochrome *c* oxidase subunit 1 (COI) region was Polymerase Chain Reaction (PCR)-amplified (Saiki *et al.* 1988) using COI primers published in Chow *et al.* (2006), (COI65F1: 5'-GGAGCTTGAGCTGGAATAGT-3'and COI1342R1: 5'-GTGTAGGCGTCTGGGTAGTC-3'). These were designed for the identification of various lobsters in the genus *Panulirus* and were optimized for amplification of the COI gene in *P. homarus homarus* and *P. h. rubellus* through a series of temperature gradients.

PCR was carried out in a final volume of 40 µl comprised of 21 µl PCR water, 4 µl 10 x buffer (Super-Therm®, Industricord cc.), 0.18 µl 25 mM $MgCl_2$ (Super-Therm®, Industricord cc.), 0.15 µl 10mM deoxynucleoside-triphosphate mixture (dNTP mix), 0.84 µl 10 mM forward primer, 0.84 µl 10mM reverse primer, 0.2 µl 5u/µl taq polymerase (Super-Therm®, Industricord cc.) and 10 µl 30 ng µl $^{-1}$ template DNA. PCRs were run in one of the following PCR machines: a Bio-Rad T100™ thermal cycler, a Applied Biosystems GeneAmp® PCR 2720/2700 or a Perkin Elmer GeneAmp 2400.

The thermo-cycling conditions for the above reaction were 5 min at 70°C for an initial denaturation, followed by denaturation at 94°C for 30 s, annealing at 60°C for 30 s, an elongation at 72°C for 1 min; steps 2-5 were set to repeat for 29 cycles, a final extension of 72°C for 10 min and a holding temperature of 15ºC.

***Gel electrophoresis*:** Gel electrophoresis was used to quantify and assess the quality of DNA after isolation as well as to visualise PCR products. Gels were run on a Labnet Power Station™ 300 plus or a Bio-Rad PowerPac™ Basic Power Supply gel system.

The quantity and quality of DNA was assessed with 1 µl loading dye (Appendix 2) which was added to 5 µl DNA in 2% agarose gels that were run in 1 X TBE (Appendix 2) at 90 volts and 400 mA constant current for 100 minutes. Ethidium bromide was added to gels for DNA visualisation under ultra-violet light (305 nm). Agarose gels viewed with a Bio-Rad ChemiDoc™ were manually inspected for band size as well as intensity as a measure of DNA quality and quantity. Large band sizes and high intensity bands were indicative of high concentrations of good quality DNA. These results were verified using a NanoDrop® ND-1000 spectrophotometer. For PCR products, the presence of a single band at 1300 bp confirmed successful PCR amplification of the COI gene. The size of the PCR products was measured against a 100 bp molecular weight marker (Fermentas, Inqaba Biotechnical Industry (Pty) Ltd) that was run simultaneously with PCR products. Note that the 100 bp molecular weight marker extends to a maximum of 1500 bp.

***Sequencing and data analyses*:** The resultant PCR products were sequenced using an ABI 3730 capillary sequencer at Inqaba Biotechnical Industries (Pty) Ltd and BigDye chemistry, or the ABI-3100 automated sequencer at the University of Stellenbosch, Central Analytical Facility (CAF). Successfully sequenced taxa were edited in BioEdit v. 7.0.9.0. (Hall 1999), aligned using CLUSTAL X (Thompson *et al.* 1994), refined manually and trimmed. The entire data set was converted into amino acids using the molecular software program MEGA v. 4 (Kumar *et al*. 2009) to check for stop codons in the reading frame of the sequence set.

Ten *Panulirus homarus homarus* individuals were sequenced and ten samples were randomly chosen from the *P. h. rubellus* data set presented in Chapter 3. Two samples of *P. h. rubellus* were chosen per locality at random to limit genetic bias. For *P. h. homarus*, a similar range across the study area was not possible due to a low abundance of these lobsters along the SE African coast and thus, low sample size in the present study.

Out-group taxa sequences, *Panulirus ornatus* (HM446347.1), *Homarus gammarus* (NTOU-M00711) and *P. japonicus* (AB071201.1), were obtained from GenBank and added to the data set to root the molecular tree.

Sequences were tested for substitution saturation using the molecular software program DAMBE (Xia & Lemey 2009). A haplotype list generated in DnaSP v. 5.10 (Librado & Rozas 2009) was used in subsequent analyses. Basic molecular indices, neutrality tests (Tajima 1989, Fu 1997) as well mismatch distribution (Rogers & Harpending 1992) analysis were calculated in DnaSP v. 5.10 and Arlequin v. 3.5 (Excoffier & Lischer 2010).

In the absence of a calibrated molecular clock for *Panulirus homarus* the time since subspecies expansion was calculated using mitochondrial COI mutation rates from congeners ranging from a lower bound, of 2.7-3.3% to an upper bound, of 9-11% (see Palero *et al.* 2008, Naro-Maciel *et al*. 2011) using the formula $\tau = 2uk$t, where u = the rate of mutation, k = the number of base pairs of the sequence and t = time since expansion. Tau (τ) was calculated in Arlequin v. 3.5 (Excoffier & Lischer 2010) and a generation time of 3 years was assumed based on their age at sexual maturity.

To test genetic partitioning between subspecies, an AMOVA was run in GenAlEx v. 6.4 (Peakall & Smouse 2006). Genetic distances between individual samples were calculated in PAUP* (Swofford 2002) and a haplotype network created in TCS v. 1.21 (Templeton *et al.* 1992). Statistical significance, where applicable was set at 95% (p <0.05).

The most appropriate model of nucleotide substitution was determined in MrModelTest v. 2 (Nylander 2004) and Modeltest v. 3.7 (Posada & Crandall 1998), using the Akaike Information Criterion (AIC) (Akaike 1974). Standard analyses were carried out to produce Maximum Likelihood (ML) (Felsenstein 1981), Parsimony (P) (Camin & Sokal 1965) and Neighbor-Joining (NJ) (Saitou & Nei 1987) trees in PAUP* (Swofford 2002), and Bayesian inference in MrBayes v. 3.1.2 (MB) (Ronquist & Huelsenbeck 2003).

For P and ML, random sequence addition and a heuristic search-run using 10 random addition starting trees, tree-bisection-reconnection (TBR) and tree branch-swapping were implemented. A confidence value was calculated for branches from 1000 bootstrap pseudoreplicates (Felsenstein 1985) and indicated at each node. In the Bayesian analysis, the Markov Chain Monte Carlo (MCMC) search method was used to search

over five million generations, sampling every 100 replicates in which the initial 10% were discarded as burn-in to approximate the probability distribution. The 10% burn-in value was determined by visual inspection of the probability distribution from a series of test runs. A 50% majority rule tree was constructed, as well as posterior probabilities estimated. MrBayes posterior probabilities and ML, P and NJ bootstrap pseudoreplicates were used to provide statistical confidence in the molecular trees.

Genetic population structure was contextualized relative to international boundaries in the SWIO region, biological (lobster reproduction, larval dispersal) and environmental factors (past and present ocean current regimes), and this information was used to construct hypotheses to explain observed phylogenetic structure, origin and subsequent dispersal of these subspecies.

RESULTS

***Sequence characteristics*:** A total of 20 sequences were analysed and an additional 3 out-group taxa sequences were included for the generation of phylogenetic trees. Sequences were trimmed from a total of 1300 bp to 591 bp as only a fragment of the COI gene was required. From this fragment, 538 sites were constant and 53 were variable sites, of which 52 were parsimonious informative sites. No stop codons were found in the reading frame of the sequence set indicating that no pseudogenes were amplified. Nucleotide substitution saturation tested in DAMBE, indicated there was little substitution saturation for the COI dataset. The Index of Substitution (Iss) was significantly lower than the Critical value of Index of Substitution (Iss.c), $p = 0.000$.

The model of evolution that best fitted the phylogenetic data set as per ModelTest was the K81uf model (Kimura 1980). Relative base frequencies for the alignment of the entire dataset were, A = 35. 65%, T = 19.15%, G = 15.80% and C = 29.39% and, substitution rates A-C = 1.0000, A-G = 226.5525, A-T = 38.2177, C-G = 226.5525, C-T = 1.0000 and G-T = 1.0000 implemented in ML and NJ. The best-fit model of evolution derived from MrModelTest was the General Time Reversible model (Tavaré 1986, Rodríguez *et al.* 1990) with relative base frequencies for the alignment of the entire dataset of, A = 35. 77%, T = 20.81%, G = 14.95% and C = 28.47% used for Bayesian inference. The rate of transitions and tranversions were 1:1 and no indels were recorded

in either model. Indels should not be found in the COI gene but may occur due to experimental error.

In the subsequent analyses, *Panulirus homarus homarus* and *P. h. rubellus* were treated as separate subspecies and are labelled accordingly, as well as treated as a single species labelled 'total'.

***Intra-subspecies variation*:** All haplotypes were unique with a total haplotype diversity of 1.00 ± 0.002. In order to limit bias, haplotypes of *Panulirus homarus rubellus* from each locality (Chapter 3) were chosen at random and, although some haplotypes were shared between subspecies, none of the shared haplotypes were generated from the random selection process and are therefore not included in the present analyses (Appendix 2). Haplotype sequences will be deposited in GenBank. Both haplotype (*h*) and nucleotide (π) diversities for *P. h. homarus* and the total species were high, while haplotype diversity was high but nucleotide diversity low for *P. h. rubellus* (Table 2.1).

Table 2.1: Intra-population indices for each subspecies as well as for the entire dataset calculated from partial COI sequence data of *Panulirus homarus* from the SWIO region

Subspecies	*N*	*h* (± SD)	π (± SD)	*M*	*K*
P. h. homarus	10	1.00 ± 0.002	0.011 ± 0.002	23	7.04
P. h. rubellus	10	1.00 ± 0.002	0.007 ± 0.000	18	4.02
Total	20	1.00 ± 0.000	0.040 ± 0.002	57	23.68

* Where *N* = sample size, *h* = haplotype diversity, π = nucleotide diversity, *M* = total number of mutations, *K* = nucleotide differences and SD = standard deviation

***Neutrality tests and mismatch distribution*:** Tajima's *D* statistic was not significant ($p > 0.05$) and values were negative for each subspecies, but not for the total species which indicated the COI marker used in the present study conformed to the assumption of selective neutrality for both subspecies but not the total species (Tajima 1989). Fu's *Fs* was significant ($p < 0.05$) and values were negative for each subspecies, as well as for the total species, indicating a departure from mutation drift equilibrium, thus supporting the hypothesis of population expansion.

Table 2.2: Neutrality tests, Tajima's *D* statistic & Fu's *Fs* scores, and their corresponding *p* values for each subspecies and for the total species tested on a fragment of the COI gene of *Panulirus homarus* from the SWIO region

Subspecies	Fu's *Fs*	*p*	Tajima's *D*	*p*
P. h. homarus	-4.601	0.000	-0.632	0.276
P. h. rubellus	-6.870	0.000	-0.856	0.209
Total	-6.433	0.005	2.482	0.957

The mismatch distribution (MMD) analyses was tested for 1) constant population growth and 2) population decline for each subspecies (data not shown) as well as for the total species (Fig. 2.2); the results are presented as a graph (Fig. 2.2) with associated statistics in Table 2.3.

Subspecies expansion dates for both subspecies were estimated to have occurred during the mid-Holocene Epoch using both models (spatial and sudden expansion models). *Panulirus homarus rubellus* exhibited later commencement of expansion than *P. h. homarus* in both cases (Table 2.3). The total species expansion time was not considered given the high level of genetic differentiation between subspecies.

Table 2.3: Mismatch distribution indices tested for two models for partial COI sequence data of *Panlirus homarus* from the SWIO region

	a) Spatial expansion assuming a constant deme size									
Subspecies	**θ_0**	**τ**	**SSD**	**p**	**HRI**	**p**	**$T_{9\text{-}11}$**	**SD**	**$T_{2.7\text{-}3.3}$**	**SD**
P. h. homarus	2.806	4.584	0.015	0.574	0.049	0.427	1305	184	4352	615
P. h. rubellus	0.001	4.124	0.048	0.112	0.104	0.198	1174	166	3915	553
Total	39.219	4.348	0.044	0.000	0.014	0.490	1238	175	4128	583
	b) Sudden expansion model (demographic change)									
Subspecies	**θ_0**	**τ**	**SSD**	**p**	**HRI**	**p**	**$T_{9\text{-}11}$**	**SD**	**$T_{2.7\text{-}3.3}$**	**SD**
P. h. homarus	2.547	4.832	0.015	0.635	0.049	0.453	1376	194	4588	648
P. h. rubellus	0.000	4.125	0.048	0.114	0.104	0.191	1175	166	3916	553
Total	22.388	1.471	0.047	0.110	0.014	0.550	419	59	1396	197

* Where SSD = sum of squares deviation, HRI = Harpendings Raggedness Index, T = time since expansion (in years before present), $T_{2.7\text{-}3.3}$ represents the lower (%) and $T_{9\text{-}11\%}$ the upper bound (%) and SD = standard deviation

The distribution of pairwise differences for the total species reflected a bimodal distribution and clearly confirmed two distinct lineages separated by a considerable number of mutations (Fig. 2.2). MMD plots for each subspecies indicated expanding populations (data not shown), supported by Fu's *Fs* and HRI. The low sample size in the present study may yield an artificial sense of population expansion and, therefore, results are tentatively discussed.

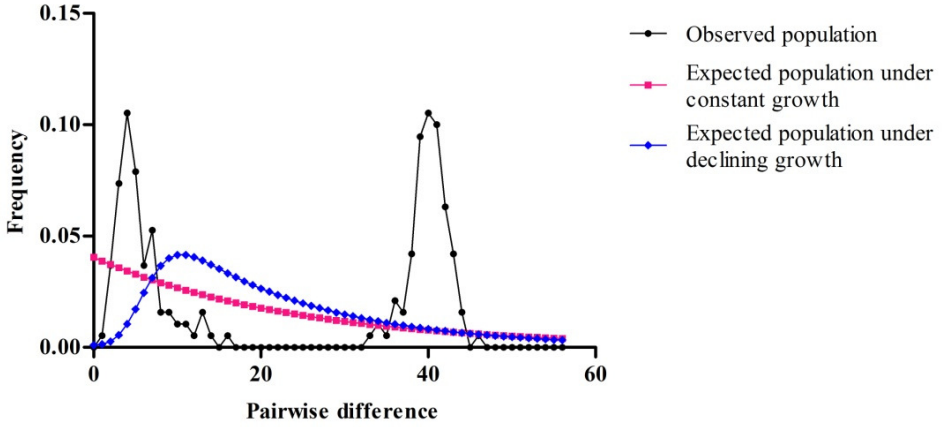

Figure 2.2: MMD plot of the entire dataset (*i.e. Panulirus homarus*, *Panulirus homarus homarus* and *Panulirus homarus rubellus*) tested as a constant population and as a declining population. The observed frequency of pairwise differences between individuals formed a bimodal distribution.

***Genetic variation between subspecies*:** A marked genetic difference (86%) and significant genetic structure were recovered between subspecies ($\Phi_{PT} = 0.860$, $p = 0.000$) and within each subspecies (Fig. 2.3).

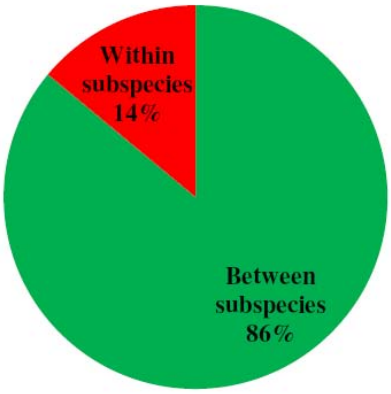

Figure 2.3: AMOVA results presented in a pie chart showing the total genetic variance of *Panulirus homarus* according to partial COI sequence data. The genetic variance has been apportioned between *Panulirus homarus homarus* and *Panulirus homarus rubellus* collected from the SWIO region.

A similar pattern of divergence (2-3%) between the two subspecies was inferred from the genetic distance matrix (Table 2.4). Genetic distances and mutations between *Panulirus homarus homarus* and *P. h. rubellus* subspecies pairs were higher than pairs consisting of members from the same subspecies. For comparative purposes the genetic distance of a single out-group taxon, *P. ornatus* was included. The genetic distances and mutations between subspecies pairs were lower than the genetic distances between subspecies and the out-group taxon.

Table 2.4: Genetic distances (%), calculated using a General Time Reversible model, between sample pairs, presented below the diagonal and average nucleotide substitutions, above the diagonal, 1-10 represent *Panulirus homarus homarus,* 11-20, *Panulirus homarus rubellus* and 21 the out-group, *Panulirus ornatus*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1		16	9	10	2	3	9	7	7	9	42	42	41	42	40	39	41	39	42	40	106
2	1.24		11	10	14	13	11	13	13	13	37	36	37	36	34	33	35	36	36	34	105
3	0.69	0. 85		7	7	8	2	6	2	4	44	41	42	41	39	38	40	39	41	39	103
4	0.77	0. 77	0.54		8	7	5	7	7	7	43	42	43	42	40	39	41	40	42	40	101
5	1.55	1.09	0.54	0.62		1	7	5	5	7	42	42	41	42	40	39	41	39	42	40	106
6	2.33	1.00	0.62	0.54	0.77		6	6	6	8	41	41	40	41	39	38	40	38	41	39	105
7	0.70	0.85	1.50	0.38	0.54	0.47		6	2	4	44	41	42	41	39	38	40	39	41	39	103
8	0.47	0.90	3.88	0.46	0.31	0.39	0.39		6	6	42	41	42	41	39	38	40	39	41	39	102
9	0.54	1.01	1.55	0.54	0.38	0.46	0.15	0.39		4	46	43	44	43	41	40	42	41	43	41	105
10	0.99	1.01	3.10	0.54	0.54	0.62	0.31	0.39	0.31		43	43	43	42	39	40	43	41	42	40	105
11	3.26	2.87	3.41	3.33	3.26	3.18	3.41	3.18	3.57	3.33		7	5	4	6	6	7	5	5	5	109
12	3.26	2.79	3.18	3.26	3.26	3.18	3.18	3.10	3.33	3.33	0.54		3	4	4	3	5	4	4	4	108
13	3.18	2.87	3.26	3.33	3.18	3.10	3.26	3.18	3.41	3.33	0.39	0.23		3	4	4	5	4	3	3	109
14	3.26	2.79	3.18	3.26	3.26	3.18	3.18	3.10	3.33	3.26	0.31	0.31	0.23		3	3	4	4	2	2	109
15	3.10	2.64	3.02	3.10	3.10	3.02	3.02	2.95	3.18	3.02	0.47	0.31	0.31	0.23		3	5	4	3	3	108
16	3.02	2.56	2.95	3.02	3.02	2.95	2.95	2.87	3.10	3.10	0.47	0.23	0.31	0.23	0.23		5	3	3	3	107
17	3.18	2.71	3.10	3.17	3.18	3.10	3.10	3.02	3.26	3.33	0.54	0.38	0.39	0.31	0.39	0.38		6	4	4	108
18	3.02	2.79	3.02	3.10	3.02	2.95	3.02	2.95	3.18	3.18	0.39	0.31	0.31	0.31	0.31	0.23	0.46		4	4	108
19	3.10	2.79	3.02	3.10	3.10	3.02	3.02	2.95	3.18	3.10	0.39	0.31	0.23	0.15	0. 23	0.23	0.31	0.31		2	110
20	3.10	2.64	3.02	3.10	3.10	3.02	3.02	2.95	3.18	3.10	0.39	0.31	0.23	0.15	0.23	0.23	0.31	0.31	0.15		108
21	8.22	8.14	7.98	7.83	8.22	8.14	7.98	7.90	8.14	8.14	8.45	8.37	8.45	8.45	8.37	8.29	8.37	8.37	8.53	8.37	

From the haplotype network, it is clear from visual inspection that *Panulirus homarus homarus* and *P. h. rubellus* form two distinct clades separated by a considerable number of mutations. The *P. h. homarus* clade is characterised by many more mutational steps between individuals than the *P. h. rubellus* clade. Furthermore, the *P. h. rubellus* clade appears to form subclades (*i.e.* BR 11, 12, SB 3, CH 20 & XX 11, 18, MB 14, PSJ 5, CH 7, SB 3).

