A hidden world beneath the sand: Testing phylogeographic and biogeographic patterns of southern African sandy beach species

By

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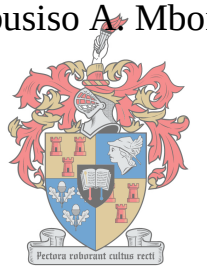

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Declaration

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Abstract

South Africa's sandy shores are listed as some of the best studied in the world, however, most of these studies have focused on documenting biodiversity and the classification of beach type and there is a distinct lack of genetic data. This has led to a poor understanding of biogeographic and phylogeographic patterns of southern African sandy beach species. Thus, in order to contribute towards plugging the phylogeography knowledge gap, the objectice of this study is to determine levels of genetic differentiation in isopods of the genera *Tylos* and *Excirolana* in the South African coast to understand their genetic diversity, connectivity and diversification processes.

Individuals (n = 214) of *T. granulatus* were sampled from nine locations along the west coast of South Africa and Namibia, almost covering the full distribution range of the species. Sequence data was obtained using the mitochondrial genes, COI and 16S. A total of ten sampling locations were covered for *E. latipes* (n = 140) and nine for *E. natalensis* (n = 171). For both species, sequence data was obtained with the mtDNA COI gene.

Sequences from the COI gene of *T. granulatus* yielded 44 haplotypes and 91% singletons. Overall, results indicated high haplotype diversity (h = 0.25 - 1.00) and low nucleotide diversity (π = 0.00 - 0.13). Further analyses revealed a strong pattern of genetic divergence characterized by two deeply divergent lineages of *T. granulatus*, with pairwise comparisons (Φ_{st}) ranging from 0.01 to 0.98 ($P < 0.05$). The genetic pattern is influenced by a phylogeographic break located between Hondeklip Bay and Kleinzee. Dating this divergence reveals a link to the Plio-Pleistocene transition that was characterized by low ocean temperatures and rapid climate and oceanographic oscillations, that also had major impacts on biogeographic and phylogeographic patterns of marine species elsewhere.

Results indicated that *E. latipes* and *E. natalensis* are sister species with monophyletic groupings. *Excirolana latipes* was characterized by a strong genetic structure across Cape Point, that appears to act as a barrier to gene flow between the western and southern lineages. Similarly, mtDNA COI revealed two distinct lineages within *E.natalensis*, although Cape Point did not appear as a significant barrier to gene flow for this species. This provides evidence that although both species have similar life-history patterns and are sympatric; their phylogeographic patterns are driven by different phylogeographic breaks. The estimates of the divergence within lineages of both *Excirolana* species (140 000 - 1.23 Ma) suggest a

strong link with the Pleistocene period. In addition, both *Excirolana* species were characterised by deeply divergent lineages, potentially indicating cryptic species.

This study revealed unknown diversities and possibilities of cryptic speciation. All three isopods were characterized by distinct lineages that should be regarded at least as Management Units (MUs) until nuclear markers and further samples are added. These MUs should be considered separately in conservation and management aims of sandy beaches. Most importantly, the outcome of this study shows the importance of integrating genetic approaches into marine conservation in South Africa.

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CHAPTER I

Introduction

1.1 The global status of sandy beaches

Escalating pressures on marine ecosystems caused by the effects of coastal developments, pollution, overexploitation, recreational activities and a rapidly changing climate (Harris et al. 2011; Reyes-Martínez et al. 2014; Poumadère et al. 2015) continue to pose great threats to sandy beaches as well as other marine ecosystems (Schlacher et al. 2008; Defeo et al. 2009; Mclachlan et al. 2013; Mead et al. 2013). With increasing human-induced impacts on the world's shorelines, goals for the conservation of biological diversity will not be met. As set by the Convention of Biological Diversity (2010), some of these goals are (i) to conserve biological diversity of ecosystems, habitats and biomes, (ii) to promote the conservation of species diversity, (iii) to promote the conservation of genetic diversity, (iv) to promote sustainable use of resources and (v) to address threats to biodiversity loss by controlling negative impacts of invasive alien species and anthropogenic impacts on various ecosystems.

With the quality of marine habitats declining worldwide (DeBoer et al. 2014), this has led to an increasing focus on marine conservation. Management and conservation of marine resources and ecosystems is well recognized globally and research has focused on a range of marine groups and ecosystem types. However, sandy beaches *per se* are still understudied and are not always well represented in conservation aims. During the VIth International Sandy Beach Symposium 2012 Workshop, Conservation Targets for Sandy Beaches were agreed upon (Harris et al. 2015) and were set for species, habitats and processes (Harris et al. 2014b). Recently, Harris et al. (2014b) took these agreements into consideration to formalise a target-setting framework to propose the first suite of conservation targets for sandy beach ecosystems.

