Genetic Population Structure of spiny lobster *Palinurus delagoae* in the south-western Indian Ocean, and the Evolutionary History of *Palinurus*

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: ..... **Date**: .....

Abstract

This study investigated the evolution of the genus *Palinurus* at the higher and lower taxonomic levels. The population genetics of the spiny lobster, *Palinurus delagoae*, was investigated by making use of a portion of the mitochondrial DNA (mtDNA) control region (547 base pairs) that was sequenced for 285 lobsters from the southeastern coast of Africa (six sites) and 49 lobsters from Walters Shoals (one site), a submerged seamount on the Madagascar Ridge. Lobsters from these two areas shared no haplotypes and differed by at least 27 mutational steps. An analysis of molecular variance showed significant genetic partitioning, and pairwise comparisons suggested that lobsters from Walters Shoals are distinct from those of other sampling areas. Along the south east African coastline there was shallow genetic partitioning between four southern sites (South Africa) and two northern (Mozambique) sites, suggesting two Management Units along the African coast. Female gene flow along the African coast may be propagated by larval dispersal in the Mozambique and Agulhas Currents and counter-current migrations by benthic juveniles along the shelf, but the mtDNA data strongly suggest that larvae at Walters Shoals have been, or are currently still retained by other oceanographic processes. The magnitude of mtDNA divergence among lobsters from the southeastern coast of Africa and those at Walters Shoals, together with the absence of any shared haplotypes between these regions, strongly suggested that these two taxa represent distinct species. The molecular data of the large subunit ribosomal RNA, 16S rRNA (481 bp), and cytochrome oxidase subunit I, COI (520 bp) were then used for a higher level phylogenetic analysis of the genus. A total of 33 individuals (five representatives from each of the six species), and two outgroups (*Projasus parkeri* and *Palinustus*

unicornutus), were subjected to maximum parsimony, maximum likelihood and Bayesian inference analyses. All analyses were conducted on both the separate data sets as well as a combination of the two genes. Bootstrap analyses of the 16S rRNA data resulted in >70% support for the monophyly of all six *Palinurus* species but no support could be obtained for any of the interspecific associations. Likewise, individual analyses of the COI gene resulted in strong support for the monophyly of the species. The combined data (parsimony analyses) increased the resolution considerably and apart from the monophyly of all six species, good bootstrap support was also obtained for associations among species. The topology for the maximum likelihood analyses displayed a more resolved and well supported tree when the basal ingroup taxon *P. elephas* was used to root the tree. The combined Bayesian analyses did not result in a well resolved topology and no significant posterior probabilities could be obtained reflecting the associations among species.

Opsomming

Hierdie studie het die evolusie van die genus *Palinurus* by hoë en laer taksonomiese vlakke ondersoek. Die bevolkingsgenetika studie op die kreef, *Palinurus delagoae*, is ondersoek deur gebruik te maak van 'n gedeelte van die mitokondriale (mtDNA) kontrole-area (547 basispare) waarvan die volgorde bepaal is vir 285 krewe van die suidoos-kus van Afrika (afkomstig van ses verskillende gebiede) en 49 krewe afkomstig van Walters Shoals (een gebied), 'n ondersese berg op die Madagaskar Rand. Krewe van hierdie twee areas deel geen haplotipes nie en verskil met ten minste 27 mutasiestappe. 'n Analise van die molekulêre variansie toon dat daar 'n beduidende genetiese verdeling tussen die twee groepe is en 'n gepaarde vergelyking toon dat krewe afkomstig van Walters Shoals verskil beduidend van krewe uit ander gebiede. Volgens die vlak genetiese verdeling tussen die vier suidelike (Suid-Afrika) en twee noordelike (Mosambiek) gebiede van die suidoos-kus van Afrika wil dit voorkom of daar twee bestuurseenhede langs die kuslyn van Afrika is. Vroulike geenvloei langs hierdie kuslyn kan dalk bevarder word deur larwale verspreiding in die Mosambiek- en Agulhas-Seestrome en teenstroom migrasie van jong bodemwonende krefies op die kontinentale plaat. Die mtDNA data stel egter voor dat kreeflarwes by Walters Shoals deur ander oseanografiese prosesse steeds (of tot onlangs toe) behou word. Die grootte van mtDNA divergering tussen krewe van die suidoos-kus van Afrika en die by Walters Shoals, sowel as die afwesigheid van enige gemeenskaplike haplotipes tussen die twee gebiede, toon met beduidende sekerheid aan dat hierdie twee taksa twee unieke spesies verteenwoordig. Die molekulêre data van die 16S-rRNA (481bp) van die groot ribosomale-subeenheid en

die sitochroom oksidase subeenheid, COI (520bp) is gebruik om 'n hoër resolusie filogenetiese analise van die genus te bepaal. Data van 33 individue (vyf individue uit elk van die ses spesies) en twee buitegroepe (*Projasnus parkeri* en *Palinustus uniconutus*) is geanaliseer deur gebruik te maak van die maksimum-parsimonie, die maksimum-waarskynlikheid en die Bayes-inferensie metodes. Alle analises is uitgevoer op beide die afsonderlike datastelle sowel as op die gekombineerde data van die twee gene. Analise van die 16S-rRNA data deur die skoenlusmetode (steekproef-hersteekproef-metode) toon meer as 70% steun vir die monofilie van al ses *Palinurus* spesies maar dit toon geen steun vir enige van die interspesifieke assosiasies nie. Net so toon individuele analise van die COI geen beduidende steun vir die monofilie van die spesies. Die gekombineerde data (parsimonie) het 'n aansienlike verhoging in die resolusie teweeg gebring en behalwe vir die monofilie van al ses die spesies was daar ook goeie steun deur die skoenlusmetode vir die assosiasie tussen spesies verkry. Die topologie vir die maksimum-parsimonie het 'n goed gesteunde en hoër resolusie boom vir die gekombineerde datastel (sonder die buitegroepe) getoon. Die gekombineerde Bayes-analise het nie 'n soortgelyke boom opgelewer nie en die assosiasie tussen die spesies is nie ondersteun nie aangesien geen beduidende *a posteriori*-waarskynlikheid verkry kon word nie.

Acknowledgements

I am grateful to everyone who has contributed in one or more ways in the completion of this project. In particular, I would like to thank my supervisors Conrad Matthee, Johan Groeneveld and Mike Cherry. Credit also goes go to the Marine and Coastal Management, Cape Town, and Nico du Plooy for collecting the material for this study. Special thanks go out to Krystal Tolley for her contribution, and the students of the evolutionary genomics laboratory at the Stellenbosch University, Zoology Department, for their laboratory advice. Financial assistance for this study was provided by the National Research Foundation (NRF) and the South African Bioinformatics Institute.

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Introduction

General Biology of *Palinurus delagoae*

The spiny lobster *Palinurus delagoae* inhabits the upper slope and deep shelf waters (150 – 600 m depth) of the south-western Indian Ocean. It is found in temperate waters (12 – 18 °C), along the African coast from eastern South Africa (30 °S) to central Mozambique (17 °S) (Fig. 1.1; Holthuis 1991). Geographically isolated populations, seemingly of *P. delagoae*, occur on the narrow south-eastern shelf of Madagascar (Berry & Plante 1973) and at Walters Shoals (32° 52' S, 45° 19' E; Fig. 1.1), a submerged seamount on the Madagascar Ridge, approximately 800 km south of the island. Along the African coast, *P. delagoae* has a long history of exploitation as a targeted species of trap-fisheries (Groeneveld 2000, Palha de Sousa 2001) and as a minor bycatch in multi-species crustacean trawl fisheries (Groeneveld & Melville-Smith 1995, Fennessy & Groeneveld 1997). Fishing off south-eastern Madagascar has been exploratory (Roullot 1988), and high seas vessels report occasional catches from Walters Shoals (pers. obs. JCG).

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Palinurus delagoae is a large (up to 4 kg), long-lived and slow-growing species, and has been the subject of many fisheries biological studies summarized in Groeneveld et al. 2006a. It is found on a wide variety of substrates, ranging from steep rock to areas of organically rich mud or sand substrata with coral fragments (Berry 1971). The species exhibits ontogenetic, long-shore and reproductive migrations, pueruli settling at > 600 m depth and then gradually migrating shorewards to adult habitats at depths of 150 – 350 m (Cockcroft et al. 1995). Egg-bearing females aggregate in shallower depths in summer, moving deeper after spawning in fall and winter (Koyama 1971, Berry 1973, Kondritskiy 1976). Juveniles along the African coast make long-shore migrations up to 500 km far, against the south-westerly flowing Agulhas current (Groeneveld 2002). All these migrations may have far-reaching implications for larval dispersal and gene-flow.

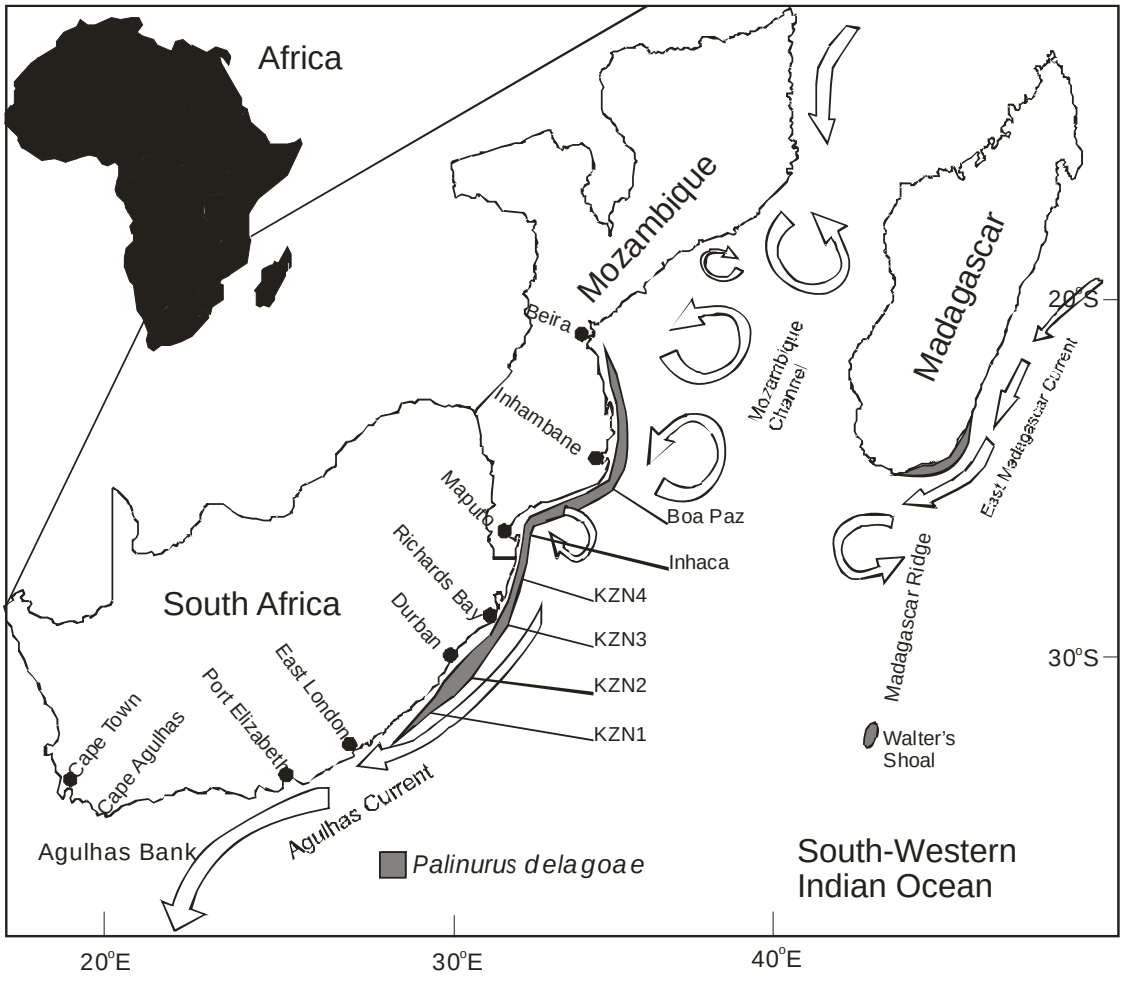


Figure 1.1. Distribution of *Palinurus delagoae* in the south-western Indian Ocean, with sampling sites indicated KZN 1-4, Inhaca, Boa Paz and Walter's Shoal. The arrows indicate the present understanding of the major sea current systems of the region after Lutjeharms (1988) and Ridderinkhof & De Ruijter (2003).

Slatkin (1987) claims the overall geographical range of a species is determined largely by a series of historical accidents, and that a species will extend its range until a barrier stops it from dispersing. For extensive gene flow to occur, the planktotrophic larval stage is generally prolonged in many marine species, which results in genetic (allozyme or mtDNA) differentiation being minimal over extensive areas (Avise 1994). Some examples of marine populations displaying this phenomenon include the sea urchin *Strongylocentrotus purpuratus* along the west coast of North America (Palumbi and Wilson 1990) and the red rock lobster *Jasus edwardsii* (Ovenden et al. 1992) in Australia. Yet according to Avise (1987), Burton (1983, 1986) and Hedgecock (1986), in other marine species with pelagic larvae, large differences do occur among populations over microgeographic and macrogeographic scales. Examples of the latter include the considerable genetic differences found between populations of the American lobster, *Homarus americanus.* This species has a pelagic larval stage of two to eight weeks, and is found between the Atlantic Ocean and the Gulf of St. Lawrence, northeast of the United States (Tracy et al. 1975).

Hedgecock (1986) proposes a few reasons why high dispersal potential gametes or larvae do not always translate into spatial population genetic homogeneity and the high estimates of gene flow. First, levels of gene flow are believed to be low due to physical barriers to larval dispersal, e.g. the influences of oceanic currents in New England and in the southeastern United States may be the cause for genetic differences reported between

regional populations in the American lobster (*H. americanus*). Second, larvae do not always display the passive dispersal propagules that they are assumed to have, but may show active migrational behavior and settlement choices in some species.

Two methods exist in estimating the levels of gene flow in natural populations. Indirect methods involve using allele frequencies and DNA sequence differences (including microsatellites) to estimate gene flow levels among populations and a direct method that uses the estimates of the dispersal distances as well as the breeding success of the dispersers to calculate the amount of gene flow occurring at that time (Slatkin 1987). On the other hand, calculating gene flow in marine environments is different from terrestrial environments because larval mixing of many marine species can be very widespread, so the potential for gene flow is high even in widely separated species (Pollock 1993). For the emergence of new species or genetically distinct populations to form, some degree of genetic isolation needs to develop and as stated by Pollock (1993) this can only be attained by semi-closed oceanographic systems linked together with larval behaviour patterns. This in turn will result in a higher level of local larval retention in the adult stock areas.