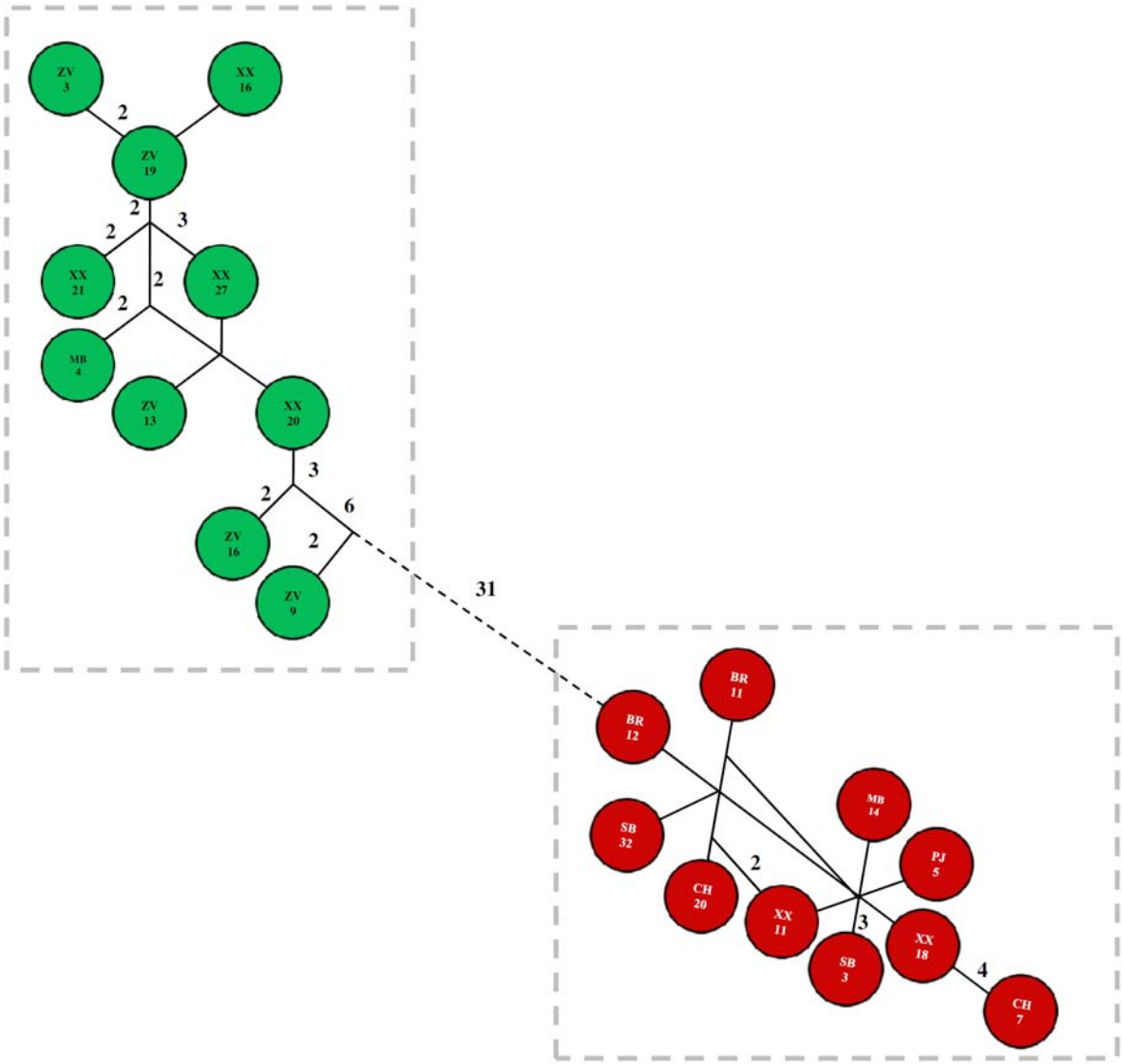

Figure 2.4: Haplotype network representing the relationship between *Panulirus homarus* individuals from the SWIO region. The size of the circle is proportional to the number of individuals sharing a particular haplotype (*i.e.* 1 cm diameter = one individual), a single line connecting haplotypes indicates a single mutational step, while numbers adjacent to the line represent the number of mutations between haplotype pairs. A solid line represents a connection with a 95% confidence interval, while a dashed line represents a connection below this confidence. Lastly, green circles indicate *Panulirus homarus homarus* individuals and red circles indicate *Panulirus homarus rubellus* individuals.

All trees were congruent and, therefore, the neighbour-joining topology is presented with associated branched lengths. Both subspecies are monophyletic with respect to the out-group taxa (BI: 1.00, M: 100%, NJ: 100%, P: 100%). There is strong support (BI: 1.00, M: 93%, NJ: 100%, P: 100%) for *P. h. homarus* and *P. h. rubellus* forming different clades and they appear to be sister taxa. In the *P. h. homarus* clade, some level of structure was indicated by two well-supported groups (bold nodes in the *P. h. homarus* clade).

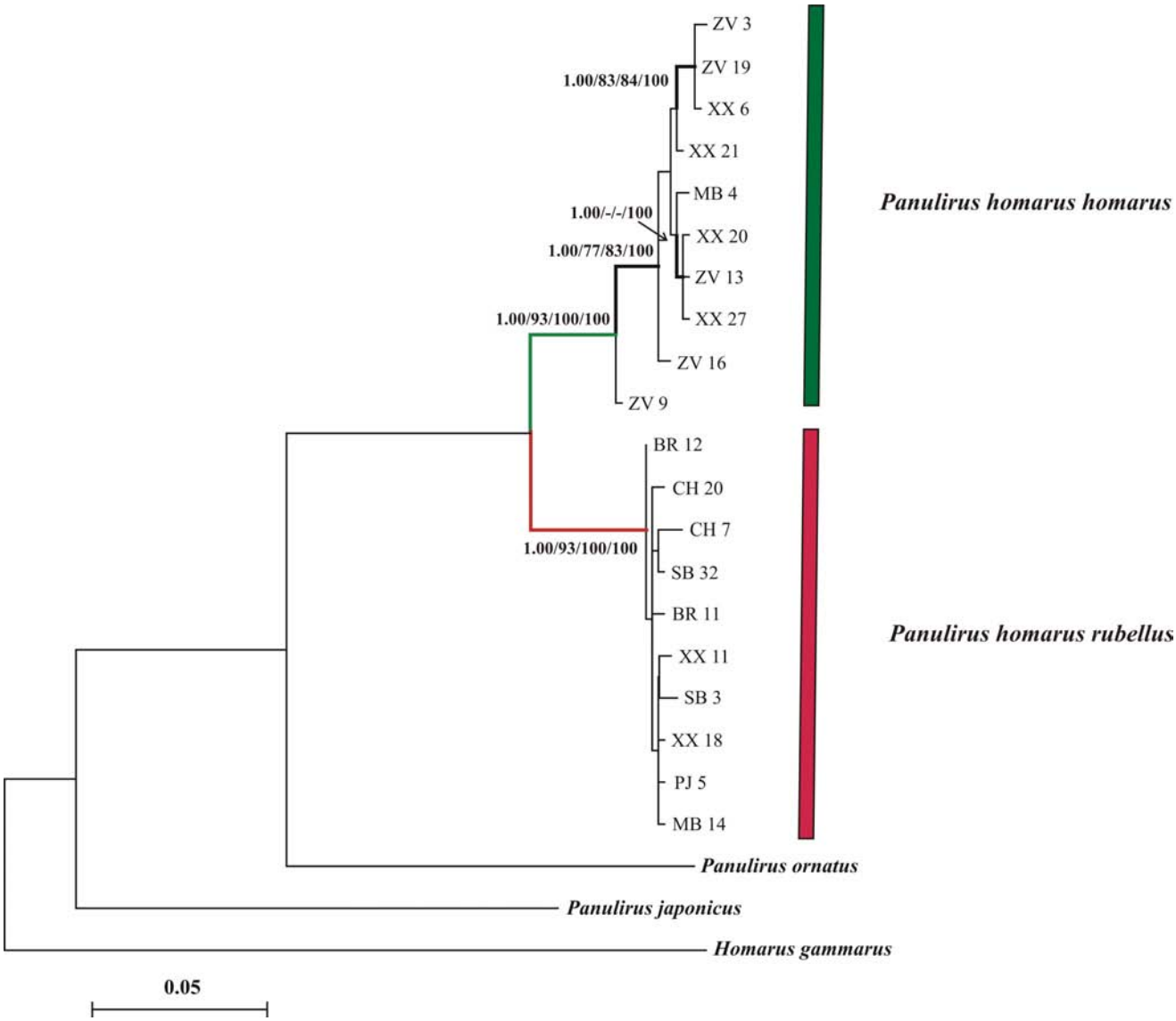

Figure 2.5: Dendogram illustrating the evolutionary relationship between *Panulirus homarus homarus* and *Panulirus homarus rubellus* in the SWIO region based on a comparison of 591 nucleotides in the COI gene. Well-supported nodes are in bold face and corresponding numbers represent nodal support (Maximum Likelihood-ML, Neighbour-Joining-NJ, Parsimony-P in %). Bayesian Inference-BI, ML, NJ and P tree topologies were congruent and therefore the NJ tree topology is presented with a scaled measure of genetic distance.

DISCUSSION

Morphological and colour differences between two forms of *Panulirus homarus*, each corresponding to a single subspecies, *P. h. homarus* (microsculpta form and green in colour) and *P. h. rubellus* (megascultpa form and red in color), were corroborated genetically using COI sequence data as per the DNA barcoding method. In recent times, DNA barcoding has become an accepted method of species differentiation in taxonomy and systematics, and is often used to supplement traditional taxonomy (Schultz 2009). As such, Berry's (1974a) previous morphological subspecies delimitation has been substantiated genetically in the present study.

In southern Mozambique, both forms *Panulirus homarus homarus* and *P. h. rubellus* occur sympatrically, inhabit similar habitats across their respective ranges, experience similar environmental stresses and have the same diet (Berry 1974a, b). As a result, morphological adaptation due to different ecological preferences was tentatively ruled out as a possible cause for morphological differences between the subspecies and these differences were, therefore, suspected to be associated with genetic differences (Berry 1974a). The present study supports the genetic difference between subspecies. The young *P. homarus* subspecies complex may represent taxa that share a number of ecological and behavioural traits, allowing their co-existence (George & Main 1967, Pollock 1992).

Intra-subspecific genetic variability

In the genetic analyses, each subspecies was treated as a separate genetic unit according to Berry (1974a) and, in addition, both subspecies were considered a single genetic unit assuming no genetic difference between subspecies. Intra-population diversity indices and MMD plots clearly indicated a high level of genetic difference between the subspecies when grouped as a single genetic unit (Total) and supported the hypothesis of two genetically distinct lineages. Intra-population diversity indices were high for the total species and, interestingly, were also high for *Panulirus homarus homarus*, suggesting a high level of genetic diversity or possibly a complex of multiple genetic lineages (cryptic subspecies). However, much work is needed to confirm if cryptic lineages do in fact exist within the *P. h. homarus* subspecies.

High levels of genetic diversity of *Panulirus homarus* across its wide distribution range are perhaps explained by the metabolic rate hypothesis (da Silva *et al*. 2011). According to this hypothesis, a strong correlation exists between nucleotide substitution and metabolic rate, suggesting that increased growth rate leads to higher rates of DNA mutation (da Silva *et al.* 2011). Temperature is a key factor influencing metabolic rate in *Panulirus homarus* (Smale 1978, Kemp & Britz 2008) and, given the largely tropical distribution of *P. h. homarus* as well as its higher growth rate than *P. h. rubellus* (Kulmiye *et al.* 2006), high levels of nucleotide diversity in the former may be explained by the metabolic rate hypothesis (da Silva *et al.*2011).

High intraspecific genetic variability often indicates a high level of population structuring (da Silva *et al*. 2011). Significantly high levels of genetic and, to a lesser extent, larval morphological diversity were recognised within the widely distributed Indo-West Pacific *Panulirus homarus homarus* (McWilliam 1995, Patek *et al.* 2001, George 2005a, Naro-Maciel *et al.* 2011). A comparison of COI variability revealed considerably greater intra-specific variability in *P. h. homarus* compared to congeners (Patek *et al.* 2001, Naro-Maciel *et al.* 2011). Furthermore, at the Marquesas Islands, *P. h. homarus* differs from the typical green variety in larval morphology and in colour (George 2005a). Interestingly, these morphotypes may also be genetically distinct species considered as a single genetic unit. In the present study, no apparent morphological or colour differences were evident between individuals of *P. h. homarus* along the east African coast. However, in crustaceans, cryptic speciation appears to be a common occurrence but is too often overlooked (Lefébure *et al.* 2006). Furthermore, Ball and Avise (1992) emphasize the importance of identifying cryptic speciation from an evolutionary perspective and, therefore, additional research may be required to clarify the genetic structure in *P. h. homarus.*

Historic demography of subspecies

Although MMD plots are generally used to infer mutation drift equilibrium and, in turn, population expansion (Harpending 1994), in the present study the MMD plot for the total species provided valuable information on the clear genetic differentiation between subspecies. Both subspecies indicated expanding populations, however, the low sample size may lower the confidence associated with population expansion inferences and are,

therefore, tentative. However, the MMD plot for the *P. h. rubellus* subspecies (164 samples) in Chapter 3 (Fig. 3.2a) closely paralleled the MMD plot for a subset of *P. h. rubellus* (10 samples) in the present study. Furthermore, although spatial as well as demographic range expansions were tested in the present study, HRI does not distinguish between the two tests and, therefore, population expansion may have resulted from either a demographic or range expansion.

Assuming population expansion underlies the historic range expansion of both subspecies in the present study, this historic range expansion may account for the present-day overlap in distribution of *Panulirus homarus homarus* and *P. h. rubellus* subspecies in southern Mozambique.

Possible hybridization?

The southern megasculpta form, *Panulirus homarus rubellus*, is abundantly distributed along the south east coast of Africa, with a low but constant abundance of the microsculpta form, *P. h. homarus* (Berry 1974a, b). Berry (1974a), thus, argued that low numbers of fecund *P. h. homarus* females suggested interbreeding must occur naturally between *P. h. homarus* females and *P. h. rubellus* males, as it was considered doubtful that *P. h. homarus* females could be fertilised by other *P. h. homarus* males (Berry 1974a). Using laboratory experiments, Berry (1974a) demonstrated that cross-fertilisation between *P. h. homarus* and *P. h. rubellus* was indeed possible. However, due to technical difficulties associated with rearing the larvae, the existence of Filial 1 (F1) progeny and reproductive viability of the offspring remains in question (Berry 1974a). Genetic techniques function to aid ecological and biological studies in such cases, particularly when traditional methods fail. Therefore, novel genetic information on the hybridization of *P. h. homarus* and *P. h. rubellus* may not only confirm whether or not offspring exist or are reproductively viable but will also add further evidence of the usefulness of genetics in both ecological and biological studies.

In the present study, some individuals bearing the morphological description of *Panulirus homarus homarus* were found to share similar haplotypes as *P. h. rubellus* individuals and *vice versa* (Appendix 2). Haplotype sharing between the two subspecies suggests that gene flow through interbreeding may have occurred. In the present study,

the southern population of *P. h. homarus* (XX) (data not shown) and, in the next Chapter, the northern population of *P. h. rubellus* (CH) did not constitute characteristic stable stationary periphery populations (von der Heyden *et al.* 2008). Instead, both populations manifested expansion and showed a high level of genetic diversity which points to the possibility of hybridization between subspecies in southern Mozambique (CH & XX) where their distribution overlaps. However, further exploration into the hybridization of subspecies was not possible owing to the molecular marker used in the present study. A mitochondrial marker is maternally inherited and offers no paternal genetic information; for inferences on hybridization both maternal and paternal information is required (Teske *et al.* 2011). Therefore, a more rigorous investigation into the hybridization of P. *h. homarus* and *P. h. rubellus* is warranted.

Subspecies differentiation

If hybridization between subspecies occurs (Berry 1974a), however, the rate at which it does is probably low as highly significant genetic structure was found between *Panulirus homarus homarus* and *P. h. rubellus.* Abundance and distribution data suggested that, although there is marginal overlap in distribution, the megasculpta and microsculpta forms seldom occur together in abundance at any given geographic location (Berry 1974a). The European and American clawed lobsters, *Homarus gammarus* and *H. americianus* are conspecifics that are also able to cross-fertilise; in an experimental study van der Meeran *et al*. (2008) demonstrated that conspecifics prefer to mate with like species when given a mate choice but will mate with a heterospecific when mate choice is limited. Similarly, if *P. homarus* conspecifics behave in this way, then mate choice of conspecifics will be favoured over heterospecifics and, should hybridization occur, the level at which it is does will be relatively low (van der Meeran *et al*. 2008).

Results of AMOVA suggested a high level of genetic structure exists between *Panulirus homarus homarus* and *P. h. rubellus*. However, local adaptation may increase true F_{st}/Φ_{PT} values (Vitalis *et al*. 2001) and may account for the high Φ_{PT} between sister subspecies in the present study. While AMOVA is not conventionally used in phylogenetic studies, the high genetic differences between the subspecies supported the genetic divergence between subspecies. This was confirmed by large levels of genetic

distances, many mutations between subspecies and clear patterns of genetic divergence inferred from the haplotype network and dendogram.

Sequence divergence

Nucleotide sequence divergence permits one to ascertain the level of divergence between taxa (Sarver *et al.* 1998). A divergence of 2% per million years is the generally accepted rate of DNA divergence between lineages in the mtDNA gene (Ball & Avise 1992). The general consensus concerning ~ 2% sequence divergence has been used to delineate species but these estimates may increase for some species such as butterflies (3.6%) or decrease in others (Tsao & Yeh 2008). Indeed, the 2% sequence divergence used to separate species has proven particularly problematic in crustaceans (da Silva *et al.* 2011). In the genus *Panulirus*, inter-specific divergence ranges from 2-3% in the COI gene (Ptacek *et al.* 2001). In the present study, nucleotide sequence divergence was in the range of 2-3% when a General Time Reversible (Tavaré 1986, Rodríguez *et al.* 1990) model was applied to the data set.

Apart from the average nucleotide sequence divergence between subspecies, genetic distances also provided a measure of taxonomic segregation between subspecies pairs. In the present study, genetic distances between many subspecies pairs were comparable to genetic differences between species pairs (Ptacek *et al.* 2001). However, classification of interbreeding sister taxa as separate species is not recommended (Mayr 1942, Ball & Avise 1992, Knowlton 2000). The possible hybridization between *Panulirus homarus* subspecies would impede complete genetic isolation between these forms, allowing some level of natural gene flow, and therefore preventing these subspecies to elevate to the rank of species according to the Biological Species Concept (Mayr 1942, Avise *et al.* 1987, Ball & Avise 1992, Knowlton 2000). However, this may only hold true if hybrid offspring are indeed reproductively viable and capable of backcrossing and introgression. In nature, even low rates of migration and gene flow between populations limit genetic isolation (Nei 1972, Silva & Russo 2000, Teske *et al.* 2011).