"Sandy beaches are coastal landforms that comprise the foredunes, intertidal and surf-zone as a single geomorphic unit: the littoral active zone" (Harris et al. 2014a). They are recognised as important sites for economic, ecological, social, cultural and recreational value (Dugan et al. 2010; Schlacher et al. 2014); however, they are infrequently included in conservation planning as beach science is a recent and emerging field (Nel et al. 2014). Based on a number

of studies recorded by the Thomson Reuters Web of Science, much focus has been placed on coastal ecosystems such as estuaries with 36 358 publications, coral reefs (20 065 publications), mangroves (11 149 publications) and rocky shores (3 157 publications) (Nel et al. 2014). As an indication of how underrepresented sandy beaches are, only 2 936 publications for sandy beaches have been recorded (Nel et al. 2014). However, sandy beaches comprise 40% of the coasts worldwide (Bird, 2000) (compared to coral reefs for example that only cover 0.09% of the oceans), which puts into perspective that as a proportion sandy beaches are understudied. A gap of knowledge and research still needs to be filled for sandy beach science in comparison to other marine habitats. For example, based on a citation analysis of the number of published literature on sandy beaches over the past 63 years (1950 - 2013), by continent, Europe had the highest number of published articles on sandy shores (see Fig. 5 & 6 from Nel et al. 2014). The Antarctic sandy shores were found to be the least well studied with only 11 papers published (Nel et al. 2014). However, when the same data was analysed by country, it was found that sandy beach research is limited to certain countries. In the United States of America, close to 600 sandy shore field studies since 1950 (15% of the total locations) have been conducted; South Africa follows with almost 400 studies (10%); and Brazil (n = 324; 8%), Australia (n = 232; 6%) and Italy (n = 144; 4%) follow to make up the top five most explored sandy coastlines in the world (Nel et al. 2014). La Cock & Burkinshaw (1996) and Brown et al. (2000) acknowledged that most managers into whose jurisdiction sandy coast beaches falls, generally have a poor understanding of the processes and management issues that maintain and affect sandy shores. Consequently, a scope of further work is required to build a clear understanding of the value, vulnerability and importance of sandy shores for effective management purposes (National Biodiversity Assessment, 2011).

1.2 The southern African coastline

1.2.1 Oceanographic characteristics of South Africa

Ocean currents play a significant role in shaping biogeographic and phylogeographic structures of marine ecosystems. Oceanographic regimes of the southern African coast are complex and I provide a brief insight into their role as drivers of patterns of biodiversity in the region. The southern African coast is defined here as stretching from Namibia, South Africa to Mozambique. From a biogeographic prospective, the southern African coast is of

great interest because of its location at the transition zone between the Atlantic Ocean and Indian Ocean, thus increasing the intricacy of species richness and endemism within this region (Awed et al. 2002; Lessios et al. 2003; Rocha et al. 2005). To a certain extent, these high levels of species richness and endemism are driven by climatic and oceanographic systems of the South African coastline.

South African coastal waters are influenced by two very different currents: the Benguela Current along the west coast of southern Africa and the Agulhas Current located along the east and south coast, with a 'transition zone' on the south west coast (between Cape Point and Cape Agulhas) (Branch et al. 1994, see Fig. 1.1). As a result of wind-driven upwelling, the Benguela Current is also highly productive, supporting abundant marine life (Griffiths et al. 2010). It is characterized by low temperatures (15 - 17^{o} C) and cold nutrient rich water (Kirst et al. 1999). In contrast, the Agulhas Current carries warm nutrient poor water onto the southern African continental shelf and deflects offshore as the shelf widens moving towards the south along the Agulhas Bank, where it retroflects when it encounters the eastward flowing southern Atlantic current, see Fig. 1.1 (Lutjeharms et al. 2000, 2010). Upwelling proceedings also occur within the Agulhas Current, but are only limited to regions nearby Port Alfred and Port Elizabeth (Lutjeharms et al. 2000). Through the different environmental parameters and also the transport of organisms in the form of larvae or adult in the water column, both currents strongly affect the biogeography of marine organisms in the southern African region (see for example von der Heyden, 2009; Teske et al. 2011).

1.2.2 Patterns of biogeography and phylogeography in southern African

Studying spatially arranged genealogies within and among closely related species (Presa et al. 2002; Beheregaray, 2008) can be used as an important tool to identify phylogeographic and biogeographic breaks within marine ecosystems (Avise, 1994; Palumbi, 1996; Hedgecock et al. 2007). Integrating biogeographic and phylogeographic applications is crucial as they both aim to understand distribution patterns of populations across different habitats. Previous work by Avise (1992, 1994) showed an overlap between phylogeographic patterns of widespread species and biogeographic boundaries. Considering that the same mechanisms that acted upon limiting species distribution also acted as a barrier to genetic flow thus creating a genetic structure, biogeographic and phylogeographic patterns may coincide (Avise, 1992, 1994).