Larval dispersal routes of *P. delagoae* and its congener *P. gilchristi* (from the southern coast of South Africa) remain unknown, although a recent study showed mitochondrial DNA panmixia in *P. gilchristi* (Tolley et al. 2005). Likewise, several other marine crustaceans show no/little genetic structure (*e.g. Jasus edwardsii,* Ovenden et al. 1992; *Nephrops norvegicus,* Stamatis et al. 2004; *Farfantepenaeus duorarum,* McMillen-

Jackson & Bert 2003, 2004), probably due to wide larval dispersal in the marine environment. On the other hand, there is growing evidence that not all crustaceans follow this pattern and fragmented gene pools have been recorded in tiger prawns *Penaeus monodon* (Duda & Palumbi 1999, Benzie et al. 2002) and several species of rock and spiny lobsters (*Jasus* (*Sagmariasus*) *verreauxi,* Brasher et al. 1992, Ovenden & Brasher 2000; *Panulirus argus,* Silberman et al. 1994, Sarver et al. 1998; *Panulirus interruptus*, Perez-Enriquez et al. 2001).

At least two factors suggest that *P. delagoae* might exhibit spatial genetic structure across its distribution. First, in some instances breeding individuals are separated by large distances and deep intervening waters, and this distribution pattern exposes larvae to three different ocean current systems, *i.e*. the Mozambique and Agulhas currents along Africa and the East Madagascar current off Madagascar (Lutjeharms 1988). Secondly, *P. delagoae* exhibits morphological variation across its range, with specimens from Madagascar (and Walters Shoals, pers. obs. JCG) more spinose than those along Africa (Berry & Plante 1973). Even the African shelf individuals are distinctly variable, as highlighted by Barnard (1926) in the initial description of the species as two varieties, *natalensis* from eastern South Africa and *delagoae* from Mozambique. A later revision of the genus in the south-western Indian Ocean synonymised the two varieties and raised them to specific rank (*P. delagoae*). The Madagascan population was also included in the latter species, but with uncertainty (Berry & Plante 1973). Interestingly a similar pattern is observed when the geographic distribution of the *Panulirus homarus* (Berry 1974), a shallow-water rock lobster, is considered. In the latter case, it is almost identical

to the situation observed in *P. delagoae* and might therefore be indicative of some vicariance event.

Aims and Objectives

The mitochondrial DNA is simple in structure and function when compared with the complex nuclear genome (Ovenden & Brasher 1994). Being inherited maternally, the mitochondrial genome has a higher rate of DNA sequence evolution than most nuclear genes (Brown 1985). The aim of this study was to determine whether genetic structure could be detected for *Palinurus delagoae* by sequencing the hypervariable region I of the mtDNA control region. Inferences regarding the contemporary genetic population structure, combined with information on life-history of the species and the oceanographic history of the south-western Indian Ocean are used to infer larval distribution patterns and recent evolutionary history. Given that the Mozambique and Agulhas currents are strong, intervening currents and juveniles do migrate along the African coast up to 500km against the Agulhas current, we would expect to find that the KZN population is poorly differentiated to the Mozambique population. As for the Walter's Shoal population, they could be distinctly different from the southern African populations due to physical barriers, the East Madagascar current and the large distance between the two areas. Given the commercial importance of this species, the genetic data could be essential for directing future management decisions.

Materials and Methods

Genomic DNA extraction and nucleotide sequencing

Samples were collected by fisheries observers stationed on a trap-fishing vessel operating off South Africa and Mozambique in 2004. Samples from Walters Shoals were obtained from a Spanish vessel fishing with trammel nets. Samples comprising lobster leg were stored in 96% ethanol for later extraction. To determine the population genetics structure of *P. delagoae*, a number of samples from various areas in the study site was required (Table 1.1). The samples obtained, which were not based on museum specimens, covered approximately 75% of the total distribution range of the species. Genomic DNA was initially extracted using the standard phenol-chloroform extraction method (Sambrook et al. 1989) but this proved to be unsuccessful. The Chelex^{TM} 100 Extraction Method was then used and this produced improved results (Tolley et al. 2005, Gopal et al. 2006). Chelex^{TM} is a polystyrene ion exchange resin that soaks up all the contaminants, leaving behind the DNA to be amplified by standard Polymerase Chain Reaction (PCR) procedures. Total genomic DNA was extracted from the leg tissue, using a standard digestion buffer containing 5% Chelex^{TM} 100 (Bio-Rad Laboratories) and 10-15 µl proteinase-K (10mg/ml). Digestions were performed for one hour. The digest was subsequently spun down, the supernatant removed, and used directly in PCR. A portion of the control region was amplified in a 25 µl reaction volume containing 5 µl of the supernatant with the genomic DNA, 0.25 µM of each primer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1 X thermophilic Buffer (50 mM KCl, 10 mM Tris-HCl, pH 9) and 0.25 units of thermostable DNA polymerase (Southern Cross Biotechnology). The primers

used for the amplification (L13473 and H14306) were designed to amplify the control region of *Palinurus gilchristi* (Tolley et al. 2005). All haplotype sequences have been submitted to Genbank (DQ269501 – DQ269660).

Table 1.1. *Palinurus delagoae* sampling localities from six regions off southern Africa and at Walters Shoals. Sample sizes (n) and sampling dates (month & year) are given.

Locality	n	Sampling Date
KZN-1	50	5/2004
KZN-2	77	1 & 3/2004
KZN-3	38	7/2004
KZN-4	46	9/2004
Inhaca	35	2/2005
Boa Paz	39	2/2005
Walter's Shoals	49	11/2004
Total	334	

Analytical Methods

Haplotype diversity (*h*) and nucleotide diversity (π) were calculated in Arlequin 2.0 (Schneider et al. 2000). Relationships among haplotypes were investigated using parsimony median-joining networks and the program Network 4.1 (Bandelt et al. 1999). Pairwise analyses of molecular variance (AMOVA) were run in Arlequin 2.0 where levels of variation (Φ_{ST}) among and within the seven sampling sites were estimated. The Tamura-Nei model of nucleotide substitution was used and the α value of the gamma distribution was estimated using maximum likelihood in PAUP* 4.0b10 (Swofford 2002). The significance of the resultant Φ_{ST} statistics were tested with 10 000 permutations. A spatial analysis of molecular variance (SAMOVA) was performed to

further test for population structure (Dupanloup et al. 2002). Isolation by distance, or the relationship between genetic and geographic distance, was investigated using the Mantel test (Mantel for Windows 1.11; Calvalcanti 2000). Fu's *Fs* test (Fu 1997) was used to test for mutation-drift equilibrium. Populations that have recently undergone a demographic change (such as expansions) are expected to be out of mutation-drift equilibrium and a significant negative value would be obtained (Fu 1997, Schneider et al. 2000). The possibility of demographic change was also investigated using mismatch distributions (Harpending et al. 1998, Schneider & Excoffier 1999). To obtain a rough estimate of the timing of demographic change, the model proposed by Rogers and Harpending (1992) was used. In the absence of a calibrated clock for lobster control region and to present a range of reasonable values, 2 mutation rates were employed (μ = 5% and 15%; per million years - Wilson et al. 1985). Generation time was set at four years. The equation $T = \tau / 2u$ was used to estimate the time since demographic change (T), whereby τ is the age of the demographic change in mutational units estimated in the mismatch distribution, and u is the sum of the of the per-nucleotide mutation rate per generation in the region sequenced (Rogers & Harpending 1992). Thus, the estimate of u varied according to range of mutational rates applied (u_1 at 5% = 0.0000547 and u_2 at 15% = 0.000164).

Results

Analyses of 547 base pairs of 334 individuals resulted in 160 haplotypes (Appendix I) of which 74% were unique (35% of all individuals had unique haplotypes). Most haplotypes differed by a single site change from each other. There were no shared haplotypes between lobsters from Walters Shoals and the African coast, and at least 27 mutational steps separate these two geographic regions. For network construction, the haplotypes from these two assemblages could not be linked with more than 95% confidence, so networks were constructed separately for African and Walters Shoals populations. A large number of haplotypes were shared among the African sampling sites and the network contained many reticulations (alternate equally likely solutions). Due to the large number of reticulations, only one of the 54 possible shortest networks is displayed (Fig. 1.2a). For Walters Shoals, only three possible shortest solutions were obtained, and these are displayed as a network (Fig. 1.2b).

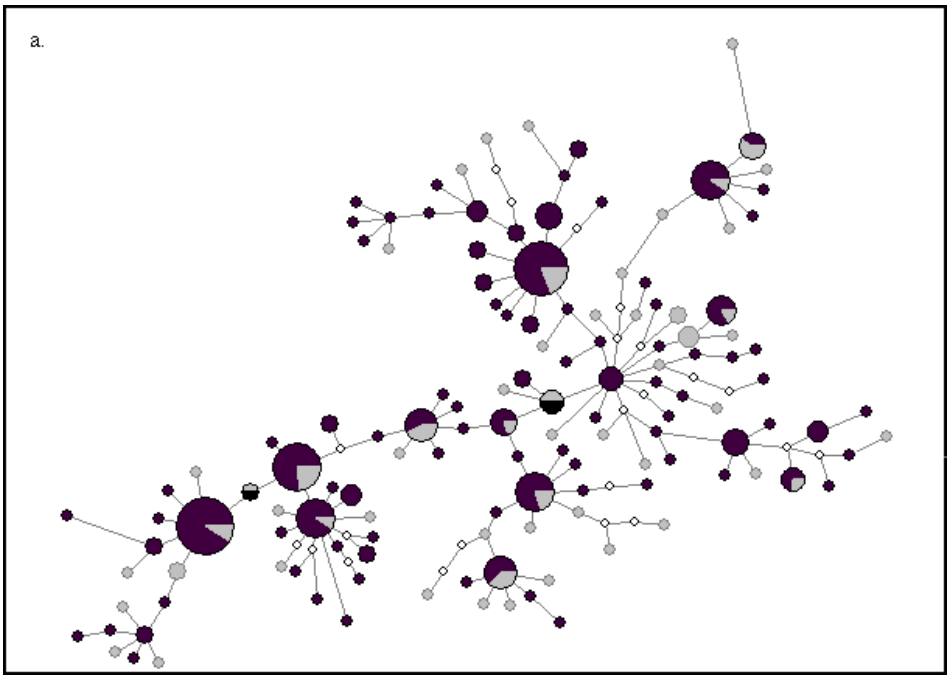
Total nucleotide diversity (π) for all sites, including Walters Shoals, was 0.022 ($\pm$ 0.011) and was lower within the individual sampling sites, ranging from 0.006 ($\pm$ 0.004) for Walters Shoals to 0.009 ($\pm$ 0.006) for Boa Paz (Table 1.2). The average haplotype diversity for all the sites combined was high (0.983 $\pm$ 0.002) and varied only slightly when sampling sites were considered separately (Table 1.2).

Table 1.2. Haplotype diversity (*h*) and nucleotide diversity (π) with 95% confidence intervals are given for the various sampling localities for *Palinurus delagoae*.

Locality	*h* (95%CI)	π (95%CI)
KZN-1	0.957 (±0.015)	0.008 (±0.005)
KZN-2	0.977 (±0.008)	0.009 (±0.005)
KZN-3	0.966 (±0.015)	0.008 (±0.004)
KZN-4	0.980 (±0.010)	0.007 (±0.004)
Inhaca	0.988 (±0.010)	0.009 (±0.005)
Boa Paz	0.999 (±0.006)	0.009 (±0.006)
Walters Shoals	0.960 (±0.012)	0.006 (±0.004)
Total	0.983 (±0.002)	0.022 (±0.011)

The Φ_{ST} value across all seven sites showed significant genetic partitioning ($\Phi_{ST} = 0.69$; $p < 0.001$), and pairwise comparisons among sampling sites showed the largest differences between the African coast and Walters Shoals (Table 1.3). The Φ_{ST} estimated among the six African sites (excluding Walters Shoals) was substantially lower ($\Phi_{ST} = 0.01$; $p < 0.05$), although some pairwise comparisons were significant between the KZN and the Mozambique sampling sites (Table 1.3). This was supported by the SAMOVA, which suggested the African coast is structured into northern (Boa Paz & Inhaca) and southern (four sites in KZN) populations (Table 1.4). Although pairwise comparisons of some sites within each of these "populations" also showed significant differences, the divergence between these two geographic groups is greater than if each site is considered a separate population (Table 1.3). The Fu's Fs test and mismatch distributions were run separately for Walters Shoals, the combined KZN samples (southern population) and combined Boa Paz & Inhaca samples (northern population). Fu's *Fs* test was significant for each of the comparisons, suggesting a recent demographic change in each population

of this species (Table 1.5). The mismatch distributions point to a population expansion in each area, as indicated by the strong expansion waves (Fig. 1.3). The Mantel test showed no significant isolation by distance regardless of whether Walters Shoals was included (r^2 = 0.91, ns) or excluded (r^2 = 0.23; ns).



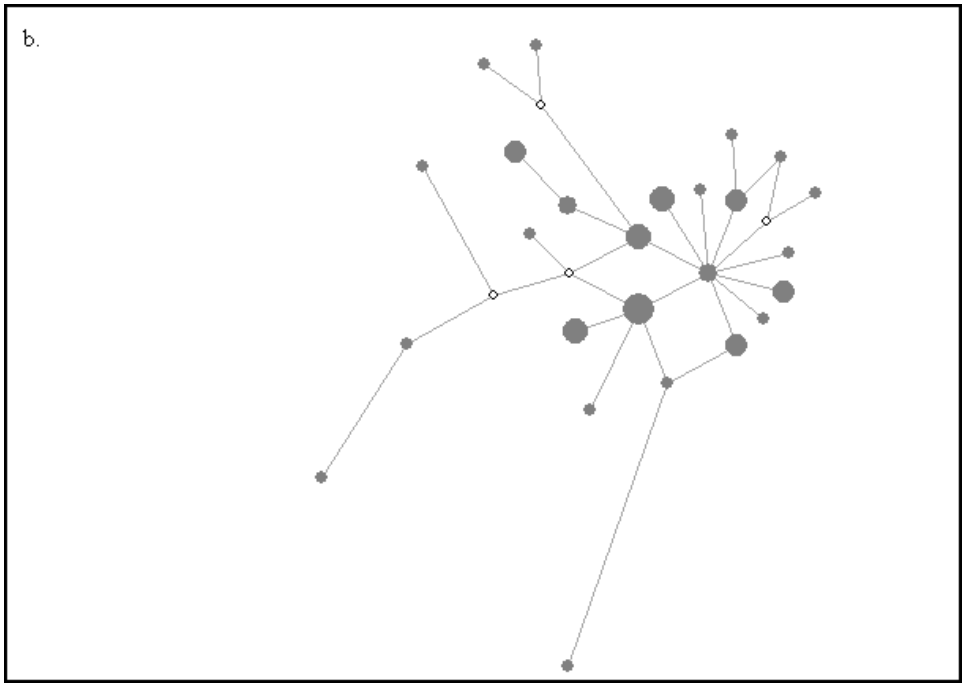


Figure 1.2. Median-joining network for *P. delagoae* from a) the African coast and b) Walters Shoals. The size of the circles is proportional to the frequency in which each haplotype occurs, and the length of the branches is proportional to the number of base changes between haplotypes. White open circles represent median vectors. The shortest branches indicate one base change. For the African populations, for each haplotype, the frequencies in the northern population (Boa Paz & Inhaca) are indicated in grey, and the southern population (KwaZulu-Natal) is indicated in black.