Two distinct lineages separated by 31 mutations were illustrated in the haplotype network. While no discernable pattern was found in the *Panulirus homarus homarus*

clade, each individual was separated from the next by many more mutational steps than in the *P. h. rubellus* clade. The higher mutation rate for the *P. h. homarus* clade has been discussed under the intra-subspecific section.

Phylogenetic analyses

Although inter-population analyses provided evidence supporting the genetic differentiation of subspecies, as did the Parsimony tree, these analyses did not incorporate a model of evolution. Therefore, to provide comprehensive phylogenetic support for subspecies divergence, phylogenetic tree-based analyses incorporating a model of evolution were undertaken to confirm the phylogeny and genetic divergence between the subspecies of *Panulirus homarus*. Each subspecies proved to be monophyletic relative to the out-group taxa, *P. ornatus*, *P. japonicus* and *Homarus gammarus*, and well-supported distance- and character-based analyses sustained the genetic differentiation between *P. h. homarus* and *P. h. rubellus.* Cryptic divergence in *P. h. homarus* lineages was also supported; however, it requires further research concerning its validity. Furthermore, *P. h. homarus* and *P. h. rubellus* formed sister taxa, and therefore share a common ancestor which is inconsistent with earlier views of a more ancient *P. h. homarus* from which two additional subspecies speciated (Pollock 1993). However, if the third subspecies (*P. h. megasculptus*) is added to the current phylogenetic picture, it may reveal a different outcome and possibly support Pollock's (1993) hypothesis.

Phylogeography

When two populations become isolated, either reproductively or geographically, each accumulates mutations and undergoes selection and genetic drift at different rates (Nei 1972, Chow *et al.* 1988). These genetically different populations give rise to what we know as subspecies, species or genera, according to their level of genetic divergence (da Silva *et al.* 2011). Specialisation of the reproductive system in different species of *Panulirus* probably prevents cross-fertilisation between species and may have contributed to the isolation of *Panulirus* species over geological time (George 2005b). *Panulirus homarus* and its subspecies comprise one of two subspecies complexes that defy reproductive isolation in the genus *Panulirus* (Berry 1974a); therefore, geographic

isolation rather than reproductive isolation may explain the genetic differentiation between these subspecies. However, along the coast of south east Africa there is no history of vicariate barriers that may have geographically isolated subspecies during periods of low sea levels (Ramsay 1985, Teske *et al.* 2011) and, therefore, larval behaviour and transport are more likely the driving force behind geographic and, in turn, genetic isolation of *P. homarus* subspecies (Pollock 1993, George 2005a).

It has been postulated that *Panulirus homarus homarus* and *P. h. rubellus* are governed by different larval transport modes (Berry 1974b, Pollock 1992, George 2005a), and this may explain their genetic distinctiveness, as well as their contemporary distribution patterns. The Agulhas Current and Indo-West Pacific current regimes have been implicated as major larval dispersal modes for *P. h. rubellus* and *P. h. homarus*, respectively (Berry 1974 a, b, Pollock 1993).

It has been suggested that *Panulirus homarus homarus* along the east African coast are derived from spill-over larvae from the West Pacific and eastern and western Indian Oceans carried by south western monsoon winds (Pollock 1993). In addition, if *P. h. homarus* larvae from the Indo-West Pacific are transported via the South Equatorial Current to the coast of Mozambique, where a series of inshore counter currents (anti-clockwise) transport larvae south (see Lutjeharms 2006), then one might expect to find *P. h. homarus* along the Mozambique coast. This hypothesis is consistent with distribution records of *P. h. homarus* (Berry 1974a, Holthuis 1991, Kulmiye *et al*. 2006). Furthermore, if *P. h. homarus* larvae, in turn, enter the Agulhas Current which flows along the east coast of South Africa, their distribution may extend as far south as the Eastern Cape in South Africa. Given the life expectancy of planktonic larvae and the seasonality of monsoon winds, a low but constant larval supply along the east African coast (Berry 1974b) is to be expected. It may also be reasonable to expect that larvae and adult *P. h. homarus* along South Africa, particularly on the Eastern Cape coast, originated from Mozambique or other East African countries.

Along the coast of southern Mozambique, it is likely that spill-over of larvae (*Panulirus homarus homarus* from the Indo West Pacific and *P. h. rubellus* trapped in inshore ocean gyres north of the origin of the Agulhas Current), may be responsible for the low abundance of subspecies occurring sympatrically. The apparent discrepancy in modes of

larval transport that have been hypothesised may have been pivotal in the geographic and, thus, genetic isolation of these subspecies.

Another example of geographic isolation between genetically distinct taxa able to cross-fertilise is the American and European clawed lobsters, *Homarus americanus* and *H. gammarus* (van der Meeran *et al.* 2008). During the Pleistocene, these conspecifics became geographically isolated when the Isthmus of Panama was formed and they currently remain isolated owing to the North Atlantic Gulf Stream (Williams 1995, van der Meeran *et al.* 2002). This presents a situation where genetic isolation was accomplished geographically between two reproductively compatible species. Similarly, *Panulirus homarus* subspecies are largely geographically isolated (as only a marginal overlap in distribution occurs); however these subspecies are yet to become reproductively isolated (Hedgecock *et al.* 1977, Chow *et al.* 1988).

If larval retention mechanisms that separate subspecies geographically are persistent over time, it is likely that new species may be formed (Pollock 1995b). However, some level of migrant spill-over in regions where *Panulirus homarus* subspecies overlap in distribution may allow them to interbreed. This will prevent their complete genetic isolation, providing the hybrid offspring are reproductively viable. Therefore, only reproductive isolation between these subspecies may give rise to separate species (Pollock 1993). Along the western Australian coast, a similar pattern of geographic isolation followed by reproductive isolation and subsequent speciation gave rise to *P. cygnus* which became genetically isolated from the more ancestral *P. longipes* population (Pollock 1992, 1995b).

Mid-Holocene subspecies expansion of Panulirus homarus homarus and P. h. rubellus

Since there are no published mutation rates for *Panulirus homarus*, an estimated evolutionary rate from congeners (Palero *et al*. 2008, Naro-Maciel *et al*. 2011) was used to approximate the period of subspecies expansion. However, in the present study, estimates provide insights that corroborate theoretical work on the phylogeography of *P. homarus* (George & Main 1967, Berry 1974b, Pollock 1993, George 2005a). It is duly noted that the sample size on which subspecies expansion time estimates have been

based are low. However, given that the subspecies expansion of *P. h. rubellus* in the present study compares well with the population expansion estimate of this subspecies in Chapter 3which was based on a much larger sample size, it may be concluded that the estimated times in the present study are indeed reliable.

In the genus *Panulirus*, groups I and II were hypothesised to have radiated earlier than groups III and IV according to adult, puerulus and larvae morphology (George & Main 1967, McWilliam 1995), biogeography and tectonic plate movements (Pollock 1992, George 1997), as well as molecular data (Chan & Chu 1996, Ptacket *et al*. 2001). In the present study, this pattern was reiterated when *P. argus* (Group I), suggested to have radiated during the Last Glacial Maxima, approximately 26 500-19 000, years ago (Naro-Maciel *et al*. 2011) was compared to *P. homarus homarus* and *P. h. rubellus* (Group IV), suggested to have radiated more recently during the mid-Holocene.

Although *Panulirus homarus homarus* appears to have expanded before that of *P. h. rubellus*, these subspecies are sister taxa and, therefore, an earlier radiation of *P. h. homarus* does not appear to represent an ancestral *P. h. homarus* according to the present study, as previously hypothesised (Pollock 1993). However, this view may be limited as only two of the three subspecies were considered.

Expansion in both subspecies was estimated to have occurred during the mid-Holocene Epoch. The turn of the last glacial maximum brought about extreme climatic changes along southern Africa (Teske *et al.* 2011). However, since the Last Glacial Maxima, the stability and gradual warming in climate may have allowed these tropical/subtropical lobsters to expand their geographic range (Ramsay 1995, Stager & Mayewski 1997, Mayewski *et al*. 2004).

CONCLUSION

Although genetically distinct, suspected hybridization between the *Panulirus* subspecies (Berry 1974a) under study may prevent these conspecifics from elevating to the rank of species, provided their hybrid offspring are reproductively viable (Mayr 1942, Ball & Avise 1992). However, given that sequence divergences between these subspecies were comparable to species, the possibility of species rank cannot be discounted.

Molecular evolution may occur faster than morphological evolution in a number of organisms and this disparity becomes particularly important, for example, in crustaceans when morphological evolution occurs at a very slow rate (Palumbi & Benzie 1991). Therefore, morphological divergence cannot be assumed to be manifested genetically. Unlike morphological phylogeny, molecular data analyses incorporate assumptions based on constant mutation rates over time (Silva & Russo 2000). Species that do not comply with the molecular clock hypothesis or in cases where morphological and molecular evolution rates are disparate, both morphological and molecular data are required to develop a sound classification and identification system.

The present investigation provided preliminary evidence for the genetic differentiation between the morphologically distinct spiny lobsters, P*anulirus homarus homarus* and *P. h. rubellus*. However, DNA barcoding should not be used solely when working with species that may be hybrids (Buhay 2009). If supplemented with nuclear markers and morphological data, this method may provide a powerful tool for species identification and classification (Avise 1994, Sarver *et al.* 1998). Although often criticised, DNA barcoding proved useful in the present study of the classification of *P. homarus* subspecies.

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CHAPTER 3

Genetic structure in the shallow-water spiny lobster *Panulirus homarus rubellus* populations in the SWIO region

ABSTRACT

The scalloped spiny lobster subspecies, *Panulirus homarus rubellus*, is widely distributed in shallow-water rocky habitats of the South West Indian Ocean where it supports various types of fisheries. This project aimed to determine whether stocks (or populations) are shared between countries (*i.e.* regional stocks), or whether they are genetically distinct local stocks, better managed on a national basis. Three regions, Mozambique, KwaZulu-Natal and Eastern Cape, comprising of two localities each were sampled. DNA was isolated from pereiopod tissue and the COI marker amplified from 164 samples which were sequenced. A suite of population genetic analyses revealed significant genetic structure between two putative regions 'MOZ+KZN' and 'EC' (Φ_{PT} = 0.104, p = 0.010). Genetic differentiation of subpopulations was supported by a total of 57 mutations as well as 1.7% nucleotide difference between subpopulations. Furthermore, a shallow level (Φ_{PT} = 0.058, p = 0.031) of female phillopatry was detected and may have contributed to the overall genetic structure observed in the present study. On the SE coast of Africa *P. h. rubellus* is distributed along a stretch of coastline adjacent to different current regimes and, therefore, potentially different larval transport modes (*i.e.* Agulhas Current and in shore counter-currents along the Eastern Cape). This has possibly driven the formation of subpopulations of *P. h. rubellus* across its African range. One of the objectives of this project was to facilitate regional fisheries management strategies of shared stocks. However, even though two distinct subpopulations were recognised in the present study, periodic gene flow across the distribution of *P. h. rubellus* occurs. Although stocks are shared between Mozambique and South Africa they are indeed genetically different from the Eastern Cape. This calls for a national management scheme for the 'EC' stock and a trans-boundary or regional management for the 'MOZ+KZN' stock that also factors in periodic gene flow between subpopulations.

Key words: Panulirus homarus rubellus, Genetic Population Genetic Structure, Shared Stocks

INTRODUCTION

Spiny lobsters have a unique lifestyle, in that they have a long-lived planktonic phyllosoma larval stage compared to other benthic crustaceans (Gurney 1942, Booth & Phillips 1994, Sekiguchi & Inoue 2002, Dixon *et al.* 2003). The phyllosoma larval stage is common in members of the Scyllaridae and Palinuridae, particularly in the genera *Panulirus* and *Linuparus* for the latter (Sekiguchi & Inoue 2002). Phyllosoma larvae are transparent, active feeders that are initially small in size, ranging from 1-2 mm (McWilliam & Phillips 1992, Pitcher 1993, Booth & Phillips 1994). Their small size allows for passive transport by off-shore oceanic currents, fronts, gyres and wind drift (Booth & Phillips 1994, Tolley *et al.* 2005, Briones- Fourzán *et al*. 2008). Thus, spiny lobsters rely heavily on the dispersal capacity and transport of their planktonic larvae for population expansion, settlement in favourable habitats and exploitation of new resources (Phillips & Booth 1994, Thorpe *et al.* 2000, Groeneveld *et al.* 2007). The long-lived larval stage, combined with the influence of major ocean currents and post-settlement on suitable rocky environments, may explain the wide geographical distribution of these lobsters (Booth 1994, Pollock & Melville-Smith 1993, Palero *et al.* 2008, Chan 2010, Groeneveld *et al*. 2012).

Typically, spiny lobsters with a longer planktonic larval duration (PLD) have the potential to settle over vast distances (Pollock 1990, Silberman *et al.* 1994, Briones-Fourzán *et al*. 2008, Butler *et al.* 2011), promoting higher levels of gene flow and, consequently, displaying less structured or panmictic populations compared to those with a shorter PLD (Silberman *et al.* 1994, Palero *et al.* 2008). However, the dispersal capacity of long-lived larvae is largely influenced by dominant ocean currents and is often curtailed by physical, chemical or biological barriers (Knowlton & Keller 1986, Todd *et al.* 1998, Thorpe *et al.* 2000, Gopal *et al.* 2006, von der Heyden *et al.* 2007a).

In some species of spiny lobsters, PLD can persist for up to 24 months (Phillips & McWilliam 1986, Booth 1994, Palero *et al.* 2008). A longer PLD fits with the expectation of less structured populations as seen in populations of *Jasus edwardsii* on the coasts of southern Australia and New Zealand (Ovenden *et al*. 1992, Silberman *et al*. 1994, Palero *et al.* 2008). To the contrary, *Palinurus delagoae* has a much shorter PLD and structured populations were found along the south east coast of Africa (Gopal

et al. 2006). *Panulirus argus* has a similar PLD compared to *P. delagoae*, however populations in the Caribbean and Brazil differed genetically owing to a physio-chemical oceanic barrier characterised by a drop in salinity between the two regions (Diniz *et al.* 2005); this reaffirmed the role of oceanic barriers to gene flow.

Panulirus homarus rubellus, a subspecies of the scalloped spiny lobster *P. homarus*, has a moderately long phyllosoma larval stage of 4-6 months (Berry 1974a). These lobsters are widely distributed in shallow water habitats along the south east coast of Africa and Madagascar (Berry 1974a). Their African distribution ranges as far north as Barra Falsa, Mozambique, and south to Port Elizabeth (PE), South Africa (Berry 1974a). Berry (1971a) described a 'wasp' abundance and distribution of *P. h. rubellus* along the African coast; comprising a lower abundance to the north (Barra Falsa and southern Mozambique), the highest abundance along the KwaZulu-Natal (KZN) coast, South Africa (Heydorn 1969), and again a lower abundance to the south along the Eastern Cape, South Africa. Incidentally, this pattern mirrors changes in sea surface temperature along the African coast, where subtropical temperatures along the KZN coast are ideal for growth and correspond with the area of highest abundance (Berry 1971a, Kemp & Britz 2008, Teske *et al.* 2011a).

The coast off south east Africa is largely influenced by the Agulhas Current; this is divided into two major regions, the northern and southern inshore regions. Each inshore region, however, is governed by two very different circulation patterns, *i.e*. the South Equatorial Current in the northern region and a major contribution to the south from the South-West Indian Ocean Subgyre (Lutjeharms 2006).

The Agulhas Current flows south along KwaZulu-Natal decreasing in intensity toward Port Elizabeth in the Eastern Cape, where it largely retroflects off the Agulhas Bank (Lutjeharms 2006). The flow of the Agulhas Current is reflected in the history and contemporary distribution of *Panulirus homarus rubellus* along the coast of south east Africa; and is also evident in the distribution of migratory marine animals (Heydorn *et al.* 1978). Therefore, it has been suggested that larvae of *P. h. rubellus* are transported by the Agulhas Current throughout its African distribution, and by the East Madagascar Current along this island's coast, both presumably being collectively entrained in the South West Indian Ocean (SWIO) Subgyre (Berry 1974a, Pollock 1993). Furthermore,

it has been hypothesised that larvae eventually return to their home grounds via the respective larval transport currents (Berry 1974a, Booth & Phillips 1994, Lutjeharms 2006). However, to date, no study has attempted to determine the population dynamics of this widely-dispersed lobster or substantiate such conjecture on larval dispersal.

Selectively neutral molecular markers are regions of the genome that are presumably void of natural selection and are, therefore, often favoured in population genetic studies (Silva & Russo 2000, Ridley 2004). As such, the neutral mitochondrial cytochrome *c* oxidase subunit 1 (COI) marker has been a popular choice and was therefore applied in the present study (Silva & Russo 2000, García-Rodríguez *et al.* 2008). The universal COI is a protein coding region of the mitochondrial genome that is currently used in various facets of biology and has been extremely valuable in animal DNA barcoding, phylogenetic and population genetic studies (García-Rodríquez & Perez-Enriquez 2006, Schultz 2009, da Silva *et al*. 2011). This marker has been useful in population genetic studies as it allows for the identification of reproductively-isolated populations that are not influenced by sex-bias dispersal (Chu *et al.* 2001, Silva *et al.* 2010). Furthermore, by using the COI marker a snap shot examination of population genetic structure can be attained (Silva and Russo 2000).

The moderately long-lived phyllosoma larval stage of *Panulirus homarus rubellus* and the influence of the Agulhas Current on larval dispersal make this crustacean a good model to elucidate gene flow in this species in the SWIO region (Pollock 1995a, Groeneveld *et al.* 2012). Moreover, *P. h. rubellus* is a target subspecies in recreational and subsistence lobster fisheries in South Africa and, therefore, there is a greater need to conserve and sustainably management these resources (Kemp & Britz 2008, Steyn *et al*. 2008). However, in the marine realm, populations are not contained by definite boundaries and stocks are often shared among countries (Knowlton 2000, von der Heyden 2007b). *Panulirus homarus rubellus* represents one such species and its distribution traverses the coasts of two countries, Mozambique and South Africa. Furthermore, its distribution covers three bioregions: the tropical Delagoa, the subtropical Natal and the warm temperate Agulhas Bioregions (Griffiths *et al*. 2008, Teske *et al*. 2011a). Each bioregion is characterised by changes in environmental conditions, notably temperature, and it is probable that these changes have driven the

formation of subpopulations along the African distribution range of this subspecies. Population genetic structure in another spiny lobster was found along the south east coast of Africa (Gopal *et al*. 2006) and several other marine organisms have shown genetic disjunction between bioregions (von der Heyden 2009, Teske *et al*. 2011a, b).

Compared to the rich body of research available on the ecology, biology and speciation of *Panulirus homarus rubellus* (Heydorn 1969, Berry 1970, 1971a, b, 1974a, b, Pollock 1992, 1993, Fielding 1996, 1997, George 2005a, b, c, Kemp & Britz 2008, Steyn *et al*. 2008, Kemp *et al*. 2009, Steyn and Schleyer 2011), genetic research on this species is largely lacking. This study is the first genetic assessment of *P. h. rubellus* populations along the south east African coast and the research output will not only facilitate the conservation and sustainable use of this resource but may also prevent their over-exploitation, as seen in many other spiny lobsters, in the future (Chu *et al.* 2001, Babbucci *et al*. 2010). The identification of genetically distinct stocks will help managers to better conserve this subspecies and ensure that diversity in the stocks is sustained (Diniz *et al.* 2005).