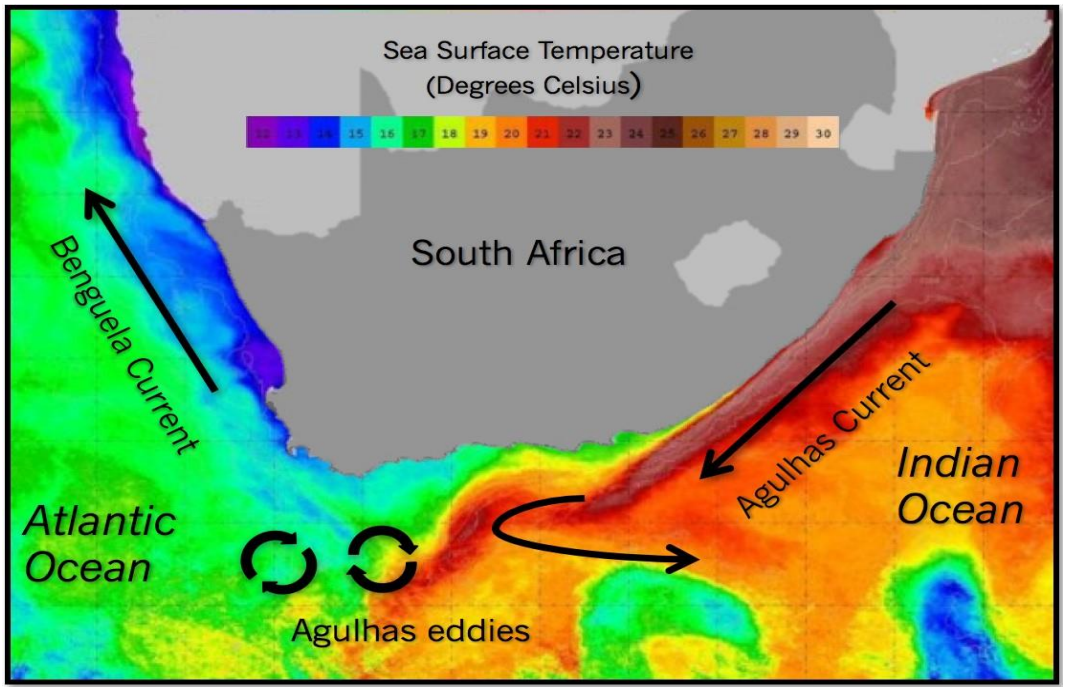

Figure 1.1: Major ocean currents and sea surface temperatures of South Africa (World Ocean Database 2009), (Nielsen, 2016).

The South African coastline is defined by five distinct coastal marine biogeographic regions: Namaqualand, South Western Cape, Agulhas, Natal and Delagoa (Lombard et al. 2004, see Fig. 1.2). Notably, within each bioregion there is a variety of localized habitats (e.g., reef, sand, mud, rocky shores), and each bioregion contains its own characteristic biota (Griffiths et al. 2010). The study of biological life with attempts to document and comprehend the spatial and temporal distribution patterns of biological diversity is termed biogeography, and thus genetic breaks across these biogeographic regions are termed phylogeographic breaks (Briggs, 1995; Avise, 2009; Gibbons et al. 2010).

There is a lack of consensus in the exact location of biogeographic boundaries in South Africa as not all species show the same biogeographical and phylogeographical patterns (Harrison, 2002; Teske et al. 2009; von der Heyden, 2009). This has led to some debate whether phylogeographic and biogeographic breaks are congruent, but there is some evidence for their congruence. In addition, some species lack divergence across phylogeographic and biogeographic boundaries (Teske et al. 2006, 2011). Further, most studies are biased towards a single taxa (although see Wright et al. 2015 for a comparative approach). This makes it

difficult to understand and to draw general conclusions on genetic and biogeographic patterns of South African marine species.