Table 1.3. Pairwise Φ_{ST} values between sampling areas for *P. delagoae* (bottom of matrix) and significance levels (top of matrix). ns=not significant.

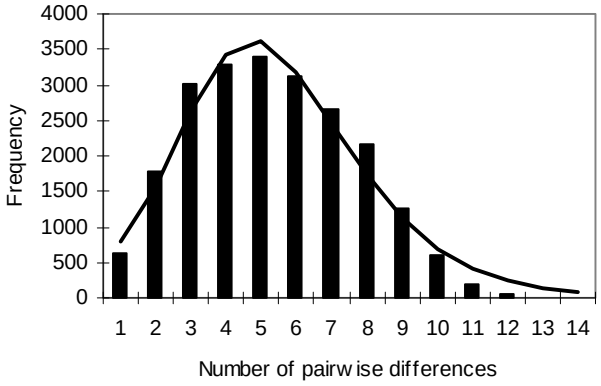
		1	2	3	4	5	6	7
1	KZN-1	---	ns	ns	ns	p<0.05	p<0.05	<0.001
2	KZN-2	0.004	---	ns	ns	p<0.05	p<0.05	<0.001
3	KZN-3	0.000	0.010	---	ns	ns	ns	<0.001
4	KZN-4	0.000	0.007	0.000	---	ns	p<0.05	<0.001
5	Inhaca	0.027	0.025	0.000	0.020	---	ns	<0.001
6	Boa Paz	0.033	0.023	0.010	0.030	0.000	---	<0.001
7	Walters Shoals	0.895	0.889	0.900	0.900	0.891	0.883	---

Table 1.4. Results of SAMOVA showing the F values for the sampling areas of *P. delagoae*. The number of hierarchical groups with the sampling areas in each group is given. Partitioning of variance (F_{SC}) is highest when there are two hierarchical groups (shown in bold).

# Groups	Sampling areas	F_{SC}	F_{ST}	F_{CT}
2	1. Boa Paz + Inhaca 2. KZN-2 + KZN-1 + KZN-4 + KZN-3	0.001	0.025	**0.024**
3	1. Boa Paz + Inhaca 2. KZN-2 3. KZN-1 + KZN-4 + KZN-3	-0.004	0.017	0.021
4	1. Boa Paz 2. Inhaca 3. KZN-2 4. KZN-1 + KZN-4 + KZN-3	-0.004	0.016	0.020
5	1. Boa Paz + Inhaca 2. KZN-3 3. KZN-2 4. KZN-4 5. KZN-1	-0.004	0.013	0.017

Estimates of τ differed only slightly according to population, all with overlapping 95% confidence intervals (CI) (Table 1.5). The τ values and their 95% CIs were used to provide a rough date of the age since the population expansions, as were the range of values for *u*. Estimates based on the lower mutation rate (5% per million years) would suggest population expansions occurred prior to the last glacial maximum (LGM), between *ca*. 30,000 - 40,000 years ago (Table 1.5). The higher mutation rate would suggest more recent expansions after the LGM, on the order of circa (ca). 9,000 – 13,000 years ago.

a.



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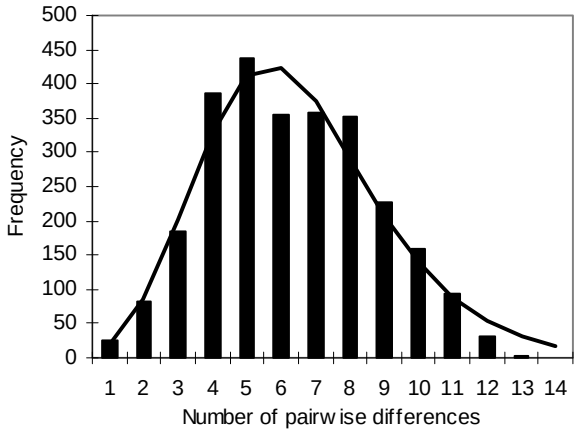

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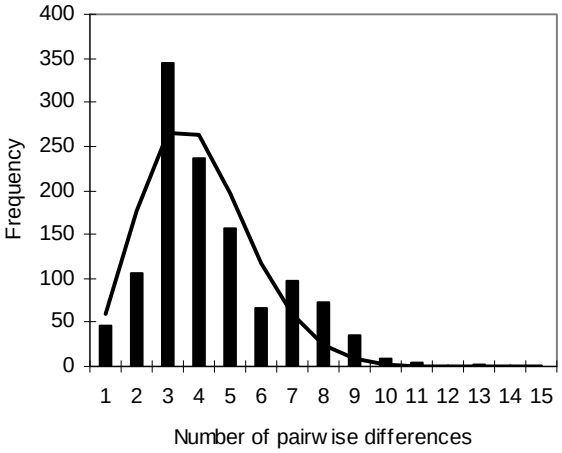

Figure 1.3. Distribution of pairwise differences between individuals for mtDNA control region for *P. delagoae* for a. southern population (KwaZulu-Natal), b. northern population (Inhaca & Boa Paz), and c. Walters Shoals. Black bars indicate the observed frequencies of pairwise differences, and the solid line is the expected distribution given a recent demographic change.

Table 1.5. Results of the F_S test (with *p* values) for *P. delagoae* from three populations defined by SAMOVA (Southern = KZN1-4, Northern = Inhaca & Boa Paz), and estimates of τ (95 % CI) from the mismatch distribution. Estimated times since expansion (T) for each population are given (rounded to nearest 100 years) for the range of mutation rates (5% and 15%) and the ranges of τ.

	F_S	τ (95% CI)	T (5%)	T (15%)
Southern coast	-25.42 (p<0.001)	3.49 (1.44 – 9.96)	32,000 (13,200-91,400)	10,600 (4,400-30,400)
Northern coast	-25.44 (p<0.001)	4.20 (2.10 – 7.52)	38,532 (19,300-69,000)	12,800 (6,400-22,900)
Walters Shoals	-16.71 (p<0.001)	2.99 (1.36 – 3.86)	27,430 (12,500-35,400)	9,100 (4,100-11,800)

Discussion

These mtDNA results suggest that the spiny lobsters at Walters Shoals are genetically distinct from those on the continental shelf off South Africa and Mozambique. Furthermore, there is shallow, but significant, genetic partitioning among lobsters from the southern (KZN 1-4) and northern (Inhaca & Boa Paz) sites of the African coast as stated in the objectives. The observed genetic structure supports earlier morphological studies that suggested two populations along the African coast (Barnard 1926, Berry & Plante 1973), and the population division suggested by the SAMOVA of a southern (KZN) and northern group (Inhaca & Boa Paz) corresponds to the var. *natalensis* from eastern South Africa and var. *delagoae* from Mozambique originally described by Barnard (1926). No specimens were available from the narrow south-eastern shelf of Madagascar, and this population can therefore not be placed with certainty. However, morphological similarities with specimens from Walters Shoals suggest a closer relationship between Walters Shoals and Madagascan populations than either has with the populations of the African shelf (pers. obs. JCG). Based on this genetic population study, and on subsequent morphological comparisons between individuals from the south-east African coast and Walters Shoals, a new spiny lobster species, *Palinurus barbarae* (Groeneveld et al. 2006b) has now been described from Walters Shoals.

Although the genetic difference observed between the African and Walters Shoals spiny lobsters could have arisen due to long term isolation (no mixing of adult/larval individuals), it is equally plausible that there was a past founder event at Walters Shoals

followed by the complete absence of gene flow. There is a deep oceanic trench between Walters Shoals and the African shelf, in which adult lobsters are not found, and this precludes adult migrations between the two areas. Any hypothesis on larval dispersive potential between the two areas would have to consider, foremost, the influences of the Mozambique Current and the two western boundary current systems of the south-western Indian Ocean, the Agulhas Current off south-eastern Africa and the East Madagascar Current off eastern Madagascar (Fig. 1.1). These currents flow along the respective shelf-edges and their inshore circulation may play a defining role in the movement of larvae and adults of many taxa (Hutchings et al. 2002). Importantly, however, knowledge of present-day oceanic processes in the south-western Indian Ocean and the life-history characteristics of spiny lobsters can provide some clues, but the interpretations of the evolutionary history of *Palinurus* are unavoidably speculative because of the highly dynamic nature of the marine environment through time.

Drift tracks of weather buoys in the south-western Indian Ocean have shown that water from the East Madagascar Current reach the Agulhas Current in only a very intermittent and irregular way (Lutjeharms 1988). Larvae from Madagascar may cross the Mozambique Channel in these water bodies, and such a one-way dispersal was suggested by Berry & Plante (1973). The authors argued that this was supported by the higher morphological variability of African shelf populations compared to the Madagascan *P. delagoae*. Nevertheless, a conjecture was proposed by Lutjeharms et al. (1981), that the East Madagascar Current retroflects into the central Indian Ocean on passing the southern tip of Madagascar (Lutjeharms 1988), and may deflect a proportion of Madagascan (and

possibly Walters Shoals) larvae away from the African shelf populations. Larvae originating from the African shelf are unlikely to cross the Mozambique Channel, because the Agulhas Current will probably entrain larvae south-westwards along the African coast, and away from Madagascar. The genetic structure detected in the present study supports the hypothesis that larval dispersal across the Mozambique channel is limited (Berry & Plante 1973), but more importantly the lack of shared mtDNA haplotypes and large Φ_{ST} values suggest that there has been prolonged historical isolation between the African shelf populations and those at Walters Shoals.

Unfortunately, no tissue samples were available from the south-eastern shelf of Madagascar to investigate the possibility of mtDNA panmixia between Madagascan and Walters Shoals populations. Such information would allow for an assessment as to whether larvae in the East Madagascar Current regularly reach Walters Shoals. Alternatively, if these two geographic areas are genetically differentiated, the conclusion would be that larvae at Walter's Shoals are retained in the vicinity of the seamount.

The spatial analysis of molecular variance (SAMOVA) suggested that sampling sites along the African coast could be partitioned into northern (Inhaca & Boa Paz) and southern (KZN 1-4) populations. This places the geographic partitioning between the two populations in the vicinity of the interface between the Mozambique and Agulhas Currents, a potential contributor to creating and maintaining the shallow genetic structure among these populations. However, some exchange between the two systems is likely as benthic juvenile migrations from KZN to Inhaca have been reported from tag-recapture

data (Groeneveld 2002). Also, water masses from the Mozambique Current feed into the Agulhas Current (Ridderinkhof et al. 2001) and larval exchange will doubtlessly occur. It is tempting to speculate why more larvae (and adults) have been retained within each current system than have been exchanged between the two systems. The Mozambique Current is described as a series of anti-cyclonic eddies moving slowly southwards along the shelf-edge of Mozambique (Ridderinkhof et al. 2001, De Ruijter et al. 2002). It is possible that some larvae are retained in this system through a combination of life-history attributes and behaviour. Characteristics that would enhance larval retention in a specific area include: 1) release of larvae inshore of strong currents, 2) a shortened larval drifting phase to reduce over-dispersal and loss, and 3) vertical migrations and horizontal swimming for positioning in the water-column. *Palinurus delagoae* females move inshore to 150 – 160 m depth to spawn (Koyama 1971) and eggs are large with larvae hatching in an advanced stage of development (Pollock & Melville-Smith 1993, George 2005), thus shortening the drifting larval phase to few stages and a short duration (information available for a congeneric indicates < ten stages and 65 days for *Palinurus elephas* compared to > 15 stages and > 200 days for *Jasus* and *Panulirus*, Kittaka 1997, Kittaka et al. 2001). Diel vertical migrations (Booth & Phillips 1994) and directed horizontal swimming of late stage phyllosomas contributed to retention of *Jasus edwardsii* larvae in an eddie (Chiswell & Booth 1999), and it is suggested that *P. delagoae* use similar mechanisms to retain a proportion of larvae in the eddies of the Mozambique Current, upstream of the southern KZN population, thus giving rise to the shallow genetic partitioning between the two populations. *Palinurus gilchristi* larvae off southern South Africa appear to be likewise retained inshore of the Agulhas Current,

where downstream drift is redressed by return migrations of juveniles (Groeneveld & Branch 2002).

The mismatch distributions and Fu's F test suggest that all three populations have undergone recent population expansions dating back between *ca*. 9,000 - 40,000 years, depending upon the mutation rate used (Table 1.5). At the faster mutation rate of 15% per million years, it would appear that *P. delagoae* underwent a very recent population expansion (*ca*. 9,000 – 13,000 years ago), possibly in connection with climatic changes since the last glacial maximum (LGM). If the mutation rate is slower (5% per million years) the date is pushed back substantially, pre-dating the height of the last glacial maximum (LGM). Given that the mutation rate could also be anything in between these values, the interpretation of the processes that could have led to a population expansion is rather difficult. Hypothetically, if the more recent date is accepted then a similar process to that hypothesized for the congener *P. gilchristi* could explain the expansion. In the latter case, a previously exposed continental shelf became inundated after the end of the LGM, providing a new and larger habitat for lobsters, allowing the opportunity for population expansion to take place. This could have been the case for Walters Shoals as well, as it was exposed during the LGM, and became inundated only within the present interglacial period (Anderson et al. 1988).