The aim of this study was to determine the genetic diversity and population genetic structure within and between populations of *Panulirus homarus rubellus* from six localities along southern Mozambique and the east coast of South Africa using mitochondrial COI sequence data. Furthermore, the phylogeography of this subspecies was inferred in an attempt to explain their contemporary biogeography; particularly in relation to dominant ocean currents and counter-currents. If genetic barriers or oceanic processes that limit gene flow between populations exist along the African distribution range of *P. h. rubellus* then, a structured population will be found. However, barring the aforementioned limiting factors a panmictic population should exist.

MATERIALS AND METHODS

***Study area*:** Six localities were carefully selected throughout the African distribution range of *Panulirus homarus rubellus* (Fig. 2.1). Three regions along east Africa were sampled, southern Mozambique, KwaZulu-Natal–South Africa and the Eastern Cape–South Africa (Fig. 2.1). Each region comprised of two localities each 1) Mozambique ('MOZ'): Chidenguele (CH), $n = 34$ and Xai Xai (XX), $n = 31$ (totalling 65 lobsters), 2)

'KZN': Blood Reef (BR), *n* = 30 and Scottburgh (SB), *n* = 44 (totalling 74 lobsters) and the 3) 'EC': Port St. Johns (PSJ), *n* = 40 and Mdumbi (MB), *n* = 25 (totalling 65 lobsters); with a grand total of 204 samples (Fig. 2.1).

Sample collection and laboratory methods follow those of the previous Chapter.

Individuals were grouped into putative populations based on the geographic locality from which they were collected and regions were delineated based on political and provincial boundaries that roughly coincided with the different bioregions (Table 3.1).

***Data analyses*:** Sequences were tested for substitution saturation using the molecular software program DAMBE (Xia & Lemey 2009). A haplotype list was generated in DnaSP v. 5.10 (Librado & Rozas 2009) where sequence sets were defined per geographic population. Basic molecular indices, including nucleotide and haplotype diversity per population, per region, as well as for the entire data set were calculated in DnaSP v. 5.10. Sequence divergence between populations (percentage and shared mutations between populations) was also calculated in DnaSP v. 5.10 and did not incorporate a model of evolution. Mismatch distribution statistics, theta (effective population size), tau and the goodness of fit tests (SSD, HRI, *p*) for these calculations were calculated Arlequin v. 3.5 (Excoffier & Lischer 2010) for each population, each region as well as for the entire dataset. Neutrality tests such as Tajima's (1989) *D* statistic and Fu's (1997) *Fs* based on 1000 simulated samples, were also calculated in Arlequin v. 3.5 for each geographic population, each region as well as for the entire data set. Tajima's *D* statistic was used to test selective neutrality (Tajima 1989), *i.e*. to test whether the mtDNA COI used in the present study was evolving neutrally in *Panulirus homarus rubellus* populations along the south east coast of Africa, and Fu's *Fs* was used to test mutation drift equilibrium from which recent demographic change (either population expansion or contraction) was inferred. Although these tests were primarily designed to test the selective neutrality of markers, in more recent years they have been used to make inferences about population sizes and expansion (Silva *et al.* 2010).The calculations of time since population expansion follows Chapter 2.

The software program Migrate-n v. 3.0 (Beerli 1998, 2004, 2006, 2008, Beerli & Felsenstein 1999, 2001) was used to determine the magnitude and direction of past

migration (gene flow). An island model of migration was implemented and slice sampling selected for theta (θ), which refers to the effective population size and migration (M). For the former, a uniform prior of 0-0.098 was implemented and, for the latter, an exponential prior of 10-200. Bayesian analysis was run to search for genealogies and a single chain implemented from a 10^7 generations of which the first 25 000 were discarded as burn-in. An adaptive heating strategy was employed with 4 starting chains (10,7,4,1) and a swapping interval of 1.

AMOVA was performed in GenAlEx v. 6.4 (Peakall & Smouse 2006) using 1000 random permutations to calculate the confidence interval. Three AMOVAs were employed, one to test total population (male and female) genetic variation, a second, to test for female philopatry (only female populations) and a third to test for genetic variation in different size cohorts. Putative populations were assigned by regarding each locality as a population *i.e*. all individuals collected from Xai Xai constituted the Xai Xai population. Size classes were determined by considering the size at sexual maturity and the time it takes to reach this size. However, males and females reach sexual maturity at different sizes and, in addition, lobster from different regions reach sexual maturity at different sizes. All factors were considered when dividing samples into size classes. A series of AMOVAs were implemented varying the putative groupings of regions in order to maximise genetic variation between putative regions. In the present, study the data was for haploid–mtDNA and, therefore, an analogue of F_{st}, Φ_{PT} was used (Peakall and Smouse 2006, McMillen-Jackson & Bert 2004a). In addition Φ_{PR} was used to denote the genetic difference between populations within a region (Peakall and Smouse 2006).

Pairwise Φ_{PT} values between populations and a Mantel (1967) test were also calculated in GenAlEx v. 6.4 (Peakall & Smouse 2006). The Mantel test was performed using 10 000 random permutations to determine whether there was a relationship between genetic divergence and geographic distance which can be used to make inferences on isolation by distance. Ocean currents were not factored in and only the distances between localites were used for the calculation. Lastly, TCS v. 1.21 (Templeton *et al.* 1992) was used to create a statistical parsimony haplotype network and was redrawn per

population as well as for the entire data set. Where applicable a significance level of 95% (p <0.05) was used in statistical analyses.

RESULTS

***Sequence characteristics*:** The number of samples of *Panulirus homarus rubellus* that were successfully sequenced was 164. All COI sequences were trimmed to 591 base pairs and used for data analyses; 538 sites were constant sites and 53 constituted variable sites, of which 52 were parsimonious informative sites. In the present study no stops codons were recorded in the reading frame of the sequence set. Stop codons in the reading frame may be indicative of pseudogenes. Substitution saturation, tested under Xia's test in DAMBE, indicated there was little substitution saturation for the COI dataset. Iss was significantly lower than the critical value Iss.c (p = 0.000).

Relative base frequencies for the alignment of the entire dataset were A = 37.06%, T = 16.44%, G = 24.88% and C = 21.62%; substitution rates, A-C = 0.5655, A-G = 14.9107, A-T = 1.4882, C-G = 1.6367, C-T = 6.9316, G-T = 1.0000; a gamma distribution shape parameter of 0.2799 and no indels were recorded.

***Intra-population variation*:** Two regions displayed significant genetic variation, 1) 'MOZ+KZN': 'MOZ' included localities from Mozambique, Chidenguele and Xai Xai, and 'KZN' included Blood Reef and Scottburgh, 2) 'EC': included Port St. Johns and Mdumbi (Table 3.1). Therefore, regional results were based on these two groupings and not on the previous regional delineation based on political boundaries and bioregions. NB: 'EC' throughout the text refers to the collective description of the Mdumbi and Port St. Johns localities and thus the subpopulation, while the Eastern Cape (written in full) refers to the province of South Africa.

A total of 95 haplotypes were recovered from 164 sequences, with a total haplotype diversity of 0.98 ± 0.003. All unique haplotype sequences will be deposited in GenBank. Haplotype (h) and nucleotide diversity (π) per region as well as per population were high, ranging between 0.97-0.99 and 0.0079-0.0208 respectively (Table 3.1). Haplotype 15 was the most frequent haplotype common in 11 individuals from all but one locality (Mdumbi) sampled. Nevertheless, 74% of haplotypes were unique.

Table 3.1: Intra-population indices for *Panulirus homarus rubellus* from the south east African coast, per population, per region as well as for the entire data set calculated from partial COI sequences

Locality	Co-ordinates	*N*	*S*	*M*	*K*	π (± SD)	*H*	*h* (± SD)
Chidenguele	X: 34.21280 Y: 24.95556	30	27	30	4.664	0.0079 (± 0.000)	24	0.9839 (± 0.013)
Xai Xai	X: 33.73417 Y: 25.11889	25	51	56	12.307	0.0208 (± 0.005)	22	0.9867 (± 0.017)
Blood Reef	X: 31.05833 Y: 29.88278	25	28	34	5.773	0.0098 (± 0.002)	23	0.9933 (± 0.013)
Scottburgh	X: 30.76056 Y: 30.28917	42	33	38	5.914	0.0100 (± 0.000)	32	0.9826 (± 0.010)
Port St. Johns	X: 29.54917 Y: 31.63889	20	50	55	11.895	0.0201 (± 0.005)	19	0.9947 (± 0.018)
Mdumbi	X: 29.21887 Y: 31.93042	22	29	33	10.545	0.0178 (± 0.001)	17	0.9740 (± 0.022)
MOZ+KZN		122	52	61	6.998	0.0118 (± 0.001)	74	0.9840 (± 0.004)
EC		42	52	58	11.300	0.0191 (± 0.002)	34	0.9870 (± 0.009)
Total		164	53	62	8.400	0.0142 (± 0.001)	95	0.9849 (± 0.003)

* Where *N* = sample size, *S* = number of polymorphic sites, *M* = total number of mutations, *K* = nucleotide differences, π = nucleotide diversity, *H* = number of haplotype, *h* = haplotype diversity, and SD = standard deviation. Note X and Y denote latitude and longitude, respectively

***Neutrality tests and mismatch distribution*:** Tajima's *D* and Fu's *Fs* are amongst the most powerful neutrality statistics used in population genetics studies, the latter being tested under the infinite sites model (Naro-Maciel *et al.* 2011). Tajima's *D* statistic was not significant ($p > 0.05$) for each population, each region as well as overall for the entire data set, indicating that the COI marker used in the present study conformed to selective neutrality (Table 3.2). Values were negative for each population, except Mdumbi, each region as well as overall for the entire data set; negative Tajima's *D* statistic are indicative of an initial large population size and in the case of population expansion, a large expansion event (McMillen-Jackson & Bert 2004a). Fu's *Fs* were significant ($p < 0.05$) and values were negative for the overall data set, each region as well as for each population, expect Mdumbi, indicating a departure from mutation-drift equilibrium. Thus, supporting the hypothesis of recent demographic change, most likely population expansion (Table 3.2). Furthermore, this indicated these populations were not in equilibrium and therefore may not be stable (Fu 1997, von der Heyden *et al.* 2007a). Fu's *Fs* was not significant ($p > 0.05$) for the Mdumbi population, indicating mutation-drift equilibrium, suggesting the Mdumbi population size has remained constant over time.

Mismatch distribution (MMD) analyses for each population, each region as well as the entire data set are represented as graphs (Fig. 3.1, 3.2) and the associated statistics are presented in Tables 3.3 and 3.4. A unimodal pattern of pairwise differences indicates

population expansion while a multimodal pattern reflects a stable population (Rogers & Harpending 1992). Therefore, the entire dataset, the 'MOZ+KZN' region and each population except Mdumbi represented expanding populations, this being evident from the overall unimodal pattern of the frequency distribution of mutations. In some populations, e.g. Chidenguele and the entire dataset (Fig. 3.1a, 3.2a), minor peaks accompanied the predominantly unimodal distribution of mutations and possibly represent different lineages (Fernández *et al*. 2012). On the other hand, Mdumbi and the 'EC' region displayed multimodal MMDs which were indicative of stable populations.

Harpendings Raggedness Index (HRI) was tested for two models, 1) a sudden range expansion (demographic change) and 2) spatial expansion (Table 3.3, 3.4). HRI was not significant for individual populations, both regions as well as for the entire dataset, supporting the hypothesis of population expansion. These results concur with the MMD graphs and Fu's *Fs* statistic.

Time elapsed since population expansion was calculated using an upper and lower boundary. For the lower boundary population expansion was estimated to commence in the late Holocene Epoch (Table 3.3); however, this seems unrealistic. For the upper boundary, population expansion was estimated to range from the mid to recent Holocene Epoch. Populations such as Chidenguele and Scottburgh far exceeded the population expansion of the 'MOZ+KZN' region as a whole; while, the Port St. Johns population was estimated to have had the most recent population expansion.

Table 3.2: Neutrality tests, Tajima's *D* statistic & Fu's *Fs* scores for a fragment of the COI gene for *Panulirus homarus rubellus* along the south east African coast with corresponding *p* values for each population, each region and the entire data set

Population	Tajima's *D* test	*p*	Fu's *Fs* Test	*p*
Chidenguele	-1.131	0.087	-18.521	0.000
Xai Xai	-0.342	0.353	-8.055	0.008
Blood Reef	-0.824	0.174	-18.454	0.000
Scottburgh	-0.788	0.206	-23.724	0.000
Port St. Johns	-0.628	0.244	-8.130	0.002
Mdumbi	1.247	0.816	-3.792	0.090
MOZ+KZN	-0.863	0.187	-24.865	0.000
EC	-0.230	0.475	-16.653	0.000
Total	-0.306	0.390	-24.485	0.000

Table 3.3: Mismatch distribution analysis tested for a sudden expansion model (demographic change), their associated goodness of fit tests and corresponding *p* values tested on a fragment of the COI gene of *Panulirus homarus rubellus* along the south east African coast

Population	θ_0	θ_1	τ	SSD	*p*	HRI	*p*	$T_{2.7\text{-}3.3\,\%}$	SD	$T_{9\text{-}11\,\%}$	SD
CH	0.005	295.312	4.996	0.0027	0.470	0.0166	0.610	4744	671	1423	201
XX	0.000	99999.000	2.750	0.0743	0.010	0.0181	0.930	2611	369	783	111
BR	0.003	99999.000	3.873	0.0064	0.410	0.0242	0.670	3678	520	1103	156
SB	0.960	103.203	4.895	0.0012	0.690	0.0108	0.690	4648	657	1394	197
PSJ	7.057	99999.000	2.211	0.0120	0.630	0.0134	0.680	2099	296	630	89
MB	13.424	81.169	1.715	0.0136	0.440	0.0156	0.700	-	-	-	-
MOZ+KZN	0.963	65.938	4.104	0.0025	0.200	0.0106	0.720	3897	551	1169	165
EC	0.000	99999.000	1.766	0.1749	0.000	0.0083	1.000	-	-	-	-
Total	3.213	1082.500	2.869	0.0057	0.340	0.0078	0.690	2724	385	817	116

* Where θ_0 = theta (effective population size) initial, θ_1 = Theta (effective population size) final, τ = Tau, SSD = sum of squares deviation and HRI = harpendings raggedness index, T = time since population expansion, range of mutation (%) ranges are in subscript and SD = standard deviation, CH = Chidenguele, XX= Xai Xai, BR = Blood Reef, SB = Scottburgh, PSJ = Port St. Johns, MB = Mdumbi

Table 3.4: Mismatch distribution analysis tested for a spatial expansion model assuming a constant deme size, their associated goodness of fit tests and corresponding *p* values tested on a fragment of the COI gene of *Panulirus homarus rubellus* along the south east African coast

Population	θ	τ	SSD	*p*	HRI	*P*
Chidenguele	0.004	4.997	0.0027	0.490	0.0166	0.680
Xai Xai	2.399	2.742	0.0233	0.050	0.0181	0.640
Blood Reef	0.006	3.870	0.0064	0.290	0.0242	0.610
Scottburgh	1.131	4.681	0.0011	0.850	0.0108	0.790
Port St. Johns	7.059	2.210	0.0120	0.590	0.0134	0.700
Mdumbi	13.523	1.470	0.0136	0.460	0.0156	0.740
MOZ+KZN	1.179	3.842	0.0025	0.330	0.0106	0.770
EC	10.347	1.766	0.0097	0. 620	0.0083	0.580
Total	3.120	2.908	0.0057	0.300	0.0078	0.750

* Where θ = theta (effective population size) initial, τ = tau, SSD = sum of squares deviation and HRI = harpendings raggedness index

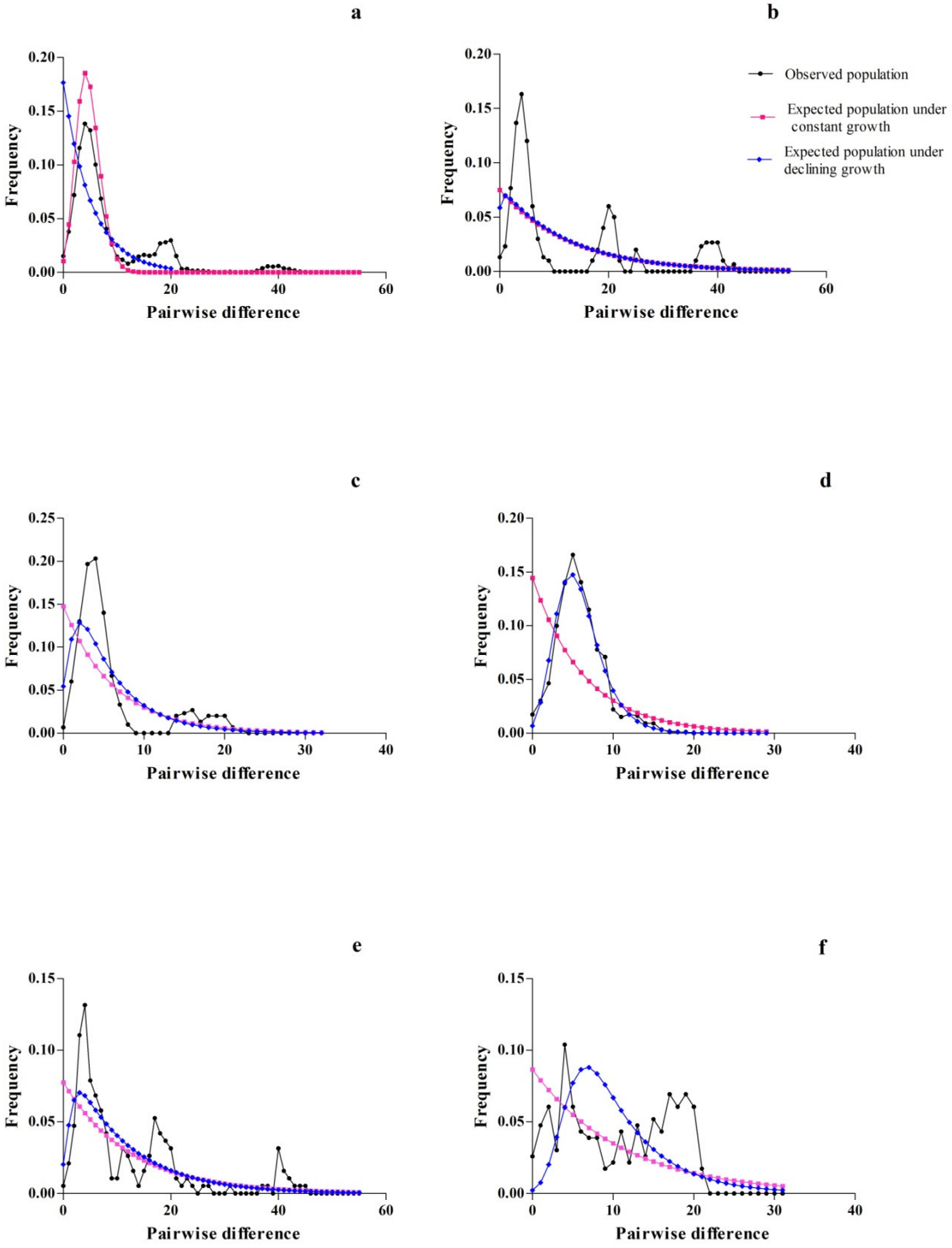

Figure 3.1: The average pairwise differences against its relative frequency (mismatch distribution plots) tested for a constant population and a declining population model for *Panulirus homarus rubellus* along the south east African coast. The different colour lines indicate, black: observed population, pink: under constant growth and blue: under a population decline. a: Chidenguele, b: Xai Xai, c: Blood Reef, d: Scottburgh, e: Port St. Johns and f: Mdumbi.