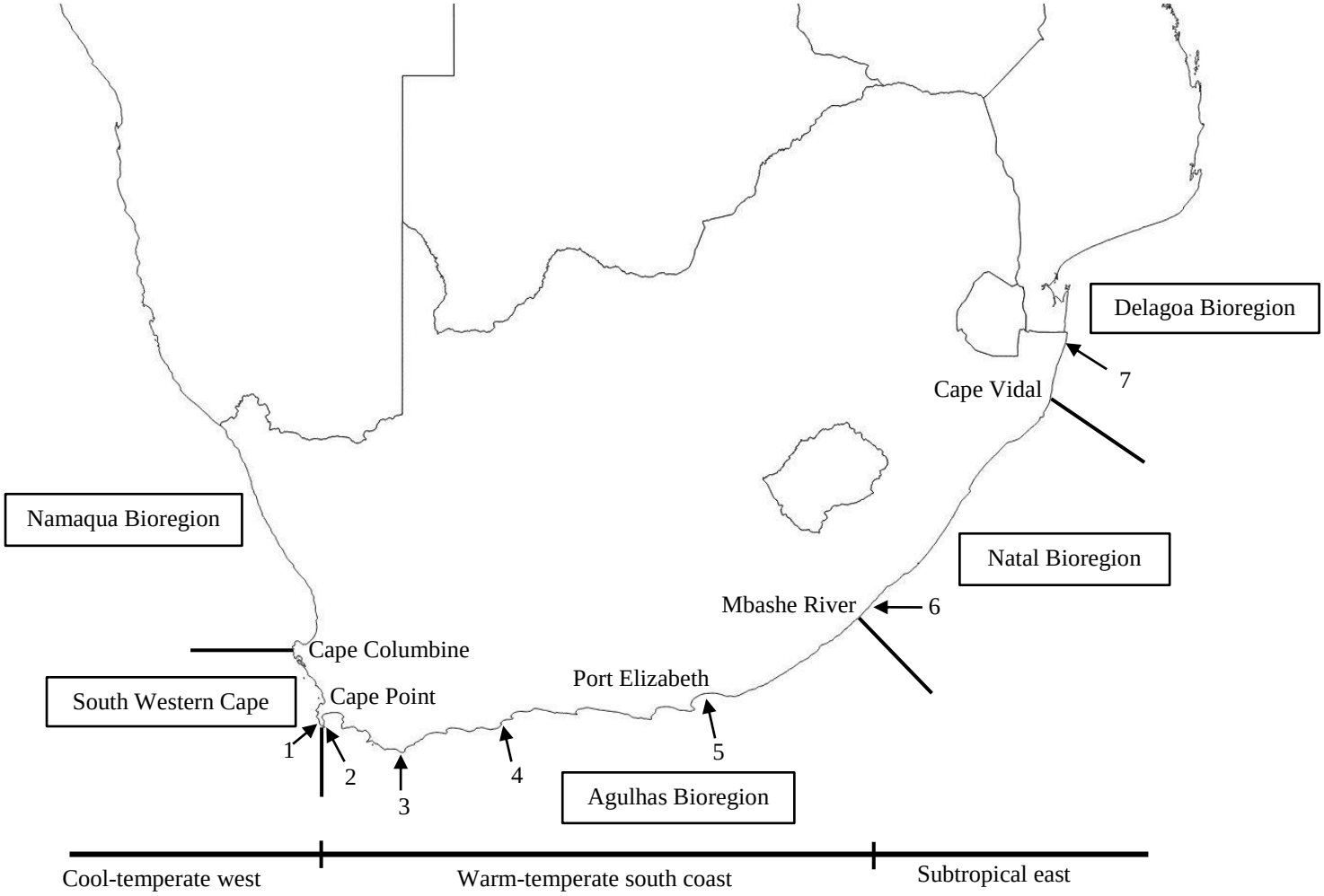

Figure 1.2: The five bioregions of South Africa indicated by black solid lines. The three major temperature regimes are also shown. Arrows with numbers represent areas that show breaks in gene flow: 1 = Cape Point, 2 = False Bay, 3 = Cape Agulhas, 4 = Mossel Bay, 5 = Algoa Bay, 6 = Wild Coast and 7 = St. Lucia/Mozambique Border (after von der Heyden, 2009).

With increasing impacts of global climate change, species distribution, range and abundance are expected to change. In the South African region, only a few studies have documented species distribution patterns in response to increasing sea surface temperatures (James et al.

2013, 2016). These studies have mainly focused on coastal and estuarine species such as the grey mullet from the family Mugilidae (James et al. 2016), where the abundance of fish species of mugilids across their biogeographic distribution (tropical, warm - water and cool - water endemics) was taken into account. To model the effects of climate change over time on these coastal species, the three groupings were related to sea surface temperatures. Results indicated a positive correlation between coastal sea temperatures, distribution and abundance. For this reason, mugilids are proposed to be some of the first fish species to respond to the global climate change (James et al. 2016). Such studies have led to an increasing attention in assessing biogeographical and phylogeographical patterns of southern African marine species. This is partly due to the necessity to establish Marine Protected Areas (Emanuel et al. 1992; Turpie et al. 2000) and the compelling need to understand potential effects of the rapidly changing climate (Anderson et al. 2012). In the South African region, several studies have identified locations that appear to reduce gene flow between populations. These include Cape Point, Cape Agulhas, Algoa Bay, Central Wild Coast and at the border of South Africa and Mozambique (von der Heyden et al. 2009; Teske et al. 2011; Wright et al. 2015), but how climate change could affect these patterns is unknown.

Studies by Teske et al. (2006) which focused on three estuarine crustacean species (*Upogebia Africana*, *Exosphaeroma hylecoetes* and *Iphinoe truncata*), as well as Teske et al. (2007) focused on the caridean shrimp (*Palaemon peringueyi*) and Teske et al. (2009) on an estuarine prawn (*Callianassa kraussi*); all provide examples of a certain degree of concordance between phylogeographic and biogeographic breaks along the South African coast line. Research on three closely related species of clinid fish *Clinus cottoides*, *Clinus superciliosus* and *Muraenoclinus dorsalis* (von der Heyden et al. 2008, 2011, 2013) revealed various degrees of population structuring between the west, south and east coast populations that corresponds with biogeographic regions.