It is interesting to observe that the congener, *P. gilchristi* displays some notable differences in population genetic structure based on the hypervariable region I of the mtDNA control region (Tolley et al. 2005) to *P. delagoae* despite their geographic

proximity. The number of polymorphic sites for *P. delagoae* and *P. gilchristi* are 121 and 52, respectively, suggesting *P. delagoae* is much more diverse than *P. gilchristi*. Both the haplotype (*h*) and nucleotide diversity (π) for *P. delagoae* ($h = 0.98$, $\pi = 0.022$) was higher than that of *P. gilchristi* ($h = 0.31$; $\pi = 0.004$). In addition, the absence of any population structure in *P. gilchristi* is in sharp contrast to the separation among *P. delagoae* populations. The higher diversity and evidence of population structure in *P. delagoae* could indicate this species has persisted longer in its present location than has *P. gilchristi*. This could be due to the hypothesis that there has been a population expansion of *P. gilchristi* that post-dates to the Last Glacial Maximum (LGM), and that the lack of population structure in that species could be due to a leading edge effect during recolonization of the Agulhas Bank. Since the end of the LGM, the sea level increased by more than 150 m, inundating most of the Agulhas Bank (Tolley et al. 2005), implying that the *P. gilchristi* population may have expanded to occupy the larger habitat as it became available. Conversely, *P. delagoae* occurs in moderately deep waters of up to 600 m and may not have been affected by the LGM. Assuming that after the LGM, only a percentage of *P. delagoae's* original habitat survived, the population expansion that this species underwent started earlier than that for *P. gilchristi*. When the mismatch distribution curves with the statistics between the two species is compared, the tau values show a significant difference (*P. gilchristi* = 1.7, *P. delagoae* = 3 - 4). This indicates that any expansion for *P. delagoae* occurred more in the distant past than *P. gilchristi* (assuming similar mutation rates). The expansion of *P. delagoae* is shifted to the right in the distribution compared to *P. gilchristi*, suggesting the expansion is older for *P. delagoae*. Furthermore, the haplotype network for *P. gilchristi* is a lot simpler with many

fewer reticulations, and is star-shaped, with most of the unique haplotypes closely related to the common central haplotype (Tolley et al. 2005), whereas the network for *P. delagoae* is far more complex indicating the recent vs. older expansion.

In summary, it appears that despite the potential for high gene flow in marine species that have larval stages carried by oceanic currents, genetic panmixia cannot be assumed. In fact, the currents themselves may serve to reduce gene flow between regions even when the distribution seems continuous. From this study and the hypotheses made, the pronounced genetic differences shown between the African coast and Walters Shoals populations resulted in the description of *P. barbarae* at Walters Shoals as the sixth species in the genus. In addition, spatial genetic structuring between the southern (KZN) and northern (Inhaca & Boa Paz) populations of *P. delagoae* along the African coast, while small, is significant. Taken together with the morphological differences in the lobsters of these two areas, it is suggested that the two regions could be considered at least separate Management Units (MUs). Further assessment covering the entire range of *P. delagoae* will help to establish whether this population structure holds, and whether the species should be managed as two separate populations along the African coastline.

Chapter Two:
Mitochondrial DNA phylogeny of the spiny lobster genus, *Palinurus*

Introduction

Taxonomy of African Lobsters

The southern African coastline is inhabited by spiny lobsters (Family: Palinuridae) representing several genera, i.e. *Jasus, Linuparus, Palinurus, Palinustus, Panulirus, Projasus* and *Puerulus* (Holthuis 1991). The Palinuridae originally evolved from the Pemphicidae in the early Mesozoic era (George and Main 1967, Pollock 1995), with two evolutionary lineages (Stridentes and Silentes) diverging approximately 150 million years ago (Pollock 1995). Two genera, *Jasus* and *Projasus* belong to the Silentes group (characterized by the absence of sound-producing stridulatory apparatus), whereas all the others are members of the Stridentes (sound-producing; Pollock 1995). The two evolutionary lineages have retained a variety of common characteristics, such as a long-lived phyllosoma larvae phase and similar adult morphology. Glaessner (1969) proposed that *Palinurus* has a fossil record dating back to the Cretaceous period, which makes it more 'ancient' than the *Panulirus* species which have been thought to have a recent post-Miocene origin (George and Main 1967, Pollock 1995).

Distribution of *Palinurus* species

Traditionally the genus *Palinurus* consists of five recognized species, all of which are found in moderately deep-water. *Palinurus elephas* has a wide geographical distribution

in the Mediterranean Sea, eastern North-Atlantic down south to Cape Bojador in Morocco (Ceccaldi & Latrouite 2000). Found among hard substrates and rocky bottoms, *P. elephas* lives between the sea shore and as deep as 150 – 160 m (Hunter 1999), where maximal abundance is between 50 and 100 m. *Palinurus elephas* is thought to be a relic of an ancestral form of *P. vulgaris*, since fossil records from the upper Cretaceous sediments in the same region have been found in an area comprising the Tethys Sea - a seaway linking the Indian and Atlantic Oceans in pre-Miocene times (Pollock 1995, Glaessner 1969). The five remaining species include *P. mauritanicus* (pink spiny lobster) distributed in the Atlantic from Ireland (Mercer 1973a) to Senegal (Vincent-Cuae 1958, Maigret 1980), and in the western parts of the Mediterranean (Fig 2.1) where it can be found from 40 to 600 m, with highest densities at 150 - 300 m (Ceccaldi & Latrouite 2000). Even though differences in their ecological niches do exist, for example depth (Ceccaldi & Latrouite 2000), more studies are needed to understand the distribution of *P. elephas* and *P. mauritanicus*, especially where they occur at the same latitude. *Palinurus charlestoni* is endemic to the Cape Verde Archipelago (Fig 2.1), and occurs at depths ranging from 100 to 400 m (Forest & Postel 1964). *Palinurus delagoae* occurs along the south-eastern coasts of South Africa and Mozambique, and possibly southern Madagascar at depths of 100 – 600 m. *Palinurus gilchristi* is endemic to the southern coast of South Africa, occurring at depths of 50 – 200m (Pollock et al. 2000, Fig. 2.1). Based on the findings in Chapter One, which deals with population genetic structure of *P. delagoae* from South Africa and Mozambique and Walters Shoals (isolated on a sea mount south of Madagascar, Fig. 2.1) a sixth species, *P. barbarae* has now been described based on genetics, morphology and distribution (Groeneveld et al. 2006). The

taxon is currently confined to a submerged seamount, Walters Shoals, south of Madagascar (Fig 2.1). The ecology of the latter species is unknown.

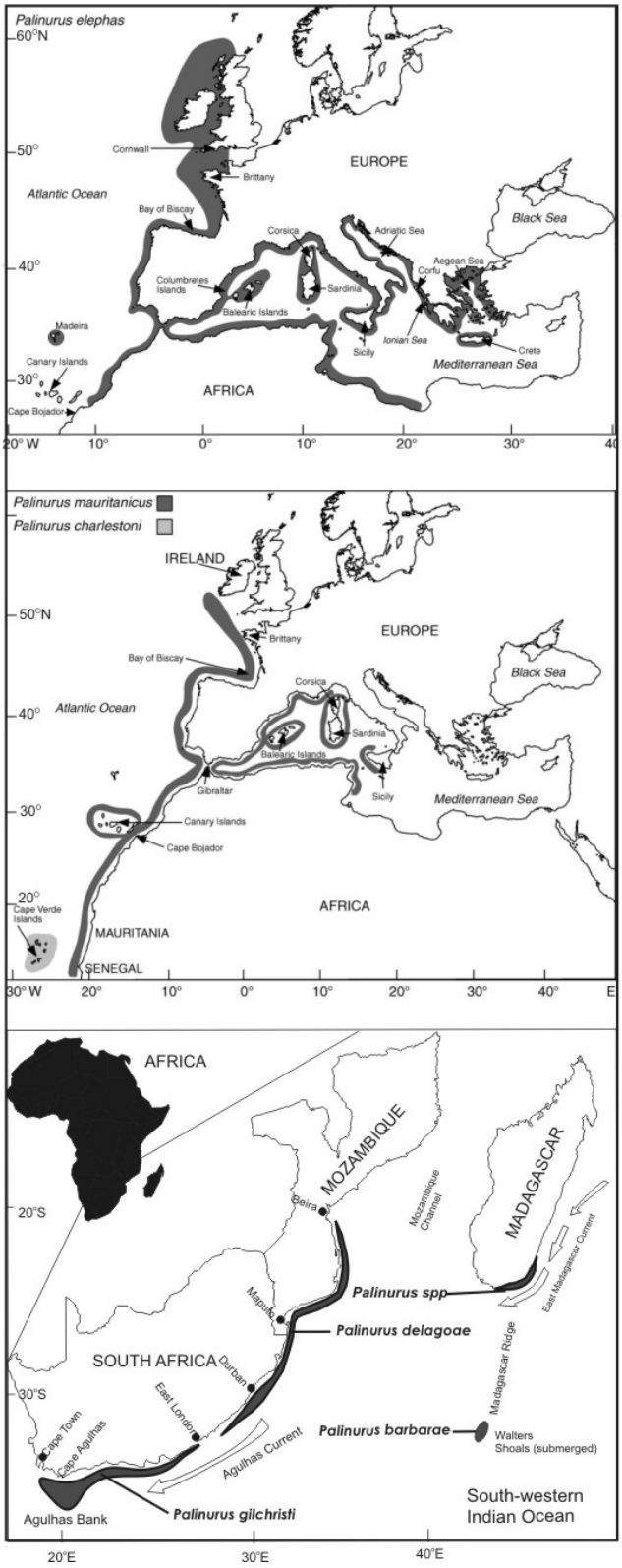

Figure 2.1 Distribution of the six extant species belonging to the *Palinurus* genus

Palinurus Morphology

The morphological differences among *Palinurus* species and even between the Atlantic and Indian Ocean taxa are limited. Nevertheless, Forest and Postel (1964) concluded that the three species found in the north-east Atlantic are distinct from the two species recognized in the Indian Ocean (*P. barbarae* was not considered). The three Atlantic species can be separated based on the shape, curve and spination of the supraorbital horns and *P. delagoae* can easily be distinguished from *P. mauritanicus* and *P. charlestoni* by the relative lengths of the carapace and abdomen. Based on the limited morphological differences between the Atlantic and Indian Ocean adults of *Palinurus*, Berry & Plante (1973) argue that on the grounds of differences in colour, morphology and geographical isolation of adults, the Malagasy population should be seen as a separate species from *P. delagoae*, especially also if *P. delagoae* is to be considered different from *P. gilchristi.* The Malagasy and southeast African populations, however, more closely related to each other (based on morphology) than either are to *P. gilchristi*. No evidence exists of any intergradation between populations of *P. gilchristi* and *P. delagoae* from Natal, although populations of *P. delagoae* from Natal and Mozambique display some morphological variations indistinguishable from those of the Malagasy population. Berry & Plante (1973) argued that this could be due to introgression between the Malagasy population and the southeast African population, caused mainly by the westward current transporting the phyllosoma larvae, suggesting that there is gene flow from the Malagasy population to the southeast African one and not to any great extent in the opposite direction. However, interestingly a similar pattern is observed when the geographic distribution of

the *Panulirus homarus,* a shallow-water rock lobster, is considered (Berry & Plante 1973). *Palinurus barbarae* is closely related to *P. delagoae* based on both morphology and mitochondrial data (*cf.* Tolley et al. 2005, Gopal et al. 2006, Groeneveld et al. 2006). When compared to *P. delagoae*, *P. barbarae* has a more spinose carapace, its posterior carapace rim ends in a smooth groove and the species has a raised angular lip. The number of peduncular spines and shape of the pleural somites further differentiates the two species.

Palinurus phylogeny

No known studies to date have comparatively addressed the genetic, larval or adult, assessments for the six *Palinurus* species, although George (2005) believes that two groups may exist when comparing the number of transverse abdominal grooves present between the three Atlantic species and the two (three – *P. barbarae* described after 2005) south-west Indian Ocean species. Phylogenetic approaches have been used with great success to test biogeographic hypotheses or to examine patterns of speciation among lobsters. The phylogenetic relationship of 21 *Panulirus* taxa, using nucleotide sequence data, revealed that two major lineages exist and that it also corresponds to the morphology of that genus (Ptacek et al. 2001). Aspects of *Jasus* speciation and phylogenies were tested by genetic analyses using allozyme frequencies (Smith et al. 1980, Booth et al. 1990) and restriction-site variation in the mitochondrial genome (Ovenden et al. 1992, Ovenden & Brasher 1994). Other phylogenetic studies done on Decapoda include the phylogenetic relationships of *Jasus* species (Brasher et al. 1992)

and genera of the clawed lobster family Nephropidae (Tam & Kornfield 1998), and the use of molecular data to explore evolutionary relationships among species of the pinnothrid crab genus *Austinixa* (Harrison 2004).

Aims and Objectives of this study

The usage of mitochondrial gene regions have previously proven useful for the reconstruction of detailed phylogenies that are independent of morphological characters and free from the environmental influence that may bias morphologically-based topologies (Simon et al. 1994). The aim of this part of the thesis is to infer a phylogeny of the *Palinurus* genus with respect to the Southern Hemisphere Indian Ocean taxa (*P. gilchristi*, *P. barbarae* and *P. delagoae*) and the Northern Hemisphere Atlantic Ocean taxa (*P. elephas*, *P. charlestoni* and *P. mauritanicus*) based on mitochondrial DNA sequences. Results of the phylogenetic analysis are based on nucleotide sequence data from two regions of the mitochondrial genome, the large-subunit ribosomal RNA (16S rRNA) and the cytochrome oxidase I subunit (COI).

Materials and Methods

Genomic DNA extraction and nucleotide sequencing

All samples of *P. gilchristi* and *P. delagoae* from South Africa and Mozambique were collected by setting baited traps. The *P. barbarae* sample from Walters Shoals was obtained from a Spanish vessel fishing with trammel nets. Samples of *P. elephas*, *P. mauritanicus* and *P. charlestoni* were sourced from individual researchers in Spain (D. Diaz, IEO, Palma) and France (D. Latrouite, IFREMER, Brest; See Appendix II). Samples comprising lobster legs were stored in 96 % ethanol for later extraction. The specimens were all collected in 2004 and 2005.

DNA extraction and amplification followed the same methods and techniques described in Chapter One. The two sets of primers used to generate double-stranded DNA are 16S rRNA-L2510 5' CGCCTGTTTATCAAAAACAT 3' and 16S rRNA H-3080 5' CCGGTCTGAACTCAGATCACGT 3' (Palumbi 1996) and L-CO1490 5' GGTCAACAAATCATAAAGATATTG 3'; and H-CO2198 5' TAAACTTCAGGGTGACCAAAAAATCA 3' (Folmer et al. 1994). The PCR thermal profile used was 95 °C for 1 min, followed by 35 cycles of 35 s at 95 °C, 30 s at 50 °C and 1 min at 72 °C, with a final extension at 72 °C for 30 s. An aliquot of the PCR product was electrophoresed on a 1% agarose gel containing ethidium bromide, and visualized by ultraviolet light. PCR products were cleaned up using Qiagen gel extraction (Qiagen) and cycle sequenced using a fluorescently labeled dye-terminator kit

(ABI, Foster City, CA). The products were purified with Sephadex spin columns and analysed on an Applied Biosystems 3100 genetic analyser.

Analytical Methods used

Sequences from all taxa and from each gene were edited using the program Sequence Navigator v1.01 (Perkin Elmer). All sequences were aligned using the default settings of CLUSTAL X (Thompson et al. 1997) and were optimized manually using MacClade 4.0 (Maddison & Maddison 1992) to ensure homology. The selected outgroup species for the phylogenetic analyses were *Projasus parkeri* (Silentes lineage) and *Palinustus unicornutus*.