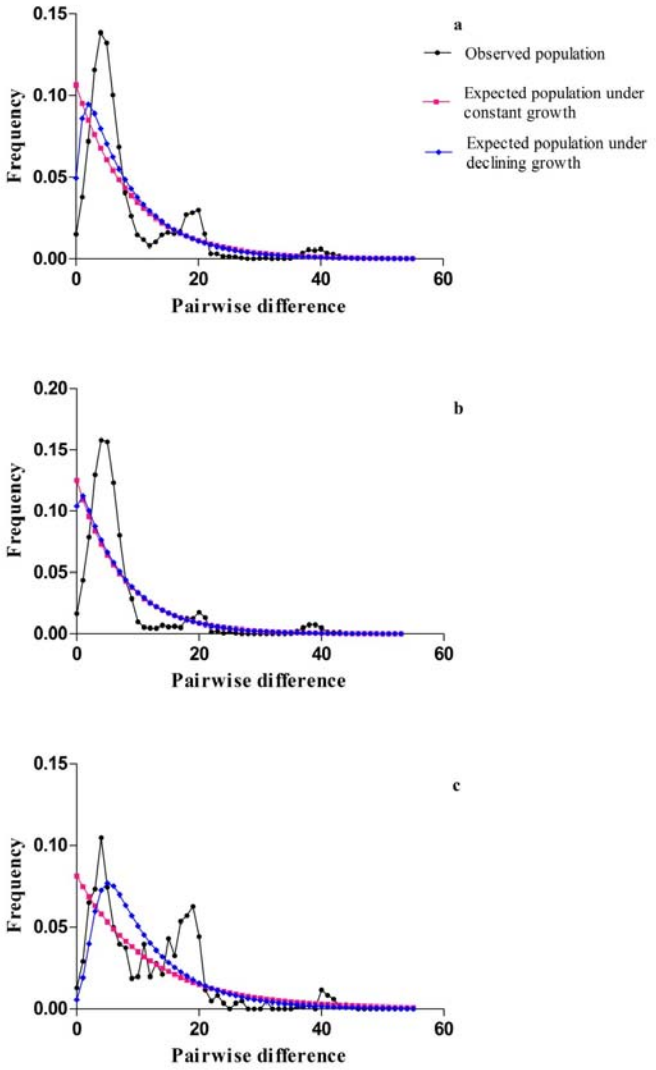

Figure 3.2: Mismatch distribution plots for the a) entire data set (all 6 populations combined) as well per region, b) 'MOZ+KZN' and c) 'EC' for populations of *Panulirus homarus rubellus* along the south east African coast.

***Inter-population variation*:** Overall gene flow mirrored the direction and flow intensity of the Agulhas Current with some areas of bi directional gene flow (Fig. 3.3A, B). With regard to Figure 2.6A stepwise migration at 'MOZ' occurred at an equal rate in both directions, while in 'KZN' there seemed to be a general trend toward Blood Reef from Scottburgh and Mdumbi appeared to proceed toward the coast of Port St. Johns. The mean rate of migration from Port St. Johns to Mdumbi was out the range of the confidence limit therefore it can be concluded that migration from Port St. Johns to Mdumbi was indeed non-significant.

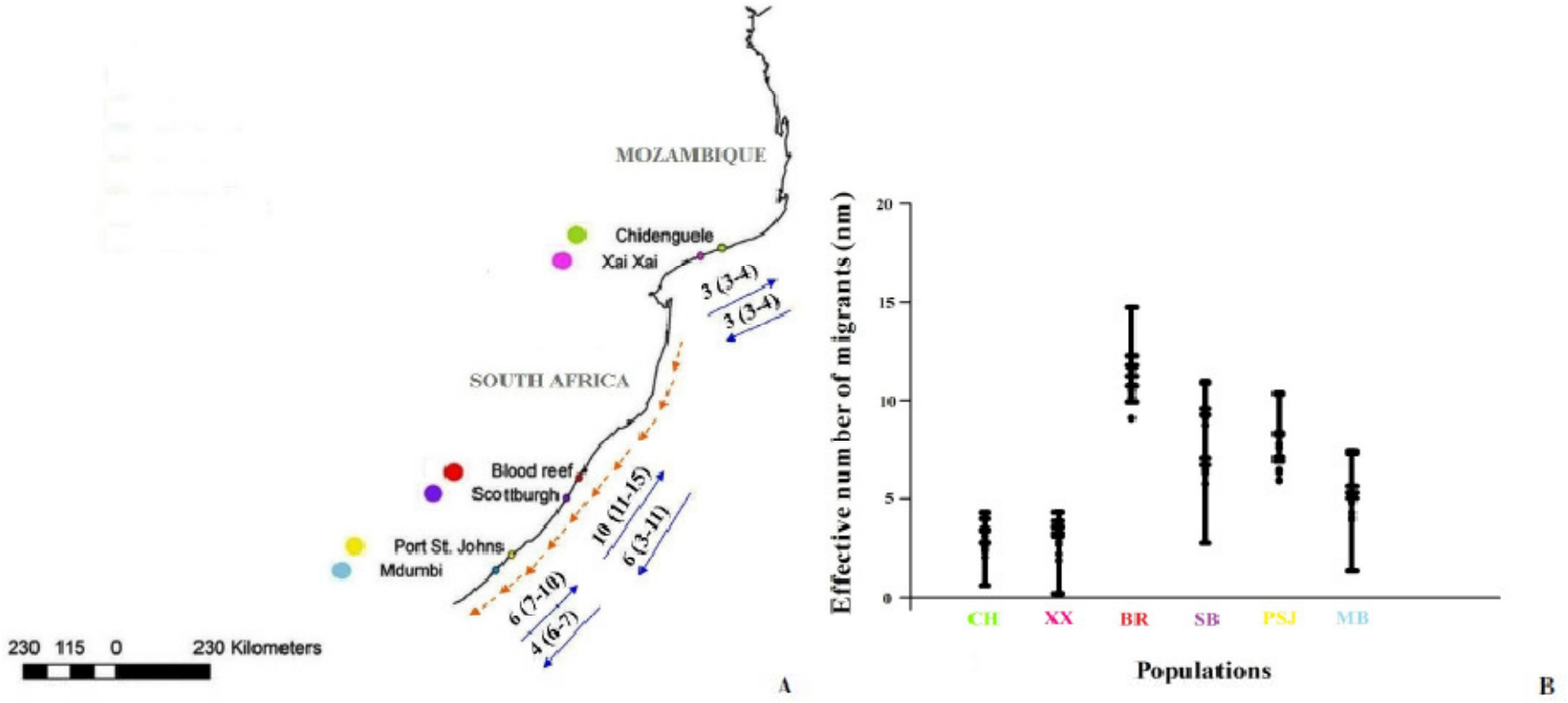

Figure 3.3: A) The direction of gene flow inferred from the relative number of immigrants (*nm*) per generation between geographic populations of *Panulirus homarus rubellus* along the south east coast of Africa, values in brackets represent the 95% confidence interval. B) Gene flow was highest along KZN (BR + SB) and decreased along the EC (PSJ + MB). The lowest number of immigrants was received at localities in MOZ. The symbols CH = Chidenguele, XX = Xai Xai, BR = Blood Reef, SB = Scottburgh, PSJ = Port St. Johns and MB = Mdumbi.

The highest number of immigrants per generation were found along the KwaZulu-Natal coast and decreased along the 'EC' (Fig. 3.3B). Blood Reef received the highest number of immigrants per generation with a much lower variance than Scottburgh, while Mdumbi received the lowest number of immigrants per generation when compared to 'KZN' and 'EC' regions. Localities in Mozambique received considerably lower number of immigrants per generation overall (Fig. 3.3B). Thus, the overall direction and intensity of gene flow was similar to the Agulhas current as gene flow was high around KZN and decreased toward the Eastern Cape.

***Total genetic variation between populations*:** AMOVAs were conducted to test genetic partitioning between populations and regions. The regional grouping that produced the highest percentage of genetic variance is presented in Fig. 3.4. AMOVA revealed an overall significant ($p = 0.010$) and high level of differentiation ($\Phi_{PT} = 0.104$) between regions and no significant ($p = 0.080$) or discernable genetic differentiation between populations within regions ($\Phi_{PR} = 0.021$). Most (90%) genetic variation occurred within populations, 8% of the genetic variance was explained by differences in two regional groupings *i.e.* 'MOZ+KZN' = Region 1 and 'EC' = Region 2 and a further 2% existed between putative geographic populations within regions (Fig. 3.4).

***Female Philopatry*:** Significant genetic structure (($\Phi_{PT} = 0.058$, $p = 0.031$) was inferred from an AMOVA comparing only female lobsters. There was a 6% genetic variation between populations but no evidence for regional structure (Fig. 3.5). Most (94%) genetic variation was found within populations.

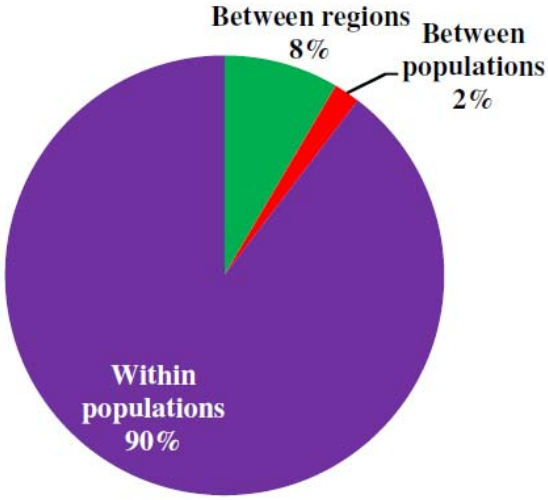

Figure 3.4: AMOVA represented as a pie graph showing the genetic variation of the entire dataset of individuals of *Panulirus homarus rubellus* along the south east African coast. The highest level of genetic partitioning existed when 'MOZ+KZN' and 'EC' were delineated as two regions.

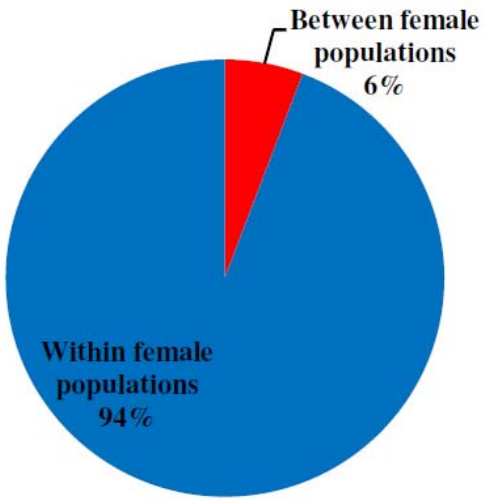

Figure 3.5: AMOVA represented as a pie graph showing the genetic variation of the entire dataset of female *Panulirus homarus rubellus* along the south east African coast. Female populations depict a shallow, significant level of female philopatry.

For the total population (male and female), pairwise ϕ_{pt} was calculated between population pairs and values ranged from 001-0.426 (Table 3.5). The Xai Xai population was not genetically differentiated from the Chidenguele population ($p = 0.056$) but was significantly different from all other populations. While the Port St. Johns population and the Mdumbi population were significantly different from each other ($p = 0.020$), they were not significantly different from Chidenguele (PSJ– $p = 0.076$ & MB–0.234) and Scottburgh (PSJ– $p = 0.053$ & MB–0.202). The highest level of genetic structure was found between Chidenguele and Scottburgh ($\phi_{pt} = 0.403$), followed by a marginally

lower genetic structure between Xai Xai and Port St. Johns (Φ_{pt} = 0.370). The Blood Reef and the Xai Xai populations were both genetically differentiated from all other populations (Table 3.5, above diagonal).

Female pairwise Φ_{PT} comparisons revealed significant genetic structure between 'MOZ' (Chidenguele and Xai Xai) and Blood Reef (Φ_{PT} = 0.074 & 0.061), 'MOZ' and Port St. Johns (Φ_{PT} = 0.314 & 0.280) and between Chidenguele and Mdumbi (Φ_{PT} = 0.078), suggesting 'MOZ' was indeed genetically differentiated from 'KZN' (Blood Reef and Scottburgh) and the 'EC' (Port. St. Johns and Mdumbi) regions (Table 3.5, below diagonal).

The genetic variation between cohort sizes revealed a high level of genetic variation 10% between cohorts (Φ_{PT} = 0.104) but the results were not significant p = 0.060 (pie graph not shown).

Table 3.5: Pairwise Φ_{PT} population comparison for *Panulirus homarus rubellus* along the south east African coast, total population (male and female) presented above the diagonal and only female populations presented below the diagonal with significant values in boldface. Pairwise comparisons were based on 1000 permutations

Population	Chidenguele	Xai Xai	Blood Reef	Scottburgh	Port St. Johns	Mdumbi
Chidenguele	-	0.014	**0.170**	**0.403**	0.003	0.001
Xai Xai	0.025	-	**0.282**	**0.022**	**0.370**	**0.063**
Blood Reef	**0.074**	**0.061**	-	**0.128**	**0.212**	0.010
Scottburgh	0.048	0.036	0.000	-	0.018	0.001
Port St. Johns	**0.314**	**0.280**	0.062	0.064	-	**0.188**
Mdumbi	**0.078**	0.025	0.002	0.000	0.080	-

DNA sequence divergence revealed marked differences between bioregions and a particularly marked difference between designated regions in the present study, *i.e.* 'MOZ+KZN' and 'EC' which differed by 57 mutations between 'MOZ+KZN' and 'EC' and 1.7% DNA divergence (Table 3.6). This corroborates the genetic divergence between regions. Sequence divergence was highest (2%) between specimens from Xai Xai and both localities in the EC region (Table 3.6). On the other hand, Chidenguele yielded the lowest level of differentiation from both localities in the 'KZN' (Blood Reef and Scottburgh). Interestingly, many neighbouring populations had high levels of sequence divergence (Table 3.6).

Table 3.6: DNA sequence divergence indicated by the number of shared mutations (above) and the average nucleotide substitution per site (%) between populations (below) for the total population (males and females) of *Panulirus homarus rubellus* along the south east African coast

	Chidenguele	Xai Xai	Blood Reef	Scottburgh	Port St. Johns	Mdumbi
Chidenguele	-	27	19	22	24	21
Xai Xai	1.5%	-	30	36	51	30
Blood Reef	0.1%	1.5%	-	29	31	29
Scottburgh	0.1%	1.6%	1.0%	-	36	29
Port St. Johns	1.5%	2.0%	1.5%	1.6%	-	30
Mdumbi	1.6%	2.0%	1.6%	1.7%	1.9%	-

A mantel test yielded isolation by distance between populations in the study area (R^2 = 0.024, p = 0.010). The haplotype network for the entire sample set, *i.e.* all populations combined contained many (74%) unique haplotypes and revealed a star-burst pattern (Appendix 1). Haplotype networks for individual populations also had many unique haplotypes (Appendix 1). Some haplotypes were shared among regions *i.e.* 'MOZ+KZN' and 'EC', as well as between individual populations.

DISCUSSION

This study was novel in two ways, first in describing the population genetic structure of *Panulirus homarus rubellus* along the African coast; secondly, the marker used in this study has not been previously tested on *P. h. rubellus* and its successful application demonstrated the utility of the mtDNA (COI) marker using primers from Chow *et al*. (2006) for this subspecies.

Genetically distinct regions

Significant genetic structure was found at the mtDNA level for the shallow-water spiny lobster *Panulirus homarus rubellus* populations collected along the south east coast of Africa. MtDNA markers have been useful in elucidating population genetic structure for other spiny lobsters, *P. argus* (Silberman *et al.* 1994, Sarver *et al.* 1998, 2000), *Palinurus gilchristi* (Tolley *et al.* 2005), *P. delagoae* (Gopal *et al.* 2006) and *Panulurus elephas* (Palero *et al.* 2008, 2011, Babucci *et al.* 2010). The COI region, for example, is valuable as it allows one to ascertain past events that may have shaped contemporary populations. It also aids in the identification of stocks, gene flow among stocks and their respective boundaries (Diniz *et al*. 2005, García-Merchán *et al.* 2011, Fernández *et al.* 2012); all aspects that are of concern in fisheries.

AMOVA recovered high levels of genetic structure between two putative regions, 1) 'MOZ+KZN' and 2) 'EC', supported by high levels of genetic divergence. The level of genetic structure found in the present study is relatively high when compared to other examples of genetic structure in marine environments (Ward 200). The high level of genetic structure in the present study suggests that a break in genetic connectivity between putative regions may exist which often occurs as a consequence of chemical, physical or biological barriers (Pollock 1995b, Bohonak 1999). The formation of subclades from the haplotype network in the previous chapter is consistent with a break in genetic connectivity between populations of *Panulirus homarus rubellus* along the south east African coast. However, since both regions are not completely genetically isolated, it is reasonable to assume the genetic barrier may be periodic (e.g. periodic upwelling events, river outflow, inshore counter-currents and eddies) or one that is currently forming.

Genetic breaks in connectivity along the south east African coast

Similar breaks in genetic connectivity have been found in other marine organisms distributed along the east coast of South Africa (von der Heyden 2009). These genetic breaks, however, vary in locality and usually differ between different species (Atkinson & Clark 2005, Teske *et al.* 2006). Thus, breaks in genetic connectivity cannot simply be defined at a particular point but rather by a genetic break 'zone' that may account for inter-species variation (Teske *et al.* 2006). The break in genetic connectivity in the present study mirrors a prominent break zone that separates the subtropical and warm temperate bioregions along the south east coast of South Africa (Teske *et al.* 2009, von der Heyden 2009, also see Fig. 2.1). Genetic divergence was recently found along this genetic break zone for an ascidian species similarly distributed along the south east South African coast (Teske *et al.* 2011b).

Another prominent genetic break zone along the east coast of South Africa occurs between the tropical and subtropical bioregions effectively differentiating the Indo-Pacific species and subtropical biota (Atkinson & Clark 2005, Griffiths *et al*. 2008, Teske *et al.* 2009, von der Heyden 2009). Genetic disjunction along this genetic break was found in the deep-water lobster, *Palinurus delagoae*, among populations in Mozambique and KwaZulu-Natal. In the present study, however, sample localities

(Chidenguele and Xai Xai) in Mozambique were much further south compared to the area studied by Gopal *et al.* (2006) and this perhaps explains why a similar defined genetic break between tropical and subtropical populations in the present study was not found. Although genetic breaks are prevalent in a number of species distributed across subtropical and tropical bioregions in south east Africa (Teske *et al.* 2007, Teske *et al.* 2009, Ridgway *et al.* 2008, von der Heyden 2009), crustaceans with an extensive larval dispersal capacity often do not conform to genetic breaks, largely owing to larval mixing in the ocean (Pollock 1995b, Thorpe *et al*. 2000, Teske *et al.* 2009, da Silva *et al*. 2011). Therefore, the geographic position of the Mozambique region (Chidenguele and Xai Xai), as well as the larval dispersal capacity of *Panulirus homarus rubellus* (Berry 1974a), may account for the lack of genetic differentiation between tropical and subtropical populations in the present study.