Genetic boundaries among marine populations are not easy to quantify, as marine populations are generally not separated by permanent or ‘hard’ barriers to gene flow. With no or few obvious physical barriers to gene flow, marine species with large ranges are expected to show genetic homogeneity over long stretches of the ocean, known as the panmixia paradigm (Dawson et al. 2011). For instance, the rocky shore dwelling *C. caffer* shows no genetic variation across its entire distribution range along the South African coastline (Neethling et al. 2008). In marine ecosystems, species-specific requirements and life-history traits play a significant role in shaping population genetic structures (Santos et al. 2006), and thus

facilitates gene flow between populations of *C. caffer* (Neethling et al. 2008). The examples above provide contrasting views on the mechanisms that drive population genetic patterns in the region, that are also not linked to life-history, except for live-bearing species that are consistently more structured than brooding or live-bearing taxa (Wright et al. 2015).

1.3 South African sandy beaches

1.3.1 Phylogeographic patterns of southern African sandy beach species

South Africa's sandy beaches are listed as some of the best studied in the world (Nel et al. 2014), however, most of these studies have focused on documenting biodiversity and the classification of beach type and there is a distinct lack of genetic data. With only a few papers published thus far on biogeographic and phylogeographic patterns of southern African sandy beach species (see examples below), there is not enough information to conclude on the genetic structure of sandy beach species in this region. Results from these studies show either no genetic structure or shallow structuring for southern African sandy beach species.

The population structure of the gastropod, *Bullia digitalis* was studied by Grant & da Silva-Tatley (1997). Their study sites covered eight localities along the South African coast and one location in Namibian. The genetic analysis of 22 protein-coding loci revealed no genetic structure between subpopulations of *B. digitalis.* This study was followed by Laudien et al. (2003) who looked at the sandy beach surf clam *Donax serra* (Bivalvia) collected along two biogeographic regions: the cold province (Benguela Current) and the warm province (Agulhas Current). Morphological data had failed to clarify whether or not populations from the two biogeographic regions belong to the same species (Laudien et al. 2003), so *D. serra* samples were collected from two selected sandy shores along the South African coastline and two from the Namibian coastline. Low genetic separation was detected, thus, the larval stage of *D. serra* appears to be efficient to allow dispersal (Laudien et al. 2003) and low connectivity, but also indicated the importance of the upwelling cell at Lüderitz as a potential genetic barrier to dispersal (Laudien et al. 2003). Following on this study, Bezuidenhout et al. (2014) found no genetic structure between four populations of *D. serra* from four localities along the South African coast. Their results further revealed low haplotype diversity which was interpreted as a sign of a recent demographic expansion.

Recently, Muteveri et al. (2015) recovered a shallow population structure between species of *Bullia rhodostoma* collected along the South African coast at eight localities within the Benguela Current and Agulhas Current. Muteveri et al. (2015) concluded that the observed phylogeographic patterns for *B. rhodostoma* showed that the species could have possibly been restricted to the South-West Coast (Agulhas Bioregion) and perhaps also the East coast and later expanded westwards after the Last Glacial Maximum.

Though few studies in the southern African region have attempted to determine biogeographic and phylogeographic patterns of sandy beach species, most of these studies have focused on a single taxa, characterized by different life histories and sampled from different areas. To my knowledge, no studies in South Africa have attempted to document genetic patterns of sandy beach species using taxa with similar life histories, sampled from the same areas.

1.3.2 Conservation status of South African sandy beaches

Almost 42% of the South African coast is sandy, 31% comprises mixed shores and 27% is rocky (Griffiths et al. 2010). Currently, 23% of the 3113 km long coastline of South Africa is under formal protection of Marine Protected Areas (Griffiths et al. 2010; Harris, 2012). The marine protection network is expected to increase with 22 new proposed MPAs published in the Government Gazette 2016, although many of these form part of offshore protected areas. Although sandy beaches cover 42% of the South African coastline, they are currently poorly protected (National Biodiversity Assessment, 2011).

Harris (2012) amongst other studies, showed that the set conservation target of 10% for marine environments globally, proposed by the Convention of Biological Diversity is too low to properly conserve sandy beaches and their species. Of the 110 known sandy beach macrofauna species in South Africa, 44% are endemic and 19% occupy only one or two of the bioregions in South Africa (see Harris, 2012 for further details). Furthermore, a large number of these endemics are found on the west and south coasts (Harris, 2012). The west coast is known to be the most threatened region in the South African coastline (Harris, 2012), with pressures in this region including diamond/mineral mining, reduced freshwater flow, coastal development, kelp or seaweed harvesting and coastal squeeze (Harris 2012). At present, the west coast is only protected by two MPAs (the Langebaan Lagoon and also the

Table Mountain MPA), which leaves the vast majority of this ~800 km coastline without formal protection.