Phylogenetic reconstruction for the 16S rRNA and COI alignments were carried out using maximum parsimony, maximum likelihood and Bayesian inference. The model that best fitted the data was selected using MODELTEST 3.06 developed by Posada and Crandall (1998). MODELTEST 3.06 utilizes both the Akaike Information Criterion (AIC, Akaike 1974) and the hLRT (Hierarchical Likelihood Ratio Test) to select an appropriate model of evolution. The AIC based on estimates of the Kullback-Leibler (K-L) distance, provides an indication of the expected distance between any model and the evolutionary process which generated the data and seems to outperform the hLRT (Posada and Crandall 2001). Based on AIC, the General Time Reversible (GTR) model was selected for the present study and was used in all model based analyses.

Parsimony phylogenies were derived for the individual DNA regions, the 16S rRNA region, the COI region and the combined analyses (16s rRNA and COI) using PAUP* 4.0b10 (Swofford 2002). The maximum likelihood analyses were performed only on the combined dataset (one with the outgroup present and one without). Parsimony and maximum likelihood analyses were performed by heuristic searches under TBR branch swapping and with 100 replicates of random taxon addition (PAUP* 4.0b10, Swofford 2002). Branch and bound searches (which guarantee to find the shortest tree) resulted in the same number of equally parsimonious trees and these trees were also the same length as those obtained using heuristic searches. Due to time constraints only heuristic bootstrap searches were performed. The consistency index (CI, Kluge and Farris 1969) and retention index (RI, Farris 1989) was used to access the relative amounts of homoplasy present in each DNA region. Even though the CI and RI measure the amount of homoplasy in a data set, the RI is considered the more dependable indicator of saturation as it is less likely to vary with topology (Nei & Kumar 2000).

Bayesian inference of phylogeny suggests it can be used as a practical substitute to conventional maximum likelihood based methods to obtain statistical support for nodes in a model based framework (Douady et al 2003, Huelsenbeck et al. 2002). Bayesian methods rely on an algorithm, Markov Chain Monte Carlo (MCMC), used for approximating probability distributions (Holder & Lewis 2003). The MCMC methodology is similar to the tree-searching algorithm, only a new tree is proposed from the original tree and the moves that change the tree must involve random choices satisfying numerous conditions. A new location in parameter space is proposed at each

step forming the next link in the chain. The MCMC algorithm has rules that decide when a tree will be accepted or rejected. This procedure is repeated millions of times creating a long chain of locations in parameter space (Holder & Lewis 2003). Given the observed data, results reveal an estimate of the probability that any particular tree is the true evolutionary tree.

Bayesian analyses were conducted with MrBayes 3.0b4 (Huelsenbeck & Ronquist 2001) on the independent mitochondrial DNA regions (16s rRNA and COI) and the combination of the two regions. The number of rate parameters set were "lset nst = 6" (GTR model following the selection by modeltest), where every site was gamma distributed, "rates = invgamma". The Bayesian analysis was run for at least $5X10^6$ generations with trees sampled every 100^{th} generation resulting in 50 000 trees saved. The run was repeated three times. The variation in the likelihood scores were visualized graphically using Microsoft ® Excel (2002) and stationarity was reached after 40 000 generations. The partition function was performed on the combined data set.

Results

Fragments sequenced

There was no evidence of insertions or deletions among the *Palinurus* sequences from the 16S rRNA and COI regions. The alignment of COI sequences was 520 bp in length and 481 bp for the 16S rRNA region. The combined dataset (COI + 16S rRNA) was thus 1001 bp in length.

Maximum Parsimony

Parsimony analyses of the 16S rRNA gene resulted in four equally parsimonious trees (length = 219 steps; CI = 0.88; RI = 0.93) and differences among the topologies were due to intra- and interspecific branch swapping (Fig. 2.2a). The individual analyses of the COI gene provided more phylogenetic resolution if measured by the number of nodes supported by bootstrap. Although eight equally parsimonious CO1 trees were obtained (length = 343 steps; CI = 0.79; RI = 0.90) the differences among the topologies were all confined to intraspecific branch swapping (Fig. 2.2b). Bootstrap analyses of the 16S rRNA data resulted in >70% support for the monophyly of all six *Palinurus* species but no bootstrap support could be obtained for any of the interspecific associations (<50%). Likewise, individual analyses of the COI gene resulted in strong support for the monophyly of the species but in this instance some support for the interspecific associations was also obtained. Because the two mtDNA genes are linked, and there were no strong conflicting nodes when the two sets of trees were compared, the data were combined for further analyses.

Parsimony analyses of the combined dataset were based on 1001 bp and 274 parsimony informative characters. A total of 160 equally parsimonious trees were obtained (length = 563 steps; CI = 0.83; RI = 0.91) but all conflict was found among terminal nodes and once again associated with branch swapping among individuals within species (Fig. 2.2c). The combined data increased the resolution considerably and associations among species were supported by bootstrap values ranging from 80% (supporting the monophyly of *P. charlestoni, P. delagoae* and *P. barbarae*) to 86% for the basal placement of *P. elephas* to all other *Palinurus* species.

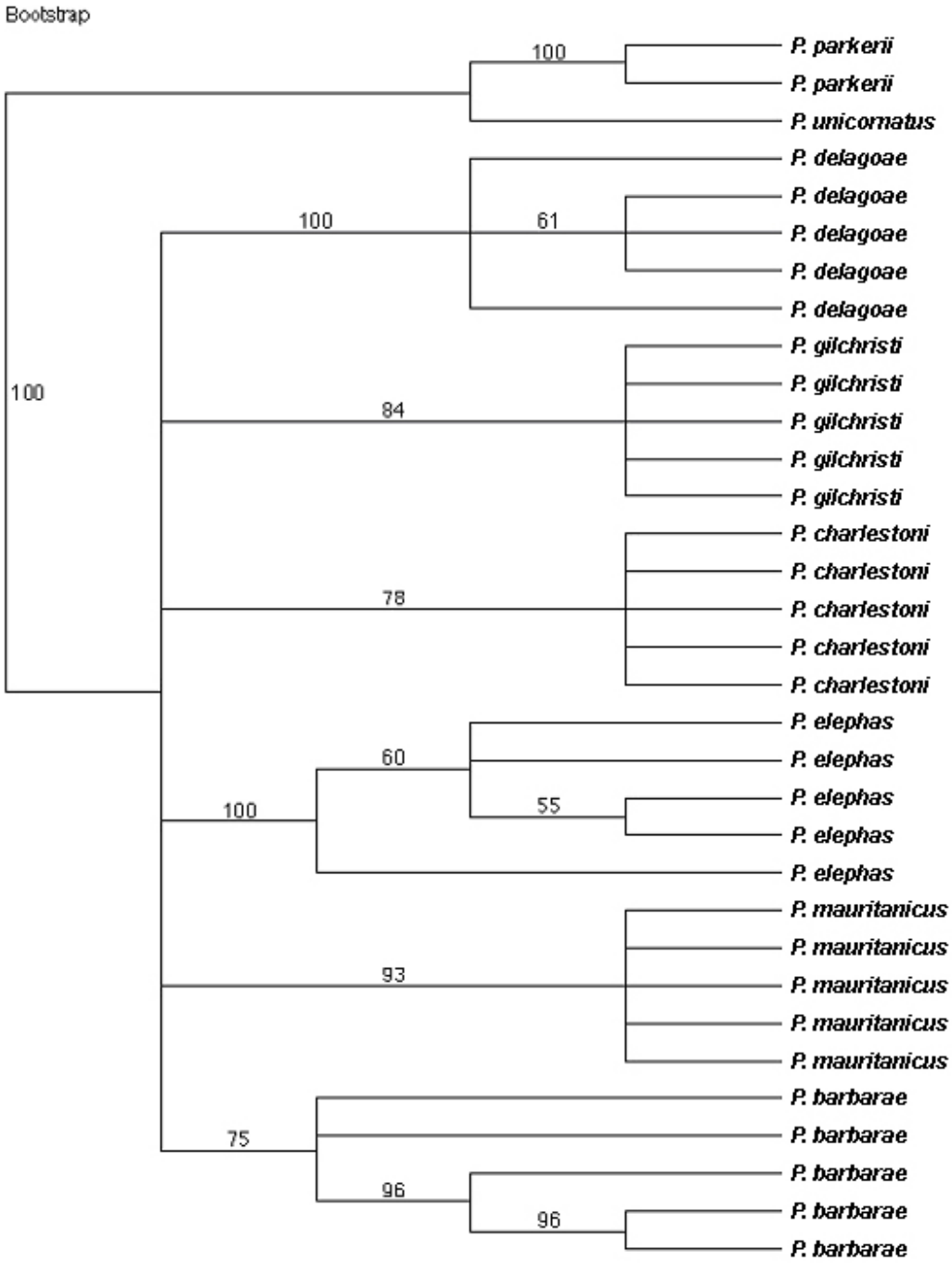

Figure 2.2a) Bootstrap consensus tree produced from 16S rRNA mtDNA sequences using maximum parsimony (tree length = 181, consistency index = 0.8122, homoplasy index = 0.1878, retention index = 0.9045). Numbers above branches are bootstrap values (100 replicates).

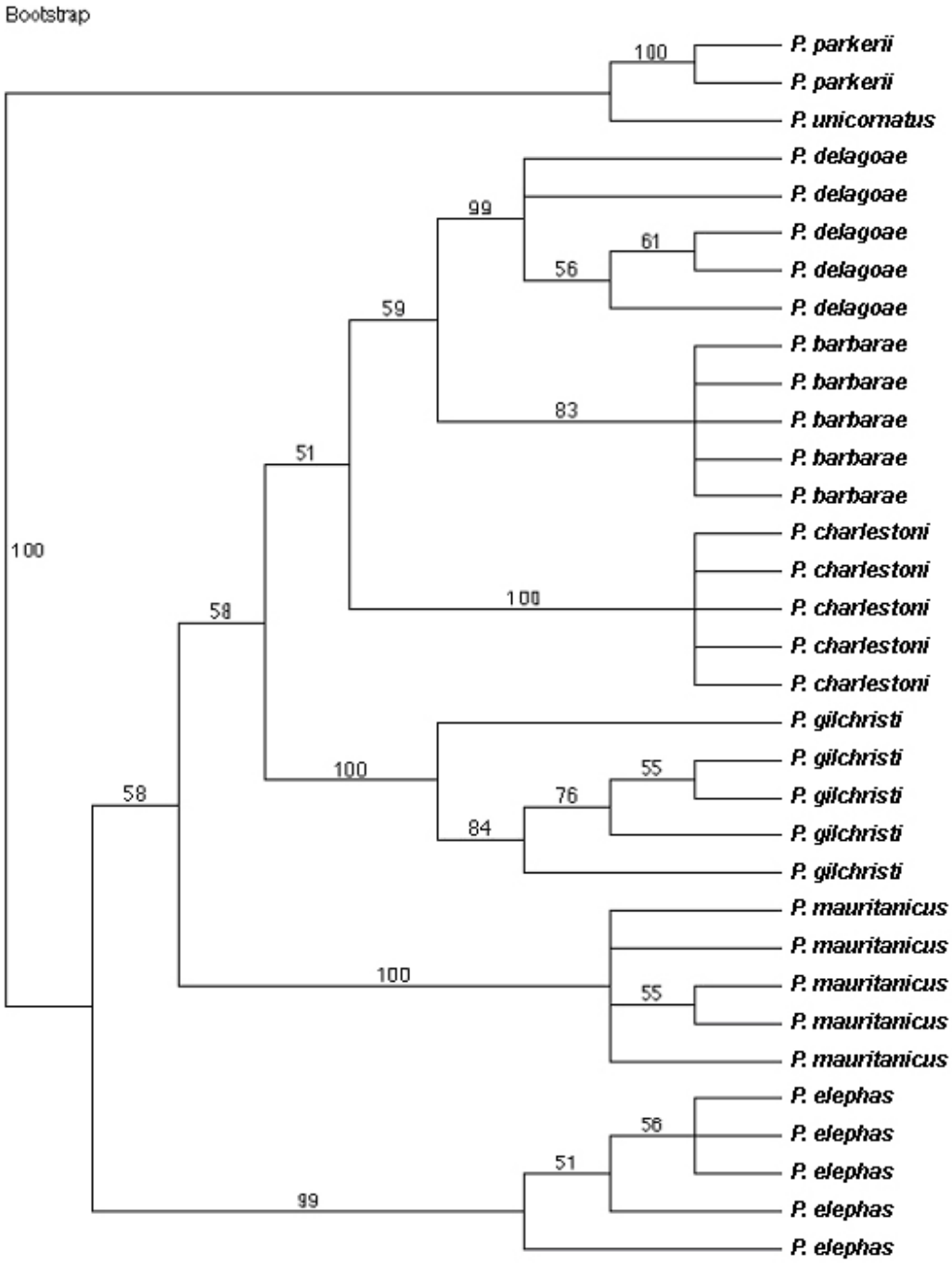

Figure 2.2b) Bootstrap consensus tree produced from COI mtDNA sequences using maximum parsimony (tree length = 302, consistency index = 0.7616, homoplasy index = 0.2384, retention index = 0.9000). Numbers above branches are bootstrap values (100 replicates).