Studies conducted on mud prawns, sand prawns and limpets also found genetic disjunction between subtropical and tropical lineages, as well as phenotypic plasticity in the respective lineages (Ridgway *et al.* 1998, Teske *et al.* 2006, 2008, 2009). This striking relationship therefore suggests temperature differences in each bioregion as a key driver behind speciation (Foll & Gaggiotti 2006, Teske *et al.* 2009, Galindo *et al*. 2010). In the present study, genetic data supporting a disjunction between KwaZulu-Natal and Eastern Cape *Panulirus homarus rubellus* stocks further corroborate differences in selected phenotypic aspects such as growth rates and size at sexual maturity (Heydorn 1969, Berry 1971b, Pollock 1995b).

Possible genetic barriers

Planktonic phyllosoma larvae of *Panulirus homarus rubellus* are passively transport by oceanic currents (Booth & Phillips 1994, George 2005c, Faubry & Barber 2012). Gene flow is, therefore, largely associated with larval dispersal in *P. h. rubellus*, as in other sedentary biota with a pelagic larval phase (Thorpe *et al.* 2000, Groeneveld *et al.* 2007, Faubry & Barber 2012). As such, genetic barriers in the present study are most likely associated with larval dispersal. Genetic barriers acting on larval dispersal may be manifested in various forms; common genetic barriers include vicariate barriers, ocean currents, inshore counter-currents, larval retention, larval behaviour, larval duration or a combination of these factors (Pollock 1993, 1995b).

A hypothesis on different modes of larval dispersal leading to genetic structure

Larval retention is a mechanism whereby larvae become entrained in oceanic gyres and counter-currents (Pollock 1995b, Yeung & Lee 2002). It has been suggested that larvae of *Panulirus homarus rubellus* larvae are conditioned for a moderately long oceanic life of 4-6 months in the Agulhas Current that feeds into the larger SWIO Subgyre in which larvae are entrained, thereafter returning to home grounds via the Agulhas Current (Berry 1971a, Pollock & Melville-Smith 1993, Pollock 1995b, Lutjeharms 2006). In the context of the present study, however, we suggest all larvae may not enter the greater Agulhas-SWIO Subgyre system and may become prematurely entrained in eddies and inshore counter-currents until competent to settle (Pollock & Melville-Smith 1993, Yeung & Lee 2002, Gopal *et al.* 2006, von der Heyden 2009). Inshore counter-currents are widespread along the south east coast of South Africa, notably along the Eastern Cape (Luteharms 2006, von der Heyden 2009). Therefore, it is likely that most individuals from the 'EC' subpopulation may be entrained in inshore counter-currents and eddies while most individuals from the 'MOZ+KZN' subpopulation may be entrained in the Agulhas-SWIO Subgyre.

If the hypothesis described applies where subsets of larvae originating from the 'EC' do not enter the Agulhas-SWIO Subgyre system and are indeed retained in inshore counter-currents and eddies, then larval mixing between the 'EC' and 'MOZ+KZN' regions will be limited. Furthermore, if a subset of 'EC' larvae have become consistently entrained in inshore counter-currents or eddies, over time, these lobsters may have faced a different set of environmental selective pressures (Pollock 1995b, Thorpe *et al.* 2000, Silva & Russo 2000). However, the effect of selective pressures may be mitigated if sufficient gene flow occurs throughout the range of *P. h. rubellus*. Moreover, the duration and occurrence of inshore counter-currents and eddies along the South African coast is periodic (Lutjeharms 2006) and, therefore, the earlier hypothesis of a periodic genetic barrier holds true if inshore counter-currents and eddies indeed impede gene flow. Therefore, different modes of larval transport, the Agulhas Current for the 'MOZ+KZN' subpopulation and inshore counter-currents for the 'EC' subpopulation may explain some level of genetic isolation and genetic structure between the 'EC' and 'MOZ+KZN' subpopulations in the present study. However,

sufficient gene flow and migration between subpopulations may mitigate complete genetic isolation and may even prevent speciation of the 'EC' and 'MOZ+KZN' subpopulations. A similar pattern of larval retention in eddies and inshore counter-currents explained shallow, significant genetic structure in *Palinurus delagoae* populations along the south east coast of Africa (Gopal *et al.* 2006). Larval retention inshore of the Agulhas Current was also noted in *P. gilchristi* (Groeneveld & Branch 2002, Tolley *et al.* 2005, Gopal *et al.* 2006).

Larval behaviour, in particular 'homing' in on oceanic features, may strongly influence the genetic structure of larval dispersers with a long PLD (Chittleborough 1974, Phillips & McWilliam 1986, Booth & Phillips 1994, Cowen *et al.* 2006, Briones- Fourzán *et al.* 2008). As suggested earlier, if the 'EC' subpopulation utilises a different larval transport mode and faces a different set of environmental selective pressures, then larval 'homing' between differentiated populations may differ. Larval 'homing' may, therefore, explain how larvae from differentiated populations are able to recognise specific environmental cues in different bioregions to which they swim. Therefore, even if larvae from the 'EC' subpopulation enter the Agulhas-SWIO Subgyre system, larval 'homing' may ensure their return to the 'EC' region and *vice versa*. However, in the present study, the 'MOZ+KZN' and 'EC' region were not strongly defined, and this suggests that while larval homing may contribute to genetic differentiation between regions, it is certainly not the only factor contributing to genetic differention between these regions. Evidence for larval 'homing' behaviour is clear when larvae of different species are entrained in a single oceanic system and each species recognises individual site specific home grounds, such as different species of *Panulirus* entrained in the North Pacific Gyre (Chow *et al.* 2012).

Genetic structure in cohorts and female philopatry

Based on pairwise Φ_{PT} values, the Blood Reef population was highly differentiated from all other localities and was suspected to have contributed to the observed regional differentiation. However, when removed from AMOVA analysis, the same regional differentiation was recovered ('MOZ+KZN' and 'EC') with similar genetic distance indices. Therefore, it can be concluded that the Blood Reef population did not bias the AMOVA results. Female lobsters have been found to migrate offshore during spawning

periods to take advantage of dominant oceanic currents to ensure the effective offshore transport of larvae (Booth & Phillips 1994, Tolley *et al.* 2005). The highly differentiated Blood Reef population consisted of 80% females and may represent a breeding cohort as this locality was the only population situated offshore (*ca.* 1-2 km offshore, 5-10 m deep).

An attempt to recover genetic structure among size cohorts revealed some level of genetic structure but the results were not significant. Lobster growth and, thus, size at sexual maturity varies widely between sexes and between different regions (De Bruin 1962, George 1963, Heydorn 1969, Berry 1971b, Jayakody 1989). Therefore, cohorts were divided based on the following factors: sex, region and size at sexual maturity. However, due to the aforementioned division, each cohort was represented by a small sample size and this may have contributed to the lack of statistical significance. Alternatively, a spurious relationship of genetic structure may have been inferred. Genetic structure between breeding cohorts has been shown in other marine organisms (von der Heyden *et al.* 2007b). However, given the variability associated with growth in *Panulirus homarus* (Berry 1971b, Kulmiye & Mavuti 2005, Kulmiye *et al*. 2006) and, thus, estimation of cohorts, the results are exploratory at best.

While no significant structure could be resolved between size classes; significant shallow structure was shown among female populations from different localities. Compared to the median level of genetic structure observed in the marine environment (F_{st} = 0.022, see Ward 2000), a relatively high level of genetic structure (ϕ_{PT} = 0.058) was recovered. On the other hand, males exhibited no genetic structure with regard to locality (data not shown). This suggests females may display a level of female philopatry and, therefore, it may be inferred that female migration is limited, while males may migrate across the geographic range of *Panulirus homarus rubellus*. Female natal homing behaviour likely allows for these female lobsters to return to home grounds where they remain resident (Phillips & McWilliam 1986, Booth & Phillips 1994, Cowen *et al.* 2006). Female philopatry may, therefore, partly account for some level of genetic differentiation observed in the present study. However, it is duly noted, that the sample size for female philopatry analysis was lower than for the total population comparisons and results are therefore discussed tentatively.

Gene flow between regions

Significantly high pairwise Φ_{PT} values encountered between regions, *i.e.* between Mozambique (Chidenguele and Xai Xai) 'KZN' (Blood Reef and Scottburgh) and the 'EC' (Port St. Johns and Mdumbi), suggest that there is some level of genetic structure between all three regions. These results are further supported by genetic divergence indices. However, no genetic structure was detected with AMOVA at the level of these three regions probably due to stochastic gene flow between some localities from different regions. Gene flow between these localities may explain the lack of genetic differentiation, based on pairwise Φ_{PT} between 'EC' and Chidenguele as well as between 'EC' and Scottburgh. In the context of the present study coastal current regimes may serve as a genetic link between these distant localities allowing for gene flow. On the other hand, many neighbouring populations were genetically distinct from one another and are likely the consequence of female philopatry. Furthermore, it has been suggested that populations separated by short geographic distances (neighbouring populations) showing high levels of genetic differentiation are typical of populations that have undergone recent population expansion (Palumbi 2003). In the present study, several populations have undergone a recent population expansion and this may also explain the genetic differentiation of some neighbouring populations. However, a finer scale local population study may serve to clarify the differentiation of neighbouring populations and the lack of differentiation between more distant ones.

An edge-effect in genetics refers to peripheral populations at the edge of a species' distribution range that are genetically distinct (Cook 1961). In population genetics an edge-effect may occur when a species range expansion is fairly recent and defined genetic boundaries have not yet been established (Teske *et al.* 2011b). It is duly noted that tests performed in the present study do not distinguish between population size and range (spatial) expansion. However, assuming that range expansion has occurred, the concept of an edge-effect applies well to a recently-derived species such as *P. homarus* (George & Main 1967). Thus, the level of genetic structure as well as the incomplete genetic isolation of both regions in the present study is likely to be a function of both an edge-effect and some level of contemporary larval exchange.

Shared haplotypes between localities support the idea of regular gene flow between populations (Appendix 2). However, haplotype-sharing did not reveal any particular pattern and many haplotypes were shared across the distribution range of *Panulirus homarus rubellus*. Nevertheless, a high level of unique haplotypes suggests that gene flow between populations may be moderate to low, at best.

Cryptic divergence

Both the Port St. Johns and Mdumbi populations individually, as well as both localities combined ('EC' population) were found to have high levels of nucleotide diversity (*ca*. 2%). In the present study sampling did not extend into the Agulhas Bioregion and, therefore, populations from the 'EC' occur in the transition zone between the Natal (subtropical) and Agulhas (warm temperate) Bioregions (see Fig. 2.1). If genetic differences have manifested in morphologically indistinguishable populations (cryptic lineages) in different bioregions, then the 'EC' population at this transition zone may represent a contact zone of subpopulations and may account for the high level of genetic diversity in 'EC' localities (Knowlton 1993, 2000). A similar pattern of high genetic diversity was also noted in the northern peripheral population (Chidenguele).

The northern distribution of *Panulirus homarus rubellus* is characterised by an overlap in distribution by another *P. homarus* subspecies, *P. h. homarus*, where both subspecies occur sympatrically in southern Mozambique. *Panulirus homarus rubellus* and *P. h. homarus* have been known to cross-fertilise and may do so in areas of over lapping distribution (Berry 1974b, Pollock 1993, George 2005b). In the present study, the Xai Xai population in southern Mozambique was characterised by a high level of nucleotide diversity (*ca*. 2%). Although hybridization has not been confirmed between *P. h. rubellus* and *P. h. homarus*, it is tempting to suspect that hybridization may be responsible for the high nucleotide diversity in southern Mozambique.

Both peripheral populations at Chidenguele and Mdumbi were also the most genetically distant from all other populations. While a mtDNA marker is suitable to draw inferences on cryptic species, it is not suitable to draw inferences on hybridization (Teske *et al*. 2011a) and, therefore, a further look into novel genetic evidence on the hybridization between these subspecies is required.

The possibility of cryptic subspecies or subpopulations cannot be ruled out given the high genetic diversity between subpopulations in the present study. A COI nucleotide diversity of 3.8% (K2P) was found between distinct subpopulations of *Panulirus pencilatus* (Chow *et al.* 2012) and comparable divergence was found between two recognised subspecies, *P. longipes longipes* and *P. l. bispinosus*. According to Wang (1975), genetic distances in the range of 0.02-0.20 are indicative of subpopulations (Guo *et al.* 2012), a range well within the values in the present study, suggesting that subpopulations or possibly cryptic subspecies exist in the present study.

High levels of genetic variability in both the Xai Xai and 'EC' populations (possibly cryptic or hybridized populations) is further supported by intra-molecular indices (see Table 3.1) indicating high levels of genetic differentiation, such as a high total number of mutations *i.e.* Port St. Johns (56) and Xai Xai (55). Moreover, MMD plots for the Xai Xai and Port St. Johns populations displayed a unimodal pattern accompanied by a series of significantly lower peaks. This pattern is indicative of high diversity (nucleotide differences) among individuals and may represent different lineages (Fernández *et al.* 2012). An overlap in distribution of cryptic lineages may contribute to the blurred genetic boundaries between subpopulations (Teske *et al.* 2011b) and the incomplete genetic isolation of subpopulations ('MOZ+KZN' and 'EC') observed in the present study.

Patterns of gene flow

The direction of gene flow helped (better) understand the effect of oceanic currents on recruitment and dispersal of larvae. The pattern of gene flow in the present study compared well with previously described abundance and distribution data for *Panulirus homarus rubellus* along the south east coast of Africa, where the highest abundance occurs along the KwaZulu-Natal coastline and lower abundances occur to the north and south of KwaZulu-Natal (Heydorn 1969, Berry 1971a, Kemp & Britz 2008, Steyn *et al.* 2008). Furthermore, stepwise population migration revealed that the highest level of migration occurred from Scottburgh to Blood Reef, followed by migration from Mdumbi to Port St. Johns. This pattern of gene flow may be due to northward migration toward more suitable, warmer environments, assuming natal homing and selective pressures in each region are not the only factors governing genetic differentiation.

The role of the Agulhas Current in gene flow

Interestingly, the pattern (intensity) and direction of gene flow closely follows the Agulhas Current and suggests the stable southward flowing Agulhas Current is a major mode of gene flow and, thus, larval transport along the south east coast of Africa (Heydorn *et al*. 1978, Lutjeharms 2006). The Agulhas Current originates somewhere between Maputo and Durban (Lutjeharms 2006) and a high level of gene flow was evident in this vicinity in the present study. The number of immigrants and, therefore, gene flow was highest along the KwaZulu-Natal coast where the Agulhas Current flows steadily. However, low numbers of immigrants were still observed north of this origin (Chidenguele and Xai Xai). In Mozambique, cyclonic eddies may spin off the Agulhas current and larvae may then be transported north, thus seeding the Mozambique localities (MacNae 1962, Berry 1971a, von der Heyden *et al*. 2008). This may explain the low levels of immigrants and, in turn, gene flow among localities in Mozambique. Inshore (cyclonic) counter-currents along the coast of South Africa may play a role in northward larval dispersal or migrant exchange on a local scale (von der Heyden *et al.* 2008). If so, this is consistent with the idea of stepwise gene flow along the South African coast (von der Heyden *et al.* 2008) and may explain the bi-directional (albeit at low levels) migration between some localities; northward larval dispersal by inshore counter-currents and southward larval dispersal by the Agulhas Current.

Lesser gene flow at localities in the 'EC' may be explained by a combination of the Agulhas Current, the geomorphology of the Eastern Cape coast line and temperature. Along the south east coast of Africa the continental shelf constricts significantly along the Eastern Cape, particularly around Port St. Johns (12.5 km) followed by a much wider continental shelf south of this region. The widening shelf area is responsible for deflecting the Agulhas Current from coastal areas (Lutjeharms 2006, Teske *et al.* 2006). If the Agulhas Current is indeed responsible for larval recruitment then the southern boundary of *Panulirus homarus rubellus* may be a consequence of low larval recruitment along the Eastern Cape concomitant with a deflecting Agulhas Current.

The Algoa Bay region and the South African south coast at large are influenced by the exclusion of warmer waters from the Agulhas Current deflecting off the Agulhas Bank and the intrusion of cooler water from the west coast, as well as periodical upwellings

(Lutjeharms 2006, von der Heyden *et al.* 2008). Therefore a steep temperature cline between the east coast and the south coast of South Africa (von der Heyden *et al.* 2008) may also contribute to lower levels of gene flow along the Eastern Cape, due to suboptimal growth temperatures (Kemp & Britz 2008). Fewer populations may have become established and continue to disperse into areas less suitable for their survival and growth. It has been suggested that the southern distribution limit of *Panulirus homarus rubellus* is governed by temperature (Berry 1971a, Kemp & Britz 2008); this together with the offshore deflection of the Agulhas Current (suggested as the primary mode of larval dispersal) likely limit the southern distribution of *P. h. rubellus*.

The northern distribution may be similarly limited by low levels of larval recruitment from the Agulhas Current; however, inshore counter-currents or eddies that may spin off the Agulhas Current ensure localities further north into Mozambique are seeded (Berry 1971a). Physiological studies have shown that the optimal growth rate for *Panulirus homarus rubellus* occurs within a temperature range common to Mozambique (Kemp & Britz 2008). However, the highest abundance of this subspecies occurs in sub-optimal temperatures in the subtropical Natal Bioregion. This, suggests that temperature is less likely to limit the northern distribution boundary of *P. h. rubellus,* which is probably governed by low larval recruitment. This reaffirms the role of larval dispersal and its influence on the distribution pattern of *P. h. rubellus* along the SE African coast.

Northern and southern population boundaries and history

The mean migration from Port St. Johns to Mdumbi did not fall within the 95% confidence limits and, therefore, suggests that no effective migration occurs from Port St. Johns to Mdumbi. This is in accordance with Mdumbi marking the southern distribution boundary of *Panulirus homarus rubellus* in the present study. The southern distribution boundary of *Panulirus homarus rubellus* is further supported by Fu's *Fs* and MMD plots that concur with a stable non-expanding southern peripheral population (Mdumbi). However, further evidence that Mdumbi is the southern distribution limit will require sampling at localities west of Mdumbi. Non-expanding peripheral populations have been found in other marine organisms and this phenomenon may be caused by the transition from suitable to less suitable habitat or changes in preferred environmental conditions (von der Heyden *et al.* 2008). In contrast, Chidenguele the

northern peripheral population, did not reflect a stable non-expanding population. At first consideration it may seem intuitive to implicate the northern distribution limit of *P. h. rubellus* to biological exclusion by *P. h. homarus*. However; both subspecies are known to occur sympatrically (Berry 1974a, b). The northern boundary sample locality was likely too far south to make inferences on the northern distribution boundary (Barra Falsa) of *Panulirus homarus rubellus*.

Peripheral populations are prone to founder effects that are characterised by low haplotype and nucleotide diversity (von der Heyden *et al.* 2008). In the present study no evidence for founder effects in either peripheral population was detected. Although Chidenguele and Mdumbi had the lowest nucleotide and haplotype diversity, respectively, a founder effect can be ruled out because these differences were not significantly lower than other populations.

All other individual populations, the 'MOZ+KZN' region, as well as the overall data set were not in mutation drift equilibrium and have therefore undergone a recent demographic change (Rogers & Harpending 1992). Demographic population changes are due to either an *'expanding or contracting population'* (von der Heyden *et al.* 2008: pp. 4815). However, a population decline is often reflected in lower genetic diversity indices (McMillen-Jackson & Bert 2004a) and, therefore, can be eliminated as a possibility. Selective sweeps, past bottlenecks, founder effects, low lineage-specific mutations and natural selection as outlined in García-Merchán *et al*. (2011), as well as an expanding population, all produce significant, negative Fu's *Fs* values and unimodal MMD plots (McMillen-Jackson & Bert 2004a, Tolley *et al.* 2005, Gopal *et al.* 2006). Selective sweeps are characterised by a high frequency of a common ancestral haplotype, as well as low haplotype diversities (McMillen-Jackson & Bert 2004b, Gopal *et al.* 2006). In the present study, a selective sweep can also be ruled out based on the starburst pattern in the haplotype network for the entire data set (Appendix 1) as well as the relatively high haplotype diversities for each individual population, each putative region and for the overall data set. High haplotype diversities also rule out the possibility of past bottlenecks (Tolley *et al.* 2005). Thus, based on the high levels of genetic diversity and a star-burst haplotype network, an expanding population is the most reasonable inference from the aforementioned statistical analyses.