1.4 Integration of genetic data into conservation and management

Fisheries, coastal developments, overexploitation and climate change are increasing pressures on biodiversity (Hoegh-Guldberg & Bruno, 2010; Mead et al. 2013; D'agata et al. 2014). Thus, the establishment of MPAs has helped to protect endangered species, ecosystems, maintain biodiversity and provide educational opportunities (Pendoley et al. 2014). Marine Protected Areas aim to protect ecosystem structure, function and integrity, enhance non consumptive opportunities, improve fisheries, expand the knowledge and understanding of marine ecosystems and help to protect endangered species, habitat variations, and ecological and evolutionary processes (McLachlan & Brown, 2006; Sowman et al. 2011; Halpern et al. 2014; Pendoley et al. 2014). A number of successful studies have shown the importance of implementing MPAs (for examples see Lubchenco et al. 2003; Kleczkowski et al. 2008; Barrett et al. 2009; Harrison et al. 2012; Kerwath et al. 2013). According the Convention of Biological Diversity (CBD) (2010), MPAs are seen as significant key mechanisms to reduce the rate of biodiversity loss. A specific protected area target was set requiring 'at least 10% of the world's ecological regions effectively conserved' with representative protected area systems established by 2010 and, in the case of marine protected areas (MPAs), by 2012 (CBD, 2004). Facing the ongoing biodiversity declines, during the tenth meeting of the CBD parties, in 2010, 20 "Aichi Targets" were agreed upon to be met by 2020. Governments have committed to conserving 17% of terrestrial and 10% of marine environments globally, especially "areas of particular importance for biodiversity" through "ecologically representative" Protected Area systems or other "area - based conservation measures", while individual countries have committed to conserve 3 - 50% of their land area (CBD, 2010; Butchart et al. 2015).

Since MPAs were first established in the 1960s and 1970s, 8.7% of the continental shelf in Kenya is protected, 8.1% in Tanzania, 4.0% in Mozambique (Wells et al. 2007), in South Africa 21.5% of the coastline lies within MPAs, however, only 9% of South Africa's coastal habitat types and 4% of the offshore habitat types are fully no protected no-take zones (Sink et al. 2012). Though MPAs have proven to be useful in the protection of many species globally, they are still insufficient to conserve biodiversity levels (genes, species and

ecosystems) on their own (Hanks & Myburgh, 2015). This is because MPA planning has focused on ecosystems and species and not necessarily on the processes that drive patterns of biodiversity. In order for MPAs to be effective, they must be connected to form a larger network (von der Heyden, 2009; Wright et al. 2015). Though larval surveys, biodiversity monitoring and fish tagging methods have been used to understand MPAs connectivity, they are not always successful (von der Heyden, 2009) and thus, it is important to incorporate the genetic component in conservation planning, as it helps to understand process that shape distribution patterns of species (Avise, 2000; Waters et al. 2003; von der Heyden, 2009; Beger et al. 2014; von der Heyden et al. 2014; Nielsen et al. 2017). In a recent paper by Wright et al. (2015), patterns of connectivity for phylogenetically diverse marine species were studied through the analysis of mitochondrial data sets. Interestingly, their results showed that the current South African MPA network is not effective in promoting population connectivity and protecting local scale processes. Furthermore, it was recommended that MPAs should be constructed in a manner that forms a network comprised of closely associated MPAs. This study provided evidence of how important it is to incorporate genetic data in marine conservation. Globally, the application of genetic techniques to conservation and management of marine resources and ecosystems is well established and research has focused on a wide range of marine taxa and ecosystem types, such as sandy beaches (Laudien et al. 2003; Ketmaier et al. 2010), rocky shores (von der Heyden et al. 2008; Marko et al. 2010), coral reefs (Ridgway et al. 2008; Almany et al. 2009), estuaries (Teske et al. 2006; Earl et al. 2010) and seamounts (Miller et al. 2010).

A further consideration to make is that marine biodiversity is vastly understudied, both globally and in South Africa (Griffiths et al. 2010). Species that are difficult to recognize based on morphology but are genetically different are defined as cryptic species (Bickford et al. 2007). Cryptic speciation is found to be very common in marine invertebrates (Knowlton, 1993), more specifically in isopods (for examples see Held & Wägele, 2005; Lefébure et al. 2006; Varela & Haye, 2012; Tourinho et al. 2016). Given that advances in molecular biology have proven to be extremely useful in the delimitation of cryptic species (Palumbi et al. 1997), this has increased our knowledge on biodiversity and its patterns of distribution. Having a broader understanding of cryptic species in particular biogeographic regions might reveal for example underestimated levels of diversity or uniqueness or endemism, which increases the conservation priority level of that particular region (Bickford et al. 2007). For example, von der Heyden et al. (2011) showed four genetic clades within two clinid species,

which led to the description of two new species (Holleman et al. 2012). From this study it can be elucidated that before the implementation of a marine reserve, one must have a full understand of the life-history traits of the target species, evolutionary history, population structure and possibilities of gene flow between these populations. A pertinent example is the unpublished data by Hawkins (2016) that revealed greater cryptic species within the genus *Eurydice*. Initially, only three species of *Eurydice* were known to occur on South African beaches: *Eurydice longicornis*, *Eurydice kensleyi* and *Eurydice barnardi*. However, molecular data revealed four new species within the genus. Genetic data allowed distinguishing the four morphospecies into four phylospecies as described by the Phylogenetic Species Concept (Hawkins, 2016). This increases the number of known *Eurydice* species from three to seven. This study raises awareness that sandy beach diversity should not be underestimated and their conservation should not be overlooked.