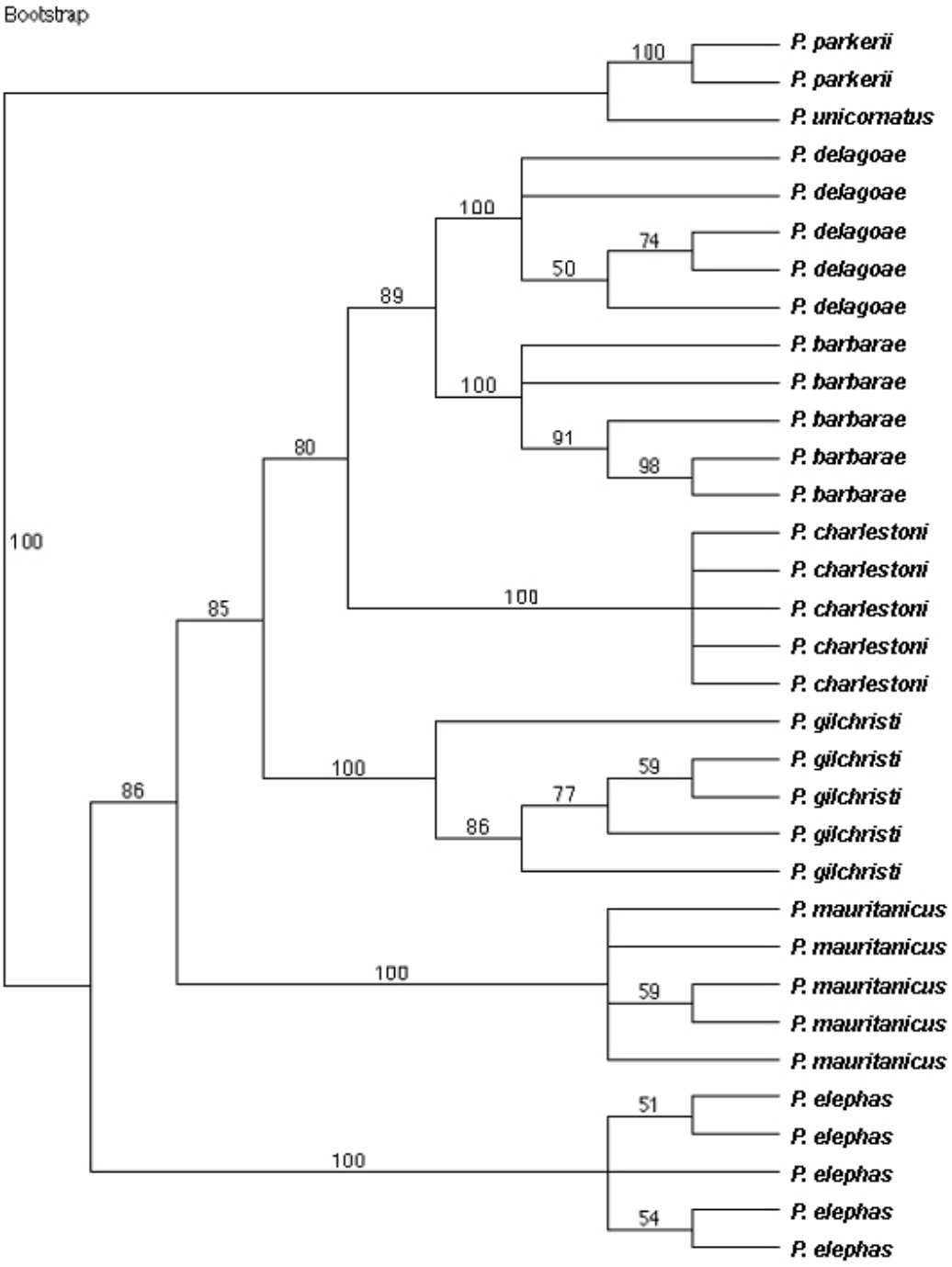

Figure 2.2c) Bootstrap consensus tree produced from the combined data using maximum parsimony (tree length = 477, consistency index = 0.7904, homoplasy index = 0.2096, retention index = 0.9071). Numbers above branches are bootstrap values (100 replicates).

Model Based Analyses

Maximum likelihood and Bayesian analyses failed to recover any significant support for the associations among *Palinurus* species. In an attempt to increase the phylogenetic resolution analyses were repeated excluding the outgroup taxa. The topologies were rooted at *P. elephas* which was always placed as the most basal taxon in the parsimony analyses (100% bootstrap). Furthermore, based on some fossil inferences *P. elephas* is also believed to be ancestral (Glaessner 1969, Pollock 1995). Despite the independent analyses performed on both the ML and the Bayesian analyses, when rooted at *P. elephas,* the tree topology resulted in an identical branching pattern than that obtained in the parsimony analyses (Fig. 2.2a, b, c). Although not significantly supported by Bayesian analyses (57-58%, Fig. 2.4c) the ML analyses recovered 76% bootstrap support for the clustering of the northern hemisphere *P. charlestoni* nested among the southern hemisphere *P. gilchristi, P. delagoae* and *P. barbarae* (Fig. 2.3a, b).

The combined Bayesian analyses did not result in a resolved topology and no significant posterior probabilities could be obtained reflecting the associations among species (Fig. 2.4a, b, c). The lack of resolving power of the Bayesian analyses is probably due to the limited number of variable characters to model the evolutionary process, as it is unlikely to be a consequence of different coalescent genes having different histories (Doyle 1992) given that both these genes are mitochondrial.

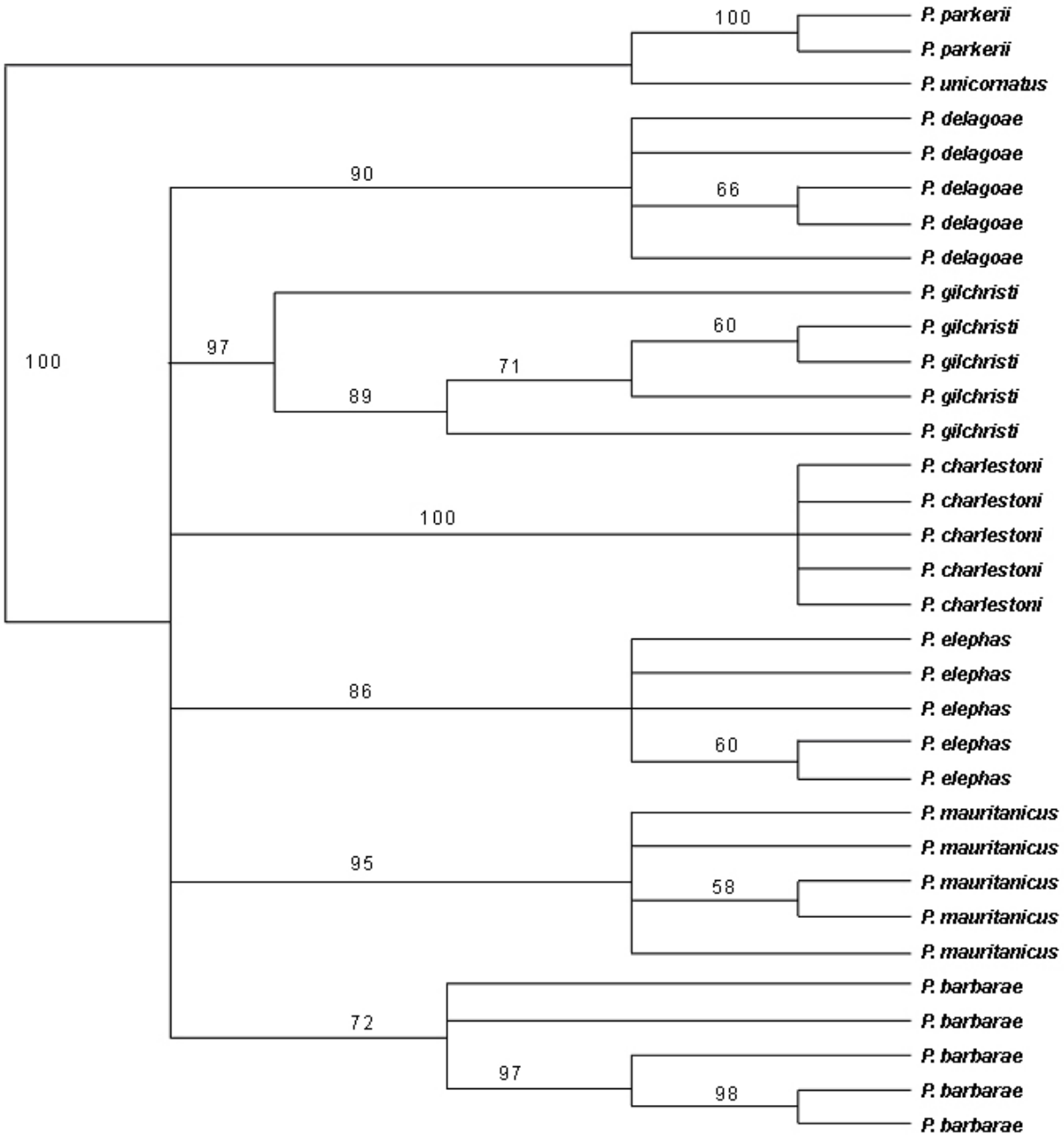

Figure 2.3a) Maximum likelihood tree for the combined sequence data set (16S rRNA & COI genes). The outgroup species were included in this analysis. The values above the branches reflect bootstrap support for the respective nodes.

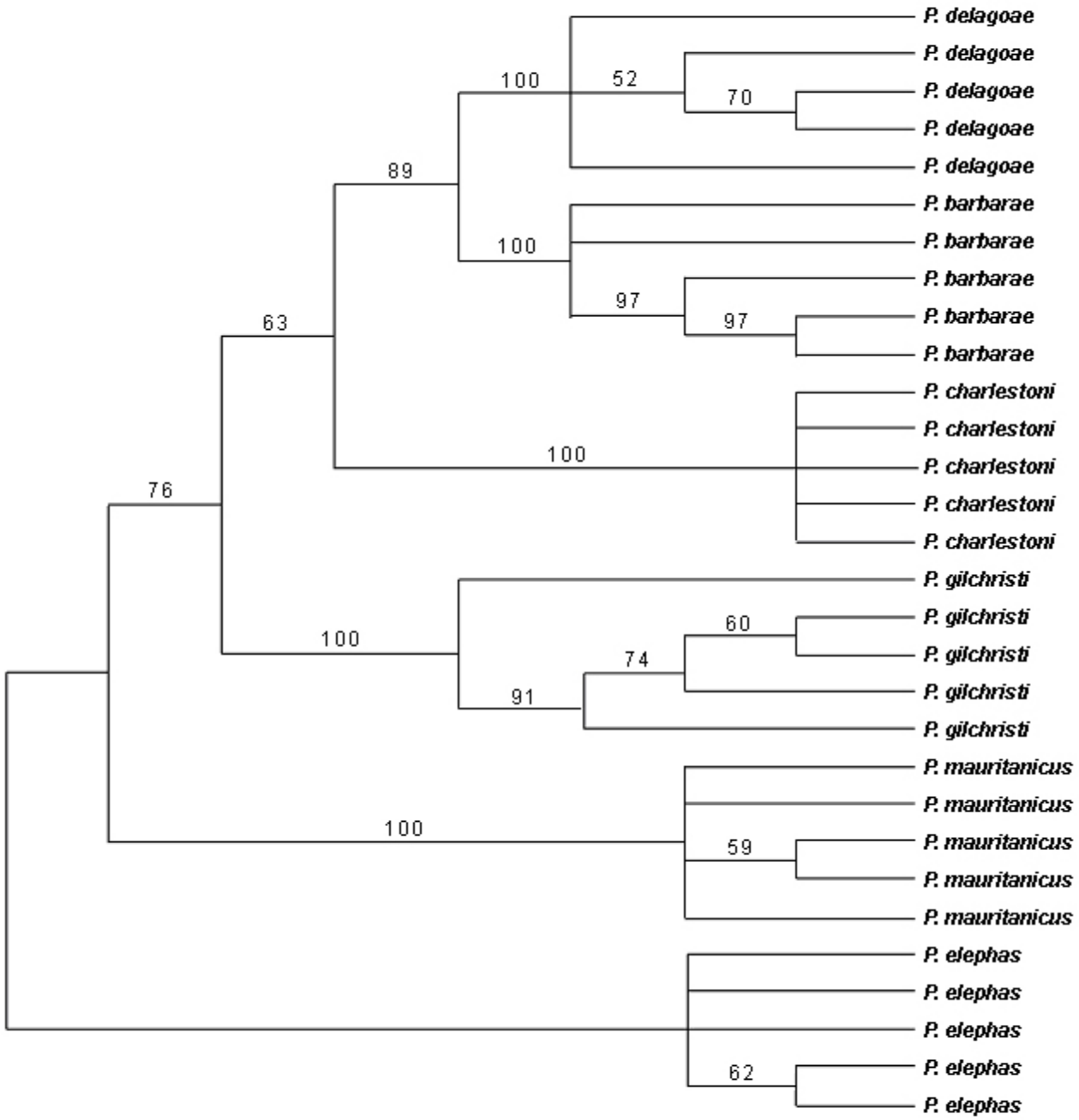

Figure 2.3b) Maximum likelihood tree for the combined sequence data set (16S rRNA & COI genes). The outgroup species were not included in this analysis. The values above the branches reflect bootstrap support for the respective nodes.

Bayesian Phylogenetics

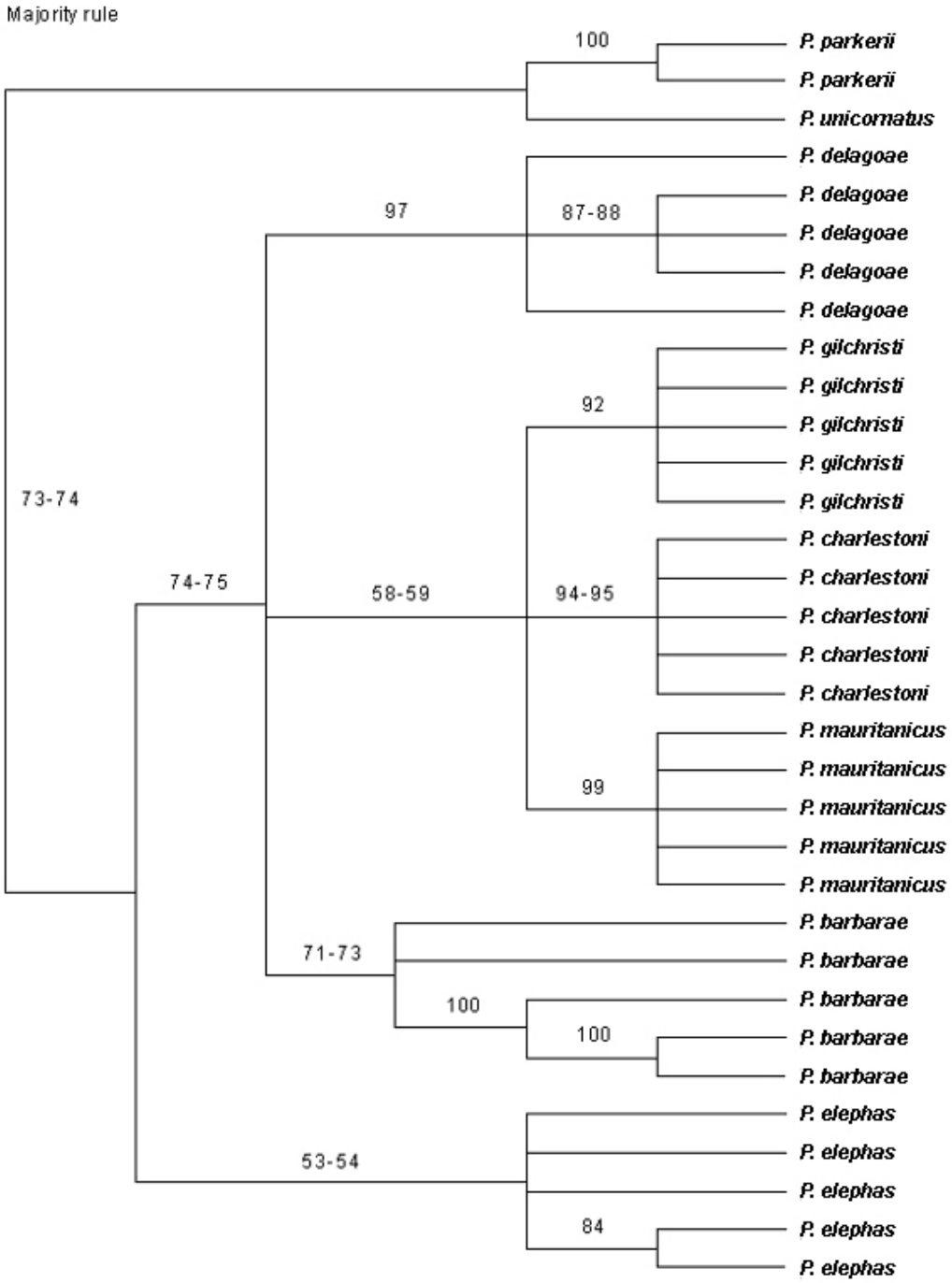

Figure 2.4a) Gene tree produced from 16S rRNA mtDNA sequences using Bayesian inference. The values above the branches indicate posterior probabilities for the respective nodes.