A unimodal distribution of MMD plots may also reflect '*heterogeneity in mutation rates among sites*' (McMillen-Jackson & Bert 2004a). However, population expansion can be further substantiated by Tajima's *D* statistic, where a negative *D* reflects an expanding population, while a positive *D* value supports the '*heterogeneity in mutation rates among sites*' hypothesis (McMillen-Jackson & Bert 2004a: pp. 105). In the present study, Tajima's *D* was negative for all individual populations, except Mdumbi, for each putative region, as well as for the entire data set. Therefore, population expansion rather than heterogeneity in mutation rates among sites is reflected in MMD plots.

Genetic diversity

High haplotype and low nucleotide diversity found in populations such as Chidenguele and Blood Reef reflect expanding populations with an initial low effective population size (von der Heyden *et al.* 2008). On the other hand, high levels of genetic diversity (high haplotype and nucleotide diversity) observed in all other populations correspond to a large original population. When initial population sizes are high then ancestral haplotypes may not retain a high frequency and, therefore, a higher frequency of unique haplotypes can be maintained (McMillen-Jackson & Bert 2004b, Fernández *et al.* 2012). The effect of genetic drift may be counteracted by an increase in the number of haplotypes and lineages due to an increase in mutations; this, in turn, decreases the stochastic loss of haplotypes and, thus, lineages (lineage sorting) and may result in high haplotype diversity (Avise *et al*. 1984). This, together with high original population sizes, may explain the lack of prominent high frequency ancestral haplotypes in population level haplotype networks (Appendix 1) and the occurrence of many (74%) unique haplotypes overall.

Localities exhibiting high genetic diversity may also point to some level of gene flow between differentiated populations (Fernández *et al.* 2012), as seen in the present study. High levels of genetic diversity may promote population proliferation, as well as adaptation to environmental change (Fernández *et al.* 2012). This reiterates the possibility of cryptic divergence between bioregions proposed for *Panulirus homarus rubellus* in the present study. However, the choice of marker in the present study cannot detection adaptation.

In summation, the high genetic diversity evident in populations of *Panulirus homarus rubellus* is likely a combination of an expanding population with high initial population sizes, some level of gene flow as well as possibly a high rate of mutation due to adaptation (McMillen-Jackson & Bert 2004a). Furthermore, García-Merchán *et al.* (2011) confirm higher levels of diversity in shallow-water lobsters compared to their deep-water counter-parts. Therefore, part of the high genetic diversity found in these shallow-water lobsters may be attributed to their habitat. Moreover, by and large decapods exhibit high mtDNA diversity, with shrimp having among the highest values (McMillen-Jackson & Bert 2004a). Intra-population indices such as haplotype diversity, although high in the present study, compare well to those of other spiny lobster mtDNA genetic studies (Ovenden *et al.* 1992, Benzie *et al.* 1993, Silberman *et al.*1994, Brooker *et al.* 2000, Tolley *et al.* 2005, Gopal *et al.* 2006).

Higher levels of genetic differentiation have been found in populations that occur at lower latitudes, as well as a trend between genetic divergence and genetic distance (isolation by distance) (Moritz & Cicero 2004). The former was shown for the blue crab and corals sampled over a latitudinal gradient where genetic variation was higher in southern vs. northern populations (McMillen-Jackson & Bert 2004b, Macdonald *et al.* 2011). In the present study, no relationship between haplotype and nucleotide diversity with varying latitude was detected; however, a pattern of isolation by distance was found. Given the large sample size, statistically significant correlations were generated for isolation by distance but may not be biologically meaningful (Waples 1998, Bohonak 1999, Palumbi 2003). Isolation by distance in oceans may be elusive and rare in marine systems due to oceanic currents and its effect on dispersal.

A molecular marker under selection has the potential to severely skew results of historic demographic change and population genetic structure (von der Heyden *et al.* 2008). Therefore the mtDNA COI marker, presumably a neutral marker, was tested for selection using Tajima's *D* statistics (Tajima 1989). Results indicated this marker was indeed under no selection and was further supported by high levels of genetic diversity characteristic of a neutral molecular marker (García-Merchán *et al.* 2011). Additionally, results from DAMBE suggest this marker was not saturated at the level of nucleotide substitution saturation and was therefore suitable for the present study.

Phylogeography of Panulirus homarus rubellus populations along the south east coast of Africa

The Holocene Epoch marked the beginning of warmer and more stable conditions (Stager & Mayewski 1997). During the early to mid-Holocene Epoch (8000 years ago) global sea levels became stable (Stager & Mayewski 1997). And, as stability during the warmer Holocene prevailed, the subtropical shallow-water lobster *Panulirus homarus rubellus* was afforded an opportunity to flourish and expand their populations.

Although mean sea level stabilised approximately 6500 years ago, this relative 'stabilisation' comprised a series of minor sea level fluctuations along the south east African coast (Ramsay 1995). In particular, the estimated expansion time of the Chidenguele population is synchronous with a 2 m deposition of bed rock in the Kosi Bay area (Ramsay 1995) a few hundred kms south of Chidenguele, Mozambique. A mid-Holocene deposition of bed rock may have created additional rocky environments that presented new suitable environments, which led to population expansion.

However, during the Holocene Epoch episodes of rapid climate change have been recorded (Mayewski *et al*. 2004) and, interestingly, several rapid climate change events coincide with individual population expansion events for various *Panulirus homarus rubellus* populations along the south east African coast. The 'MOZ+KZN' region expanded approximately 3987 years ago (using the lower bound mutation rate) in accordance to a cooling trend in southern Mozambique (Mayewski *et al*. 2004). This would have allowed these subtropical lobsters an opportunity to invade cooler tropical waters. An overall warmer and wetter southern Africa around 4000 years ago may have indeed driven various other population expansion events (Mayewski *et al*. 2004).

The recent population expansion of *Panulirus homarus rubellus* corroborates with a recently derived *Panulirus homarus* subspecies (George & Main 1967, Pollock 1993). The time elapsed since population expansion can only be calculated if a population is indeed expanding, thus the time estimated since population expansion was only possible for one of the 'EC' populations *i.e.* the one at Port St. Johns and its recent colonisation is consistent with the hypothesis of a recent genetic break in connectivity between the 'EC' and 'MOZ+KZN', as suggested earlier.

CONCLUSION

Significant genetic structure was recovered among the shallow-water lobster, *Panulirus homarus rubellus*, populations along the south east coast of Africa. Two genetically distinct subpopulations were recognised, 'MOZ+KZN' and 'EC', suggesting a break in genetic connectivity along the transition zone between the subtropical Natal and warm temperate Agulhas Bioregions. A common pattern of genetic disjunction along this transition zone has been found for a number of other marine organisms (von der Heyden 2009). In the present study, a genetic break in connectivity was attributed to a possible genetic barrier to larval dispersal, as gene flow was concomitant with larval dispersal for *P. h. rubellus*.

Given that gene flow and, therefore, larval dispersal in the present study closely paralleled the Agulhas Current, the genetic barrier was further associated with larval transport. It has been suggested that different modes of larval transport have driven the formation of subpopulations.

Past phylogeographic events, as well as present-day larval retention mechanisms (inshore counter-currents and eddies), may have led to the genetic differentiation of the 'MOZ+KZN' and 'EC' subpopulations. However, moderate levels of contemporary gene flow, the moderate larval duration of *Panulirus homarus rubellus*, and sufficient oceanic mixing may mask this genetic break. Furthermore, the recently derived *Panulirus homarus* subspecies complex is probably influenced by an edge-effect that may have contributed to the lack of definite differentiation between subpopulations.

The genetic divergence between putative regions was consistent with genetic divergence between other subspecies and even some species using a COI marker. Teske *et al.* (2006) emphasize that many crustacean species along the South African coast consist of multiple cryptic lineages or cryptic species complexes rather than existing as a single evolutionary unit. It is, therefore, likely, *Panulirus homarus rubellus* may represent yet another case of cryptic divergence.

A moderately long PLD and the aid of extensive oceanic currents do not necessarily translate into high levels of gene flow and genetic panmixia (Gopal *et al.* 2006). While it may seem intuitive to correlate PLD with genetic structure, growing evidence

suggests that larval behaviour as well as effective population sizes may greatly influence genetic structure or lack thereof (Faubry & Barber *et al.* 2012). Larval behaviour, in particular larval 'homing' toward environmental or physiological cues, as well as female philopatry may highly reduce levels of gene flow in species with an extensive larval dispersal capacity (Faubry & Barber *et al.* 2012). Thus, the relationship between gene flow and dispersal largely depends on the study organism and the choice of molecular marker (Bohonak 1999).

From a management perspective, despite consistent patterns of differentiation between regions some level of gene flow does occur and therefore clear management considerations will require further research. The genetic pattern observed in the present study is exploratory and much work remains to confirm the level of population genetic structure, the existence of cryptic lineages and the possibility of hybridization.

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CHAPTER 4

General Conclusions, Management Recommendations and Future Research

This project was developed with the aim of aiding fisheries management of *Panulirus homarus* stocks in the SWIO region. However, techniques and recommendations used in the present study have implications that go beyond this particular fishery. Indeed, if recommendations from the present project are successfully implemented to *P. homarus* fisheries in South Africa, it may encourage resource managers to adopt similar genetic techniques to aid in the management of other fisheries.

GENERAL CONCLUSIONS

The mtDNA (COI) marker used in this study demonstrated its wide application from a population genetic to a phylogenetic and phylogeographic perspective.

This study represents the first molecular phylogenetic analysis and delimitation of the *Panulirus homarus rubellus* and *P. h. homarus* subspecies. The DNA barcoding approach was employed and the first DNA barcoding sequence for *P. h. rubellus* is now available. *Panulirus homarus homarus* is widely distributed throughout the Indo-West Pacific and, although DNA barcodes are available for this subspecies, no barcode existed for *P. h. homarus* in Mozambique until now. Previous studies (Ptacek *et al*. 2001, George 2005) and the present study concurred that genetic variability in *P. h. homarus* is high throughout its distribution and, therefore, DNA barcodes for this subspecies from different regions will allow for a comprehensive comparison of the individuals in this subspecies.

Panulirus homarus homarus and *P. h. rubellus* were shown to be genetically distinct lineages that were 2-3% divergent from one another. This genetic differentiation in *P. h. homarus* and *P. h. rubellus* corroborated the previous morphological taxonomic delimitation (Berry 1974). Over time, subspecies have probably become geographically isolated from their shared common ancestor, as well as from each other (Pollock 1995, Thorpe *et al.* 2000, Silva & Russo 2000). Each subspecies probably displays different genotypes due to an accumulation of mutations and genetic drift in each respective subspecies (Slatkin 1981, Reuschel & Schubart 2006). *Panulirus homarus rubellus* and *P. h. homarus* were found to be sister taxa and were monophyletic with respect to the out-goup taxa. This is in contrast to an earlier view of a more ancient *P. h. homarus*

from which *P. h. rubellus* speciated. This may, of course, be subject to change if the third subspecies is added to the present phylogenetic picture.

Nevertheless, although the genetic differentiation between subspecies was comparable to differences between species, their taxonomic rank cannot be raised to species level based on the Biological Species Concept (Mayr 1942) as suspected hybridization (Berry 1974) between these subspecies may allow for some level gene flow. Some individuals displayed morphology similar or identical to *Panulirus homarus homarus* but with the genotype of *P. h. rubellus* and *vice versa* and this served as preliminary genetic evidence of hybridization between subspecies. However, mtDNA is not an ideal marker for determining hybridization as it lacks paternal genetic information and these results are, at best, speculative.

Historic range expansion of both *Panulirus homarus homarus* and *P. h. rubellus* may explain their present distributional overlap in southern Mozambique, a suspected hybridization zone. Abundance and distribution data suggested that the overlap in distribution of *P. h. homarus* and *P. h. rubellus* is marginal and, therefore, if hybridization does occur then the level at which it occurs is expected to be low.

The present study also represents the first genetic study on *Panulirus homarus rubellus*, endemic to the SWIO. The genetic structure of populations of *P. h. rubellus* across its African distribution was investigated and two genetically distinct subpopulations were recognised. Given the moderately long larval duration and the potential for extensive larval dispersal of *P. h. rubellus*, it is interesting that this spiny lobster failed to satisfy the expectation of genetic panmixia. Genetic panmixia has been demonstrated for a number of crustacean species with a moderately long planktonic larval duration (PLD) such as *P. interruptus* and *Palinurus gilchristi*, both of which have similar PLDs to *P. h. rubellus* (Tolley *et al*. 2005, García-Rodríquez & Perez-Enriquez 2006). Therefore, *P. h. rubellus* represents an exception to the 'general' trend of genetic panmixia in species with a moderately long PLD. Indeed, recent literature has shown this trend to be less common than previously considered.

Considering there are no vicariate barriers along the southern African coast and that larval transport plays a crucial role in the gene flow of *Panulirus homarus rubellus,* the

genetic structure detected may be associated with larval transport. Gene flow closely paralleled the pattern of the Agulhas Current and it was therefore considered the primary mode of larval transport for *P. h. rubellus (*Chapter 3). However, since distinct subpopulations were found, a hypothesis was proposed to explain genetic differentiation in the subpopulations whereby a subset of larvae may have become prematurely entrained in inshore counter-currents and gyres, thereby isolating the 'EC' subpopulation. Therefore, the different larval transport modes and retention mechanisms described in Chapter 3 may be responsible for the genetic structure of *P. h. rubellus* populations along the south east African coast.

However, low levels of contemporary gene flow between subpopulations suggest the genetic barrier constraining larval dispersal may be stochastic (mediated by inshore counter-currents that vary in duration and occurrence) or one that is still forming. Nevertheless, larval transport may be sufficient to isolate subpopulations which differed by 1.7% sequence difference. Shallow, yet significant, female philopatry, natal homing and different selective pressures in the different putative regions may have contributed to the overall genetic differentiation observed in subpopulations.

Gene flow in populations of *Panulirus homarus rubellus* along the SE African coast corroborated previous abundance and distribution records of *Panulirus homarus rubellus* where it is highest in abundance in KwaZulu-Natal, decreasing to the north in southern Mozambique and in the south in the Eastern Cape. The deflection of the Agulhas Current, as well as unsuitable environmental conditions, explained the southern distribution boundary of *P. h. rubellus*. However, the northern distribution lacked a well-defined boundary but this may be due to a sampling artefact.

Evidence for a possible edge-effect (Cook 1961), together with estimated dates of subspecies/population expansion, suggested that genetic isolation of both subspecies and the genetic barrier driving the formation of subpopulations of *P. h. rubellus* may have occurred recently, *ca*. mid-Holocene Epoch. Time elapsed since subspecies expansion for the shallow-water (group IV) *Panulirus homarus* (*P. h. homarus and P. h. rubellus*) was compared with *P. argus* (group 1) and, deep-water counterparts *Palinurus gilchristi* and *P. delagoae* (Tolley *et al*. 2005, Gopal *et al*. 2006, Naro-Maciel *et al*. 2011). The estimated expansion of *P. homarus* compares well with a later radiation by

group IV *Panulirus* species, as well as a later colonisation of shallower water by lobsters.

The estimated time of expansion for both subspecies, as well as for several *Panulirus homarus rubellus* populations dates back to the mid-Holocene Epoch and is consistent with an overall trend of warmer and more stable environmental conditions. Moreover, the Holocene Epoch was marked by an increase in sea level as well as the deposition of bedrock that may have created new environments, allowing these subtropical and tropical shallow-water lobsters to successfully expand their range.

Chow *et al*. (2012) recently detected cryptic speciation in the pronghorn spiny lobster, *Panulirus penicillatus.* Limited gene flow across a prominent oceanic genetic break in connectivity-the East Pacific Break (EPB) -led to the genetic differentiation of these lobsters. Similarly, it is suggested that the distribution of *P. homarus rubellus* populations traversing a prominent biogeographic and phylogeographic break situated between the KwaZulu-Natal and Eastern Cape coasts may be a consequence of cryptic divergence. Genetically distinct populations of *P. penicillatus* on either side of the EPB genetic break differ not only genetically but also exhibit minor difference in colour. Differences in colour in other spiny lobster species and subspecies (Chan & Chu 1996, Sarver *et al*. 1998, 2000, Chan 2010) have been shown to be supported by genetic differences similar to the *P. h. homarus* and *P. h. rubellus* subspecies in the present study.

MANAGEMENT RECOMMENDATIONS

Despite the rising interest in lobster fisheries around the world, in South Africa, limited efforts have been made to move away from conventional conservation practices for their sustainable management (Caddy & Cochrane 2001). Fisheries around the world have adopted new and innovative ways in which to manage their stocks, one such advancement has been the application of genetic techniques in fisheries (Ward & Grewe 1994, Ward 2000, Diniz *et al*. 2005). The use of genetics in fisheries management is primarily employed to identify groups of interbreeding individuals, also known as genetic breeding units akin to ‘stocks’ in fisheries management and ‘populations’ in

genetics (Thorpe *et al.* 2000, Crivello *et al*. 2005, Waples & Gaggiotti 2006, Babbucci *et al*. 2010).

Genetically distinct subpopulations have been identified for other spiny lobsters occurring across wide distribution ranges: *Panulirus argus* subspecies from Brazil to North Carolina (Sarver *et al*. 1998, 2000), *Jasus edwardsii* throughout New Zealand and across the Tasman Sea (Thomas 2012) and *Palinurus delagoae* in the SWIO (Gopal *et al*. 2006), and this genetic information has the potential to facilitate the effective management of these stocks. Similarly, by determining the population genetic structure and genetic diversity of *P. homarus rubellus* across its distribution range, key management questions may be addressed.

The application of genetic techniques aimed at identifying genetic structure in populations of marine organisms was initially explored in the 1930s (Ward 2000). Since then, genetic techniques have improved, become more readily available and the interpretation of data has been aided by innovative computer software. So, why is this long-recognised technique not being exploited in fisheries? Perhaps a bridge in communication between fisheries scientists and fisheries managers is required. The use of genetics in fisheries management has clearly demonstrated its worth (Ward 2000) but the challenge of implementing these invaluable techniques in our fisheries remains.

Implications of the present study: *Regional vs. National management*

The present study provided compelling evidence for the genetic distinctiveness between *Panulirus homarus homarus* and *P. h. rubellus.* However, in regions where *P. h. homarus* overlaps in distribution with *P. h. rubellus*, no distinction is made between subspecies in the fisheries. Based on genetic data, it is proposed that both subspecies be treated as separate management units. However, in light of the low abundance of *P. h. homarus* along the South African coast and the low abundance of *P. h. rubellus* in Mozambique, separate management of these subspecies may not be feasible in these countries. We therefore advise that, for South Africa, management and permit regulations focus solely on *P. h. rubellus* for which sufficient population genetic data have been generated (Chapter 3). Furthermore, a phylogenetic approach provides no

reliable indication of population level differentiation in *P. h. homarus* and, therefore, appropriate management suggestions cannot be made regarding this subspecies.