1.5 Project Aims

Distribution patterns of southern African sandy beach species across environmental gradients have not been well documented and there is a current lack in knowledge of biogeographic and phylogeographic patterns of sandy beach species in the southern African region. Thus, in order to contribute towards plugging the sandy beach phylogeography knowledge gap, the aims of this project were to focus on determining the genetic structure of three isopod species. These species can be found across several bioregions (Namaqualand, South Western Cape and Agulhas) and thus make it possible to better disentangle biogeographic and phylogeographic patterns of sandy beach species in South Africa. The chapters are arranged as follows:

Chapter II examines the phylogeographic patterns of the Giant Beach Pillbug *Tylos granulatus*, which is endemic, on the west coast of South Africa and Namibia.

Chapter III defines and compares the phylogeographic patterns of two sympatrically distributed *Excirolana* species across most of their range.

CHAPTER II

Phylogeographic patterns of the Giant Beach Pillbug, *Tylos granulatus*, along the west coast of southern Africa

2.1 The genus *Tylos*

Isopods of the genus *Tylos* Audouin, 1826, belong to the family Tylidae (Kensley, 1974; Brown & Odendaal, 1994), which also includes a second genus, *Helleria* Ebner, 1968 (Schmalfuss & Vergara 2000). These oniscidean isopods occupy the upper intertidal zone of sandy beaches. The genus *Tylos* currently contains 21 recognized species that are distributed on sandy beaches globally (Hayes, 1970; Kensley, 1974; Brown & Odendaal, 1994). Based on morphology, a number of *Tylos* species in the past have been incorrectly identified as *Tylos latreillii* Audouin, 1826, an isopod species that was originally found in Egypt (Audouin, 1826). This led to a misconception that *Tylos* included highly dispersive species (for example see Schultz, 1970), which contradicts their biological characteristics (Hurtado et al. 2014). *Tylos* lacks a planktonic larval stage, which makes them direct developers (Schultz 1970; Kensley, 1974; Brown & Odendaal, 1994), suggesting highly restricted dispersal abilities. Furthermore, adult *Tylos* species have limited swimming abilities and thus avoid entering the ocean (Brown & Odendaal, 1994). This is partially because they can only survive for few hours when fully submerged underwater (Schultz 1970; Kensley, 1974; Brown & Odendaal, 1994). However, Schultz (1970) and Kensley (1974) explained that juveniles of certain species could potentially disperse from one beach to another through means of surfing by rolling into a ball that will be washed off through wave action.

Tylos granulatus is known to have a 24 - hour cycle that is in line with the cycle of the tides (McLachlan & Brown, 2006). They remain buried and inactive during the day near the previous high tide zone to avoid desiccation, but emerge to feed on kelp, detritus and algae along the high tide mark (Hamner et al. 1969; Schultz 1970; Kensley, 1974; Hayes, 1977). Before dawn, they burrow back into the sand, thereby preventing desiccation and also being washed away during the following high tide (Hamner et al. 1969; Schultz 1970; Kensley, 1974; Hayes, 1977). Different species of *Tylos* burrow to different depths, as do populations of the same species on different beaches (Brown & Odendaal, 1994).

Of the 21 recognized species globally, two are endemic to southern African sandy beaches: *Tylos granulatus* and *Tylos capensis* (Krauss, 1843; Schultz, 1970; Kensley, 1974; Brown & Odendaal 1994). The first description of *T. granulatus* and *T. capensis* was by Krauss in 1843 where both species were recorded in Table Bay. Donn & Cockcroft (1989) recorded *T. granulatus* as far as Cape Cross in Namibia. Later, Branch et al. (1994) put the *T. granulatus* distribution along the west coast of Namibia to sandy beaches near Cape Point in South Africa, with *T. capensis* distribution stretching eastwards from Cape Point to Port Elizabeth. The two species show no overlap in their distribution ranges (Kensley, 1972).

Kensley (1974) established that *T. granulatus* can burrow to depths up to 500 mm into the sand and *T. capensis* to 300 mm. Odendaal et al. (1994) found that *T. granulatus* in certain beaches could burrow to one metre or more. Brown & Trueman (1994) reported that *T. granulatus* from Yzerfontein were found at only 10 - 20 cm below the sand. However, no matter how deep they may be, it is easy to tell whether *Tylos* are present on a beach by looking for the burrows they retreat into by day above the high tide mark and exit holes at night, see Fig. 2.1.