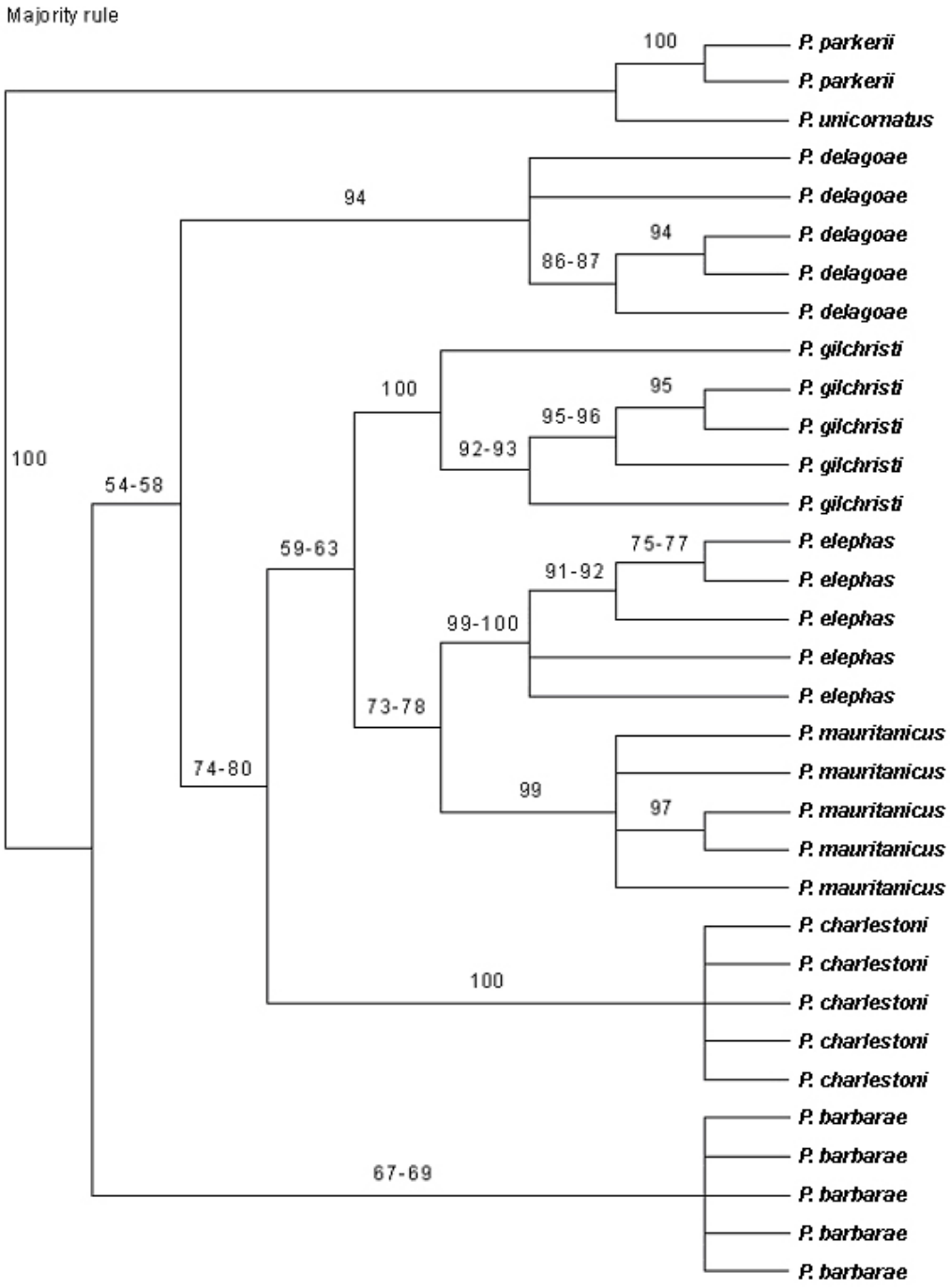

Figure 2.4b) Gene tree produced from COI mtDNA sequences using Bayesian inference. The values above the branches indicate posterior probabilities for the respective nodes.

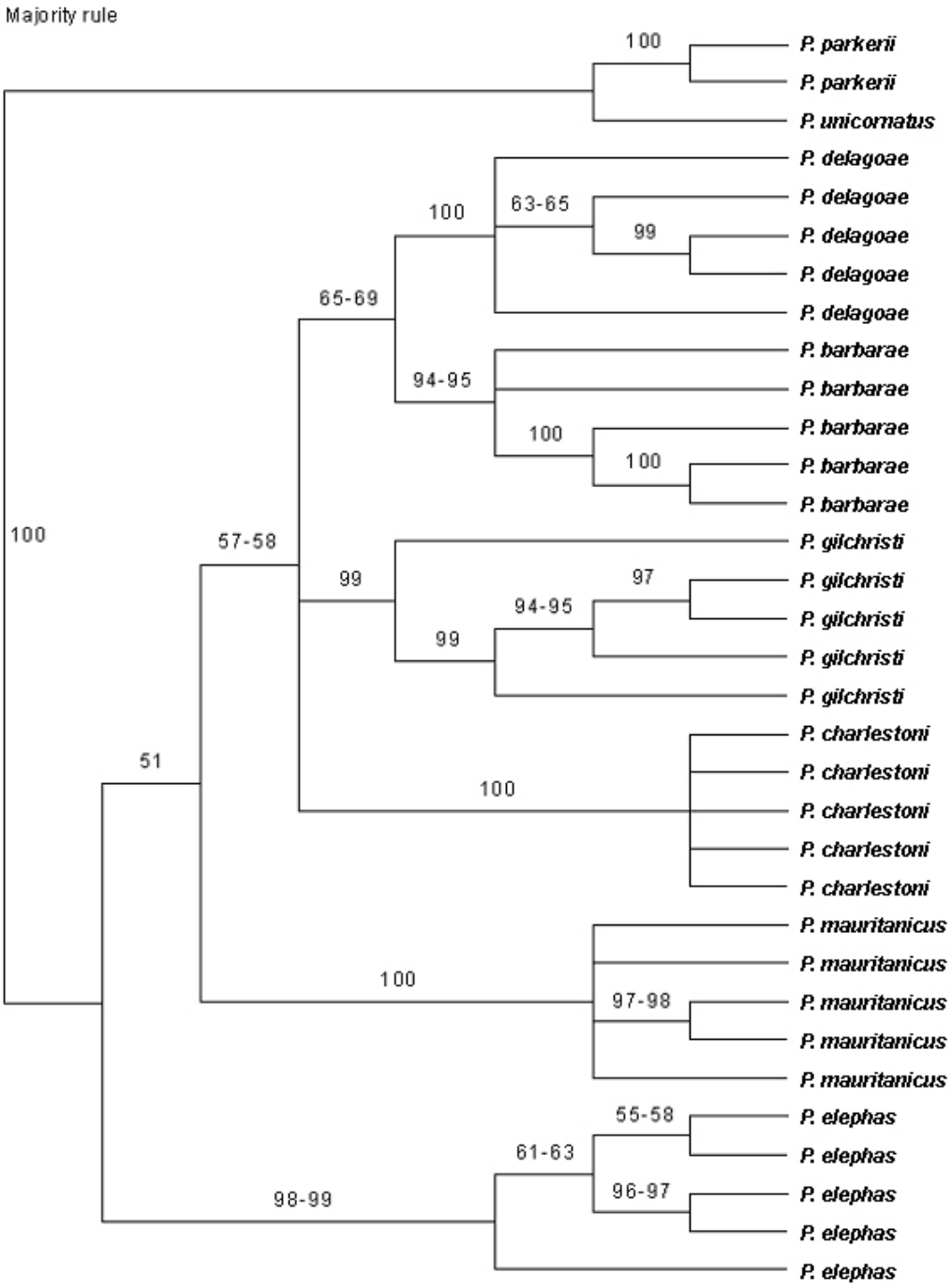

Figure 2.4c) Gene tree produced from both 16S rRNA & COI mtDNA sequences examined using Bayesian inference. The values above the branches indicate posterior probabilities for the respective nodes.

Palinurus phylogeny

Parsimony and model based phylogenetics gave strong support for the monophyly of the genus *Palinurus* and also supported the monophyly of each of the six species. Based on the combined parsimony tree, and in some instances also drawing from the model based analyses, it seems reasonable to suggest that the north Atlantic species are paraphyletic with the Cape Verde Island endemic, *P. charlestoni*, always clustering nested among the south-western Indian Ocean species. The monophyly of the latter clade including *P. gilchristi*, *P. charlestoni*, *P. delagoae* and *P. barbarae* is strongly supported by parsimony bootstrap (84%) and ML (76% when rooting the topology at *P. elephas*). Corrected sequence distance analyses are congruent with this finding and indicated that the closest *Palinurus* species in our phylogeny represent *P. charlestoni* and *P. gilchristi* with a combined nucleotide divergence of 3.01% (Table 2.1). Interestingly, nuclear sequences of the 28S rRNA region for four of the six species included in this study (Cannas et al. unpublished; DQ377979, *P. charlestoni*; DQ377982, *P. gilchristi*; DQ377980, *P. elephas*; DQ377981, *P. mauritanicus*) also suggest a close relationship between *P. charlestoni* and *P. gilchristi* (0.32%) whereas 1.29% sequence divergence separate the geographically close *P. mauritanicus* and *P. charlestoni*.

Table 2.1. Average GTR corrected sequence divergence values among species sequenced in the present study. The combined data were used. Values above the diagonal represent sequence divergence and those below are the standard deviations.

	P. parkeri	*P. unicornutus*	*P. delagoae*	*P. gilchristi*	*P. charlestoni*	*P. elephas*	*P. mauritanicus*	*P. barbarae*
P. parkeri	-	28.10	26.64	26.04	25.97	26.12	25.48	26.54
P. unicornutus	0.00	-	27.76	28.57	28.61	27.62	28.12	27.97
P. delagoae	0.00	0.00	-	3.71	3.56	7.86	6.10	3.32
P. gilchristi	0.01	0.01	0.00	-	3.01	6.70	4.69	4.26
P. charlestoni	0.00	0.00	0.00	0.00	-	7.54	5.00	3.77
P. elephas	0.00	0.00	0.00	0.02	0.00	-	8.13	8.24
P. mauritanicus	0.00	0.00	0.00	0.01	0.00	0.00	-	6.05
P. barbarae	0.01	0.01	0.01	0.01	0.01	0.07	0.07	-

Discussion

Molecular data have not previously been used to address phylogenetic hypotheses for all members of the genus *Palinurus*. Mitochondrial COI and 16S rRNA genes are the most commonly used molecular markers to infer species level phylogenetics relationships in rock lobsters due to availability of universal primers (Schubart et al. 1999, 2000). In the present study the COI gene appeared to be more useful for addressing species relationships in *Palinurus*. The 16S rRNA gene appears to have less phylogenetic utility for addressing species level relationships in *Palinurus,* as most of the resolution was confined to supporting the monophyly of species and several polytomies were found among them.

Model based approaches for analyzing phylogenetic data have been used more frequently in recent years to supplement or in some instances replace parsimony. In the present study it seems that parsimony outperforms the model based analyses. The results displayed well-supported tree topologies for the parsimony analyses compared to maximum likelihood and Bayesian analyses, which was not the case in other similar studies. Simmons et al. (2003) compared the accuracy of Bayesian support values to parsimony-based jackknife values for mitochondrial genomes from higher teleost fishes, and found that both measures of support differed significantly from an ideal support index; parsimony underestimates support values whereas Bayesian methods overestimate support values and exceeds the magnitude by which parsimony underestimates support. Also both methods performed poorly when taxon sampling increased and character

sampling was not increased. These results tend to suggest that support values are not always accurate and shouldn't be interpreted as probabilities that clades are correctly resolved. Another recent study done by Simmons et al. (2006), tested whether it is beneficial for the accuracy of phylogenetic inference to sample characters that are evolving under different sets of parameters using bayesian MCMC (Markov Chain Monte Carlo) and parsimony approaches. Here, results showed that the Bayesian analysis outperformed parsimony when heterogeneous simulation parameters were incorporated into a substitution model, but that parsimony outperformed Bayesian MCMC when heterogeneous simulation parameters were not included in Bayesian substitution models. However, branch-length heterogeneity was generally disadvantageous for phylogenetic inference using both parsimony and Bayesian approaches. Parsimony was found to be more conservative than Bayesian analysis in that it resolved fewer incorrect clades. Although Simmons et al. (2006) regards parsimony methods as more consistent than Bayesian ones, Douady et al. (2003) holds that discrepancies do exist between nonparametric bootstrap proportions and Bayesian posterior probabilities, which generally lead to difficulties in interpreting extremely conflicting results. This study applied the nonparametric bootstrap re-sampling procedure to the Bayesian approach: the results showed that the relation between posterior probabilities and bootstrapped maximum likelihood percentages is highly variable, but very strong correlations always exist when Bayesian node support is estimated on bootstrapped character matrices. Moreover, being more conservative, the bootstrap approach might be less prone to strongly supporting a false phylogenetic hypothesis.

Both methods of phylogenetic reconstruction (maximum likelihood and Bayesian analyses), used on the two gene regions either separately or combined, failed to produce high resolution topologies among species of *Palinurus*. *Palinurus elephas* appears to be the most primitive species as well as the ancestor of the group despite occurring in shallow waters. Even though *P. mauritanicus* has a similar geographical distribution to *P. elephas*, the depths at which they occur are different. The ancestor of *P. elephas* could have occupied a broad Tethys Sea distribution in the North Atlantic and moved through the Levant (Suez) into the Indian Ocean, and depending on the prevailing temperatures, it could have lived in shallow waters (as at present) and/or deeper (>200 m). A similar explanation was proposed by George (2005) for the *Panulirus* ancestor *P. argus*. The genus originated in the broad Tethys Sea and the *Panulirus* stock was initially divided longitudinally by the collision of Africa with Eurasia, resulting in the isolation of the Indo-west Pacific element and the final separation of the east Pacific from the Atlantic elements occurring with the Pliocene closure of the Panama Seaway. Due to these geographical events, many new habitats were formed for incipient species as well as the emergence of oceanic islands, climatic changes and the formation of more discrete current systems.