Along the south east African coast, *Panulirus homarus rubellus* was presumed to be a single stock occurring across a wide distribution range (Berry 1974, Pollock 1993). However, based on the present results, two putative regions influenced by different coastal regimes (*i.e.* Agulhas Current and inshore counter-currents or eddies) may have driven the formation of subpopulations along this wide geographic range (Berry 1971, Pollock 1993, Lutjeharms 2006).

In the context of the present study, while a *Panulirus homarus rubellus* stock is shared between South Africa and Mozambique, they are differentiated from the Eastern Cape stock. This suggests that a national management scheme for the 'EC' *P. h rubellus* stock may be required and a trans-boundary or regional management for the 'MOZ+KZN' *P. h rubellus* stock. However, considering that there is a low level of contemporary gene flow between subpopulations, effective management will require a scheme tailored for each subpopulation that factor in gene flow.

The pattern of gene flow between populations of *Panulirus homarus rubellus* along the South African coast closely paralleled the Agulhas Current and, if gene flow is indeed concomitant with the Agulhas Current, managers may find data from Agulhas Current projects (e.g. ACEP & ASCLME) useful to predict inter-annual levels of variation and success in larval recruitment.

While a regional scheme is recommended for the 'MOZ+KZN', the low larval recruitment and, thus, low abundance of *Panulirus homarus rubellus* along the coast of southern Mozambique may in fact pose a problem to the idea of regional management.

Lastly, the anecdotal hybrid zone in southern Mozambique (Berry 1974) and the provisional evidence of this hybrid zone will require concerted joint management by South Africa and Mozambique. But, further investigation of this is needed before any management suggestion can be made in this regard. The management and conservation of both species may positively contribute to the management and maintenance of the hybrid zone.

Management and conservation is needed throughout the distributional range of *Panulirus homarus* and conservation efforts should be aimed at sustaining viable stock size and genetic diversity for the future.

FUTURE RESEARCH

The choice of molecular markers is an important criterion to consider when developing a study; however, different levels of resolution are required to address different research questions. The resolution of molecular markers varies widely according to this selection (García-Rodríguez & Perez-Enriquez 2006). In the present study, the mtDNA COI was selected as this marker offered both a perspective of the population level structure as well as a phylogenetic view of *Panulirus homarus* subspecies.

Mitochondrial DNA genes have long been presumed neutral markers, however, recent studies suggest that these markers may be prone to selective pressure and their use as neutral markers should be tentative (Bazin *et al*. 2006, Babbucci *et al*. 2010, Palero *et al*. 2011). Neutral markers may also be affected by selective processes when subjected to linkage disequilibrium (Vitalis *et al*. 2001). In addition, mtDNA provides no information on male-mediated gene flow as this gene is maternally inherited. Moreover, in light of the many caveats associated with using COI as a sole marker in defining species boundaries, as outlined by Herbert *et al*. (2004) and Moritz and Cicero (2004), an additional nuclear marker would help to address the aforementioned concerns.

Significant genetic structure was found in populations of *Panulirus homarus rubellus* along the south east African coast using a mtDNA (COI) marker. While an overall level of genetic structure was revealed, elucidation of even finer scale population differentiation remains. Haplotype networks formed numerous reticulations (an alternative genetic link; see Appendix 1) and this reflected the low resolving power of the COI marker at a finer-scale population level. In population genetics, microsatellites are markers of choice and are routinely used in combination with mtDNA markers (Silva & Russo 2000, Selkoe & Toonen 2006). Similarly, the addition of a suite of microsatellites to the present data set may provide more comprehensive information at the population level (Sarver *et al*. 1998, Yang 1998, Palero *et al.* 2009, Babbucci *et al*. 2010). Microsatellites are repetitive, non-coding sequences of nuclear DNA that have a

high occurrence of mutations (Brooker *et al.* 2000, Diniz *et al.* 2005, Selkoe & Toonen 2006) and are, therefore, rapidly evolving markers (Cabezas *et al.* 2009). These markers are currently applied in fisheries and display considerable potential in fisheries management (Ward 2000).

It has been suggested that larvae of *Panulirus homarus rubellus* along the African coast are transported by the Agulhas Current and become entrained in the SWIO Subgyre. Similarly, it has been suggested that larvae are transported by the East Madagascar Current along the Madagascan coast and also become entrained in the SWIO Subgyre (Berry 1974, Pollock 1993). If this is so, then one might expect to find a lack of differentiation between African and Malagasy *P. h. rubellus* populations. However, it has been suggested that different modes of larval transport and retention led to the formation of genetically distinct subpopulations of *P. h. rubellus* along the SE African coast and similarly subpopulations may exist between Africa and Madagascar. Little is known about the genetic stock structure, abundance and distribution or management of *P. h. rubellus* along the Madagascan coast. The addition of Malagasy data would be valuable not only to the Madagascan fishery but may also, in turn, benefit the South African *P. h. rubellus* fishery. Thus, a genetic assessment of populations encompassing the entire distribution of *P. h. rubellus* would determine the population dynamics of this valuable lobster resource.

The genetic structure between African and Malagasy populations will not only be interesting from the population level but also from a phylogenetic and phylogeographic perspective. Therefore, the addition of both a species level marker, as well as population level markers, may help determine whether or not mainland (Africa) and island (Madagascar) populations of *Panulirus homarus rubellus* are genetically structured, as well as allow for inferences on gene flow between the island and mainland. Furthermore, Malagasy populations could be placed in a phylogenetic context relative to the existing phylogeny of *P. homarus*. In addition, if the third member of this subspecies trio, *P. h. megasculptus*, is added to the present data it will provide a complete phylogenetic assessment of the *P. homarus* subspecies complex.

The identification of genetically distinct populations can influence the way a fishery is managed; however, one of the challenges inherent in fisheries is hybridization (Ward

2000). The Internal Transcribed Spacer region (ITS) region, as well as several other nuclear introns, have been successfully used to elucidate hybridization in a number of species (Chan *et al*. 2007) and, more recently, in the pygmy angelfish (DiBattista *et al*. 2012). Hybridization has been suspected to occur at both regions of overlapping distribution of *Panulirus homarus homarus*. Therefore, hybridization between *P. h. homarus* and *P. h. rubellus* and, between *P. h. homarus* and *P. h. megasculptus* may be explored and will provide a better picture about hybridization between *P. homarus* subspecies.

If hybridization indeed occurs between *Panulirus homarus* subspecies, these hybrid zones will become important in the conservation and management of this species. Hybridization has the ability to alter and, in the long-term, possibly eliminate the genetic signature of sub/species. Hybridization may even lead to a reduced fitness of lineages (von der Heyden 2009). Furthermore, habitat preferences may allow different subspecies to occur sympatrically without competitive exclusion, a conformity that may not apply to hybrids (von der Heyden 2009). In fact, if hybrids are conferred with a selective advantage they, may very well, competitively exclude purebred or native populations (van der Meeran *et al*. 2008). The unknown behaviour and genetics of hybrid specimens reiterates the need to identify and accordingly manage hybrid zones.

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APPENDIX 1

Haplotype networks constructed with both TCS and Network v. 4.6.1.0 produced networks that were highly reticulated. Haplotype networks for each population and for the overall data set were constructed. However, considering the high genetic variation in the data set, the confidence interval of 95% was adjusted in some instances so as ensure the connection of all haplotypes. Where necessary, the confidence interval was set at a maximum of 30 mutational steps.

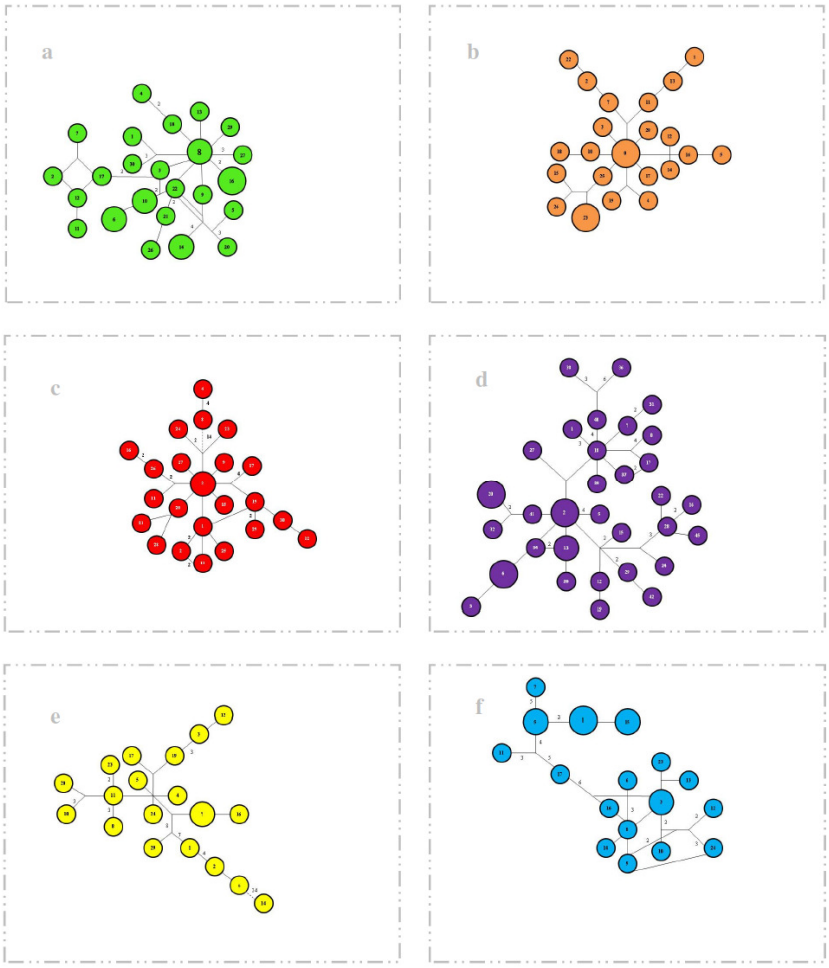

Figure 5.1: Haplotype networks for individual populations of *Panulirus homarus rubellus* along the south east coast of Africa, circles are representative of haplotypes and the size of the circle is proportional to the haplotype frequency. Solid lines represent haplotype connections with a 95% confidence and dotted lines a confidence limit below 95 %. The number of mutational steps between haplotypes is indicated by numbers between haplotype pairs, the absence of a number is assumed as a single step. a) Chidenguele, b) Xai Xai, c) Blood Reef, d) Scottburgh, e) Port St. Johns and f) Mdumbi.

The haplotype network for the entire data set was implemented in Splits tree and forms a star burst pattern. Given the low resolution of the COI marker the haplotype network was highly reticulated and therefore Spilts tree was used to minimise the bias that occurs when redrawing large, highly reticulate networks.

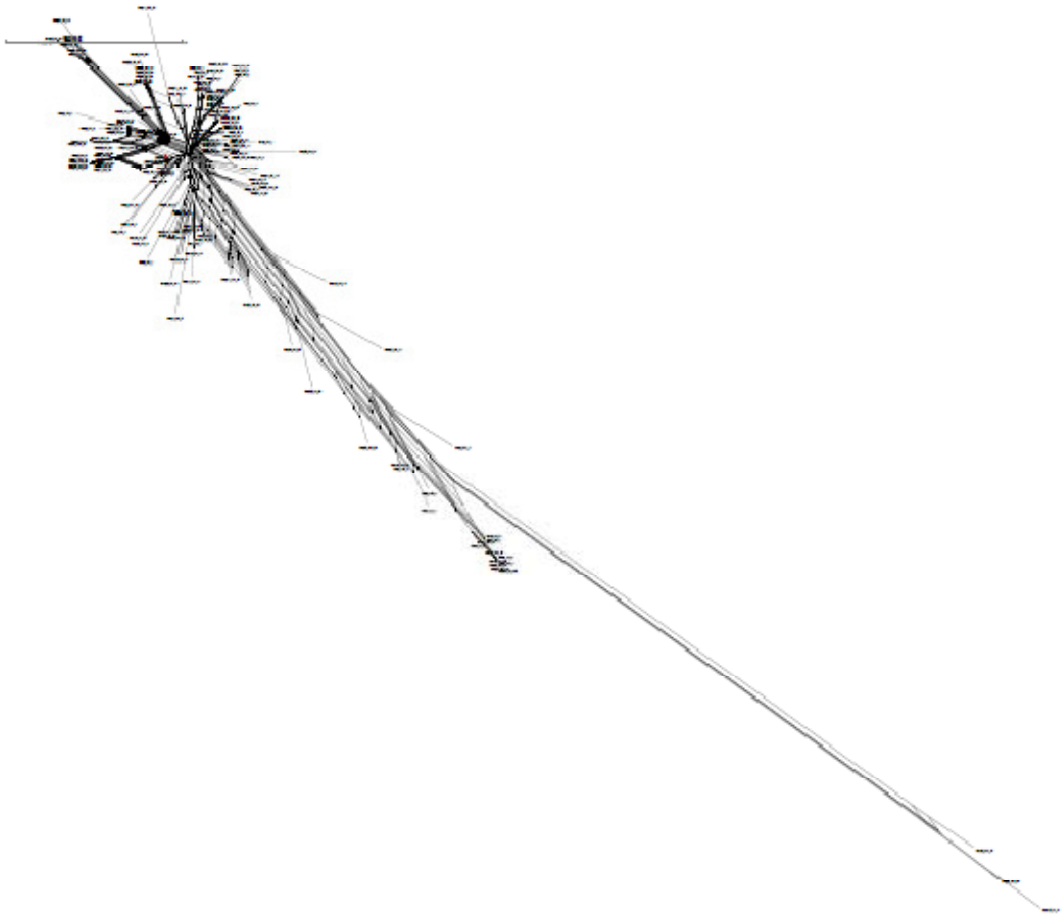

Figure 5.2: Haplotype networks for the entire data set of populations of *Panulirus homarus rubellus* along the SE coast of Africa. A general star-burst pattern can be inferred with a subset of differentiated individuals, consistent with the idea of two distinct subpopulations.

APPENDIX 2

Table 5.1: Haplotype identities, number and locality at which samples were obtained and the number of samples sharing a particular haplotype

Haplotype_ID	Frequency	Samples
Hap_1	1	**ZV_1**
Hap_2	1	**ZV_2**
Hap_3	1	**ZV_3**
Hap_4	1	ZV_5
Hap_5	4	**ZV_6**, **ZV_8**, ZV_17, **CH_21**
Hap_6	3	ZV_7, BR_20, SB_34
Hap_7	1	**ZV_9**
Hap_8	1	**ZV_10**
Hap_9	10	**ZV_11**, **ZV_15**, CH_10, CH_27, BR_3, BR_18, SB_2, SB_11, SB_21, MB_8
Hap_10	2	**ZV_12, XX_27**
Hap_11	1	**ZV_13**
Hap_12	1	ZV_14
Hap_13	1	**ZV_16**
Hap_14	1	**ZV_18**
Hap_15	1	**ZV_19**
Hap_16	1	**ZV_20**
Hap_17	1	CH_1
Hap_18	2	CH_3, XX_1
Hap_19	6	CH_5, XX_4, SB_41, PJ_11, MB_3, MB_21
Hap_20	2	CH_6, SB_26
Hap_21	1	CH_7
Hap_22	8	CH_8, CH_28, XX_19, SB_20, SB_30, SB_35, SB_44, MB_23
Hap_23	3	CH_9, SB_7, SB_33
Hap_24	3	CH_12, XX_12, MB_16
Hap_25	3	CH_13, CH_32, BR_30
Hap_26	1	CH_14
Hap_27	1	CH_15
Hap_28	2	CH_16, PJ_4
Hap_29	3	CH_17, CH_18, PJ_12
Hap_30	11	CH_19, CH_23, CH_29, XX_2, BR_21, BR_22, SB_6, SB_9, SB_10, PJ_7, PJ_9
Hap_31	1	CH_20
Hap_32	5	CH_22, XX_16, SB_13, SB_23, PJ_24
Hap_33	2	CH_24, BR_13
Hap_34	5	CH_25, XX_28, SB_29, SB_43, MB_24
Hap_35	4	CH_26, XX_8, XX_29, BR_1
Hap_36	1	CH_30
Hap_37	1	CH_31
Hap_38	1	CH_33
Hap_39	1	CH_34
Hap_40	1	XX_3
Hap_41	1	XX_5
Hap_42	1	**XX_6**
Hap_43	2	XX_7, PJ_16
Hap_44	2	XX_10, SB_12
Hap_45	1	XX_11
Hap_46	2	XX_13, SB_18
Hap_47	2	XX_14, SB_22
Hap_48	1	XX_15

Hap_49	1	XX_17
Hap_50	1	XX_18
Hap_51	1	**XX_20**
Hap_52	1	**XX_21**
Hap_53	1	XX_22
Hap_54	7	XX_23, XX_24, XX_30, BR_4, PJ_6, MB_15, MB_25
Hap_55	1	XX_25
Hap_56	1	XX_26
Hap_57	1	**XX_31**
Hap_58	1	BR_2
Hap_59	1	BR_8
Hap_60	1	BR_9
Hap_61	1	BR_11
Hap_62	1	BR_12
Hap_63	1	BR_15
Hap_64	1	BR_16
Hap_65	1	BR_17
Hap_66	1	BR_19
Hap_67	1	BR_23
Hap_68	1	BR_24
Hap_69	1	BR_25
Hap_70	1	BR_26
Hap_71	1	BR_27
Hap_72	1	BR_28
Hap_73	3	BR_29, SB_19, PJ_8
Hap_74	1	SB_1
Hap_75	1	SB_3
Hap_76	1	SB_5
Hap_77	1	SB_8
Hap_78	1	SB_17
Hap_79	1	SB_15
Hap_80	1	SB_16
Hap_81	1	SB_24
Hap_82	1	SB_27
Hap_83	2	SB_28, PJ_3
Hap_84	1	SB_31
Hap_85	1	SB_32
Hap_86	1	SB_36
Hap_87	1	SB_37
Hap_88	1	SB_38
Hap_89	1	SB_39
Hap_90	1	SB_40
Hap_91	1	SB_42
Hap_92	1	SB_45
Hap_93	1	PJ_1
Hap_94	1	PJ_2
Hap_95	1	PJ_5
Hap_96	1	PJ_10
Hap_97	1	PJ_14
Hap_98	1	PJ_17
Hap_99	1	PJ_19
Hap_100	1	PJ_20
Hap_101	1	PJ_23
Hap_102	1	**PJ_25**
Hap_103	1	PJ_29

Hap_104	3	MB_1, MB_2, MB_19
Hap_105	1	**MB_4**
Hap_106	2	MB_5, MB_22
Hap_107	1	MB_6
Hap_108	1	MB_7
Hap_109	1	MB_9
Hap_110	1	MB_10
Hap_111	1	MB_11
Hap_112	1	MB_12
Hap_113	1	MB_13
Hap_114	1	MB_14
Hap_115	1	MB_17
Hap_116	1	**MB_18**

*The abbreviation key for localities are the same as in the study area map (Fig. 2.1), *Panulirus homarus homarus* samples are in bold and *Panulirus homarus rubellus* in standard format.

APPENDIX 3

Gel electrophoresis reagents

10 X TBE stock solution

53.89 g Tris-HCL

24.96 g Boric acid powder

1.86 g EDTA

Make up to 500 ml with distilled water, adjust pH to 8.3. Autoclave before use.

Ethidium bromide stock (10 mg/ml EtBr)

10 mg EtBr

1 ml distilled water

Loading dye solution

0.1% (w/v) bromophenol blue

0.02% (w/v) xylene cyanol FF

15% (w/v) Ficoll 9Type 400 (Pharmacia) in water