On the other hand, the radiation of the *Palinuridae* appears to have emerged from the northward collision of the African plate with Eurasia in the Miocene which probably closed the Red Sea region isolating the Indian Ocean 'complex' (represented by *P. gilchristi, P. delagoae, P. charlestoni*, and *P. barbarae*). Initial separation may have

occurred as the seaway shallowed and before the actual collision took place, perhaps in the Oligocene (George 2005). The ancestor of the Indian Ocean 'complex', due to prevailing currents, would have passed through the Red Sea and moved south along the African coast, under the North East monsoon conditions (i.e. southward currents), eventually reaching the Mozambique and Agulhas Currents. Both these currents are so strong that the original larvae may have been shunted downstream, giving rise to *P. gilchristi*. George (2006) proposes that continual rising of the Himalayas intensified both wind and current strengths increasing the Agulhas Current to the extent that it allowed *P. charlestoni* to pass into the South Atlantic, moving at depths below the *Jasus lalandii* depth range (> 60 m) and up as far as Cape Verde Islands, passing via the Benguela Current and South Equatorial. In the vicinity of the Cape Verde Islands, the current systems weakened leaving *P. charlestoni* to adapt to eddy systems at the right depth around the steep volcanic islands. *Palinurus gilchristi*, then is the ancestral and relict species of the Indian Ocean 'complex' and *P. delagoae* split off in response to a different habitat suitability at deeper depths but similar bottom temperatures less influenced by the Agulhas Current. The biological characteristics of the four sister taxa (represented by *P. gilchristi, P. delagoae, P. charlestoni*, and *P. barbarae*) are comparable but are different from *P. elephas* and *P. mauritanicus* with respect to morphological differences, particularly since Berry & Plante (1973) found extremes of variation in the shape, curvature and spination of the supraorbital horns of one of the Indian Ocean species which overlapped the variations in the four Atlantic species.

If one places the species geographically on a map of Africa in order of the phylogenetic results obtained, it can be said that the *Palinurus* genus has circumnavigated Africa. It is rather peculiar that *P. mauritanicus* and *P. charlestoni* are such close geographical neighbours off Mauritania and Cape Verde Islands, but theoretically *P. mauritanicus* diverged via the African west coast and *P. charlestoni* had traveled clockwise around Africa.

As far as tree topologies are concerned, this study proved to be very challenging, as our statistical methodological approach (maximum parsimony) gave better resolution trees compared to our model based method (Bayesian analysis). In previous crustacean studies as well as studies of other taxa, Bayesian and/or maximum likelihood analyses prove to be superior to maximum parsimony (Machordom & Macpherson 2004, Simmons et al. 2006). Atypical results have been found in the present study, proving that newer analytical methods (i.e. Bayesian Inference) may not always display superior results, depending on the type of data being used.

In summation, the molecular phylogeny has begun to clarify the relationships within the genus *Palinurus*. The phylogenetic results represent a first step towards understanding the pattern of speciation in *Palinurus* solely on the basis of molecular characters. Our phylogeny based on genetic data could be used as a framework for better understanding the significance of the morphological characters of the species. By using molecular markers, we were able to discover that this spiny lobster genus, *Palinurus* possibly evolved by circumnavigating Africa.

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Appendix I – Frequencies of *Palinurus* spiny lobster control region haplotypes from the coasts of KwaZulu-Natal (South Africa) and Mozambique, and the Walters Shoals.

Haplotype	South KZN	South-Central KZN	Central KZN	North KZN	Inhaca	Boa Paz	Walters Shoals	Total
KG1	1	1	2	0	1	0	0	5
KG2	4	5	5	4	2	1	0	21
KG3	0	1	1	0	0	0	0	2
KG4	4	6	2	1	2	2	0	17
KG5	7	8	2	3	1	1	0	22
KG6	1	1	0	0	0	0	0	2
KG7	0	1	0	1	0	0	0	2
KG8	0	1	1	1	1	0	0	4
KG9	2	1	0	1	2	1	0	7
KG10	0	3	1	1	0	0	0	5
KG11	2	3	3	2	0	1	0	11
KG12	0	1	0	4	0	0	0	5
KG13	0	1	0	1	0	0	0	2
KG14	0	2	0	0	2	1	0	5
KG15	2	2	0	1	2	1	0	8
KG16	1	3	1	3	2	0	0	10
KG17	0	2	0	0	0	0	0	2
KG18	2	0	1	0	0	0	0	3
KG19	6	1	1	2	1	0	0	11
KG20	1	0	4	0	0	1	0	6
KG21	2	0	0	0	0	0	0	2
KG22	1	0	0	1	0	0	0	2
KG23	1	0	0	1	0	0	0	2
KG24	1	0	0	2	0	0	0	3
KG25	0	2	2	0	0	0	0	4
KG26	0	0	2	0	1	1	0	4
KG27	0	1	1	0	0	0	0	2
KG28	0	2	0	1	0	0	0	3
KG29	0	2	0	0	0	0	0	2
KG30	0	0	0	1	1	0	0	2
KG31	0	0	0	0	0	0	3	3
KG32	0	0	0	0	0	0	3	3
KG33	0	0	0	0	0	0	2	2
KG34	0	0	0	0	0	0	2	2
KG35	0	0	0	0	0	0	6	6
KG36	0	0	0	0	0	0	4	4
KG37	0	0	0	0	0	0	4	4
KG38	0	0	0	0	0	0	3	3
KG39	0	0	0	0	0	0	4	4
KG40	0	0	0	0	0	0	3	3
KG41	0	0	0	0	1	1	0	2
KG42	0	0	0	0	2	1	0	3
KG43	0	0	0	0	1	1	0	2
KG44	0	1	0	0	0	0	0	1
KG45	0	1	0	0	0	0	0	1
KG46	0	1	0	0	0	0	0	1
KG47	0	1	0	0	0	0	0	1
KG48	0	1	0	0	0	0	0	1
KG49	0	1	0	0	0	0	0	1

KG50	1	0	0	0	0	0	0	1
KG51	0	1	0	0	0	0	0	1
KG52	0	1	0	0	0	0	0	1
KG53	0	1	0	0	0	0	0	1
KG54	0	1	0	0	0	0	0	1
KG55	0	1	0	0	0	0	0	1
KG56	1	0	0	0	0	0	0	1
KG57	1	0	0	0	0	0	0	1
KG58	0	1	0	0	0	0	0	1
KG59	0	1	0	0	0	0	0	1
KG60	0	1	0	0	0	0	0	1
KG61	1	0	0	0	0	0	0	1
KG62	1	0	0	0	0	0	0	1
KG63	1	0	0	0	0	0	0	1
KG64	1	0	0	0	0	0	0	1
KG65	1	0	0	0	0	0	0	1
KG66	1	0	0	0	0	0	0	1
KG67	0	1	0	0	0	0	0	1
KG68	1	0	0	0	0	0	0	1
KG69	1	0	0	0	0	0	0	1
KG70	1	0	0	0	0	0	0	1
KG71	0	0	1	0	0	0	0	1
KG72	0	0	1	0	0	0	0	1
KG73	0	0	1	0	0	0	0	1
KG74	0	0	1	0	0	0	0	1
KG75	0	0	1	0	0	0	0	1
KG76	0	0	1	0	0	0	0	1
KG77	0	0	1	0	0	0	0	1
KG78	0	0	1	0	0	0	0	1
KG79	0	0	1	0	0	0	0	1
KG80	0	0	0	1	0	0	0	1
KG81	0	0	0	1	0	0	0	1
KG82	0	0	0	1	0	0	0	1
KG83	0	0	0	1	0	0	0	1
KG84	0	0	0	1	0	0	0	1
KG85	0	0	0	1	0	0	0	1
KG86	0	0	0	1	0	0	0	1
KG87	0	0	0	1	0	0	0	1
KG88	0	0	0	1	0	0	0	1
KG89	0	0	0	1	0	0	0	1
KG90	0	0	0	1	0	0	0	1
KG91	0	0	0	1	0	0	0	1
KG92	0	0	0	1	0	0	0	1
KG93	0	0	0	1	0	0	0	1
KG94	0	0	0	1	0	0	0	1
KG95	0	0	0	0	0	0	1	1
KG96	0	0	0	0	0	0	1	1
KG97	0	0	0	0	0	0	1	1
KG98	0	0	0	0	0	0	1	1
KG99	0	0	0	0	0	0	1	1
KG100	0	0	0	0	0	0	1	1
KG101	0	0	0	0	0	0	1	1
KG102	0	0	0	0	0	0	1	1
KG103	0	0	0	0	0	0	1	1
KG104	0	0	0	0	0	0	1	1
KG105	0	1	0	0	0	0	0	1

KG106	0	0	0	0	0	0	1	1
KG107	0	0	0	0	0	0	1	1
KG108	0	0	0	0	0	0	1	1
KG109	0	0	0	0	0	0	1	1
KG110	0	0	0	0	0	0	1	1
KG111	0	1	0	0	0	0	0	1
KG112	0	1	0	0	0	0	0	1
KG113	0	1	0	0	0	0	0	1
KG114	0	1	0	0	0	0	0	1
KG115	0	1	0	0	0	0	0	1
KG116	0	1	0	0	0	0	0	1
KG117	0	1	0	0	0	0	0	1
KG118	0	1	0	0	0	0	0	1
KG119	0	1	0	0	0	0	0	1
KG120	0	0	0	0	1	0	0	1
KG121	0	0	0	0	1	0	0	1
KG122	0	0	0	0	1	0	0	1
KG123	0	0	0	0	1	0	0	1
KG124	0	0	0	0	1	0	0	1
KG125	0	0	0	0	1	0	0	1
KG126	0	0	0	0	1	0	0	1
KG127	0	0	0	0	1	0	0	1
KG128	0	1	0	0	0	0	0	1
KG129	0	0	0	0	0	1	0	1
KG130	0	0	0	0	0	1	0	1
KG131	0	0	0	0	0	1	0	1
KG132	0	0	0	0	0	1	0	1
KG133	0	0	0	0	0	1	0	1
KG134	0	0	0	0	0	1	0	1
KG135	0	0	0	0	0	1	0	1
KG136	0	0	0	0	0	1	0	1
KG137	0	0	0	0	0	1	0	1
KG138	0	0	0	0	0	1	0	1
KG139	0	0	0	0	0	1	0	1
KG140	0	0	0	0	0	1	0	1
KG141	0	0	0	0	0	1	0	1
KG142	0	0	0	0	0	1	0	1
KG143	0	0	0	0	0	1	0	1
KG144	0	0	0	0	0	1	0	1
KG145	0	0	0	0	0	1	0	1
KG146	0	1	0	0	0	0	0	1
KG147	0	0	0	0	0	1	0	1
KG148	0	0	0	0	0	1	0	1
KG149	0	0	0	0	0	1	0	1
KG150	0	0	0	0	0	1	0	1
KG151	0	0	0	0	0	1	0	1
KG152	0	0	0	0	0	1	0	1
KG153	0	0	0	0	0	1	0	1
KG154	0	0	0	0	0	1	0	1
KG155	0	0	0	0	0	1	0	1
KG156	0	0	0	0	1	0	0	1
KG157	0	0	0	0	1	0	0	1
KG158	0	0	0	0	1	0	0	1
KG159	0	0	0	0	1	0	0	1
KG160	0	0	0	0	1	0	0	1
Total	50	77	38	46	35	39	49	334

Appendix II – Geographical data of lobster individuals used for the phylogenetic analysis

Species Name	Geographical Area	Latitude	Longitude	Collector
Palinurus delagoae	Northern KZN	26°54′05′′	33°01′02′′	Nico du Plooy
	Central KZN	28°38′15′′	32°26′06′′	Nico du Plooy
	South KZN	30°36′00′′	30°44′00′′	NIco du Plooy
	Boa Paz	25°14′′05′′	33°56′75′′	Nico du Plooy
	Inhaca	26°10′13′′	33°04′43′′	Nico du Plooy
Palinurus gilchristi	South coast of Africa	35°52′97′′	20°24′32′′	Nico du Plooy
	South coast of Africa	34°46′05′′	23°16′83′′	Nico du Plooy
	South coast of Africa	34°46′05′′	23°16′83′′	Nico du Plooy
	South coast of Africa	34°50′00′′	25°00′00′′	Grant van der Westhuizen
	South coast of Africa	34°40′00′′	25°38′00′′	Hentie Heyns
Palinurus charlestoni	Ilha do Sol	16°00′00′′	24°00′00′′	David Diaz
	Ilha do Sol	16°00′00′′	24°00′00′′	David Diaz
	Ilha do Sol	16°00′00′′	24°00′00′′	David Diaz
	Ilha do Sol	16°00′00′′	24°00′00′′	David Diaz
	Ilha do Sol	16°00′00′′	24°00′00′′	David Diaz
Palinurus barbarae	Walters Shoal	33°12′0′′	43°50′00′′	JC Groeneveld & C Griffiths (Identifiers)
	Walters Shoal	33°12′0′′	43°50′00′′	JC Groeneveld & C Griffiths (Identifiers)
	Walters Shoal	33°12′0′′	43°50′00′′	JC Groeneveld & C Griffiths (Identifiers)
	Walters Shoal	33°12′0′′	43°50′00′′	JC Groeneveld & C Griffiths (Identifiers)
	Walters Shoal	33°12′0′′	43°50′00′′	JC Groeneveld & C Griffiths (Identifiers)
Palinurus mauritanicus	Viviers de Locarec/Furic, Penmarc'h	48°20′00′′	09°00′00′′	David Diaz
	Viviers de Locarec/Furic, Penmarc'h	48°20′00′′	09°00′00′′	David Diaz
	Viviers de Locarec/Furic, Penmarc'h	48°20′00′′	09°00′00′′	David Diaz
	Viviers de Locarec/Furic, Penmarc'h	48°20′00′′	09°00′00′′	David Diaz
	Viviers de Locarec/Furic, Penmarc'h	48°20′00′′	09°00′00′′	David Diaz
Palinurus elephas	Illes Medes	42°30′00′′	03°12′00′′	David Diaz
	Illes Medes	42°30′00′′	03°12′00′′	David Diaz
	Illes Medes	42°30′00′′	03°12′00′′	David Diaz
	Illes Medes	42°30′00′′	03°12′00′′	David Diaz
	Illes Medes	42°30′00′′	03°12′00′′	David Diaz