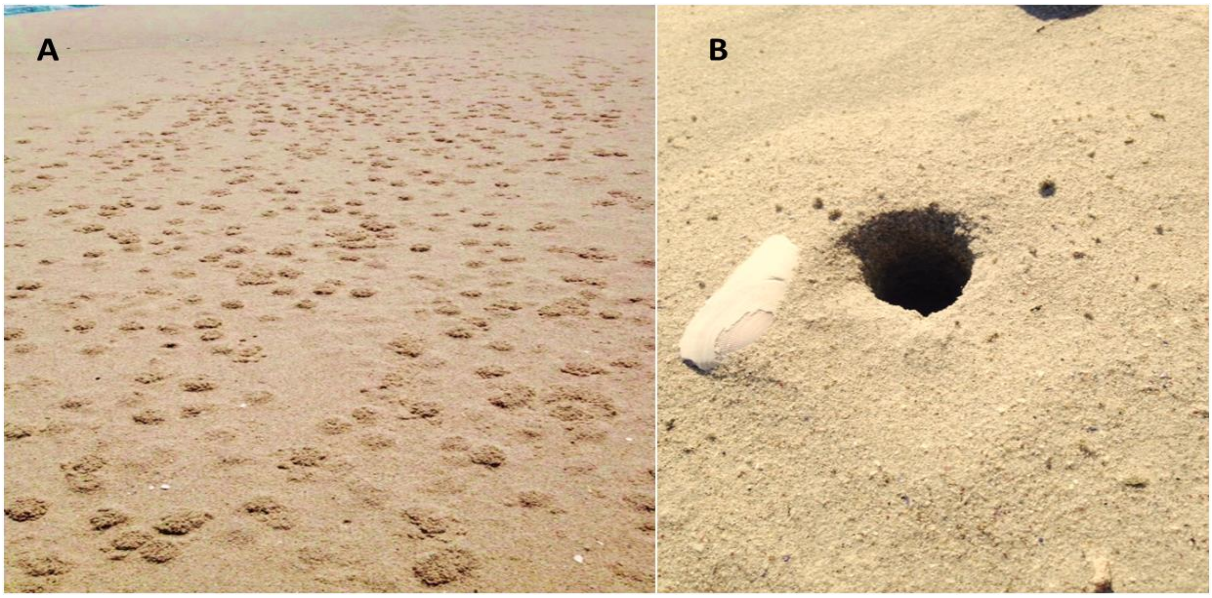

Figure 2.1: Characteristic molehills (A) and exit holes (B) of *Tylos granulatus* (Pictures taken in Doringbaai, 2016).

2.2 Is *Tylos granulatus* an endangered species?

Sandy beach invertebrates usually have a high tolerance to change and can easily adapt to environmental fluctuations, including those prompted by anthropogenic activities (Brown, 2000). Brown & McLachlan (1990) further explain that even after sandy beach populations have been destroyed, recolonization is highly common in most sandy beach species; however, there appears to be an exception for the Giant Beach Pillbug, *T. granulatus*. Populations of *T. granulatus* used to thrive in high abundance along the west coast of South Africa and Namibia, however, their abundance has drastically declined and local extinction of this species has been reported from some South African beaches. The following paragraphs will provide a list of documented threats to the decline of *T. granulatus*.

Tylos granulatus are very susceptible to pollution as they are semi-terrestrial species (meaning they are exposed to pollution from both land and the sea, Brown & Odendaal, 1994). In 1974, after crude oil was washed off onto the shores of Bloubergstrand, a few of the *T. granulatus* individuals that emerged at night were all almost instantly killed (Kensley, 1974) as their pleopods (respiratory systems) were clogged leading to death (Kensley, 1974). In 1986, populations of *T. granulatus* were recorded as far south as Cotton Beach in Strand (Fig. 2.2), and now populations of this species have completely disappeared in this region (pers. comm.). When comparing historical distribution ranges of *T. granulatus* from the year 1986 - 2008 and following this up with surveys conducted in 2015 and 2016, the distribution range appears to have become narrower (see Fig. 2.2 and Appendix 1).

With increasing pressures along the South African coastline, Brown (2000) noted that *T. granulatus* might be becoming an endangered species and identified off-road vehicles as the main cause of population declines of *T. granulatus*. Brown & Odendaal (1994) cited that thriving populations of *T. granulatus* that once occurred in Hout Bay, South Africa, in the 1950's went through complete local extinction after construction of a road and a parking area in the forefront of foredunes. *Tylos granulatus* populations have also completely disappeared in False Bay, which may be linked with the removal of kelp (Brown & Odendaal, 1994).

Tylos granulatus is likely to be highly impacted by habitat transformation and coastal development, climate change and coastal squeeze (Brown & McLachlan, 1990; Dugan et al. 2008). For example, *T. granulatus* needs dunes and the supralittoral habitat to survive; however, building of walls on the shoreline due to increasing sea level rise compresses the intertidal zones. This coastal squeeze will result with the disappearance of the high tide mark (the high tide zone will become the middle zone and the middle zone will become the low

tide zone) (Schlacher et al. 2007; Dugan et al. 2008). With pressures such as sea level rise and coastal development accelerating at a fast rate, coastal squeeze is a threat of most concern thus appropriate mitigation steps need to be urgently taken into account to conserve sandy beach ecosystems (McGranahan et al. 2007; Mendoza-González et al. 2012; Seto et al. 2011).

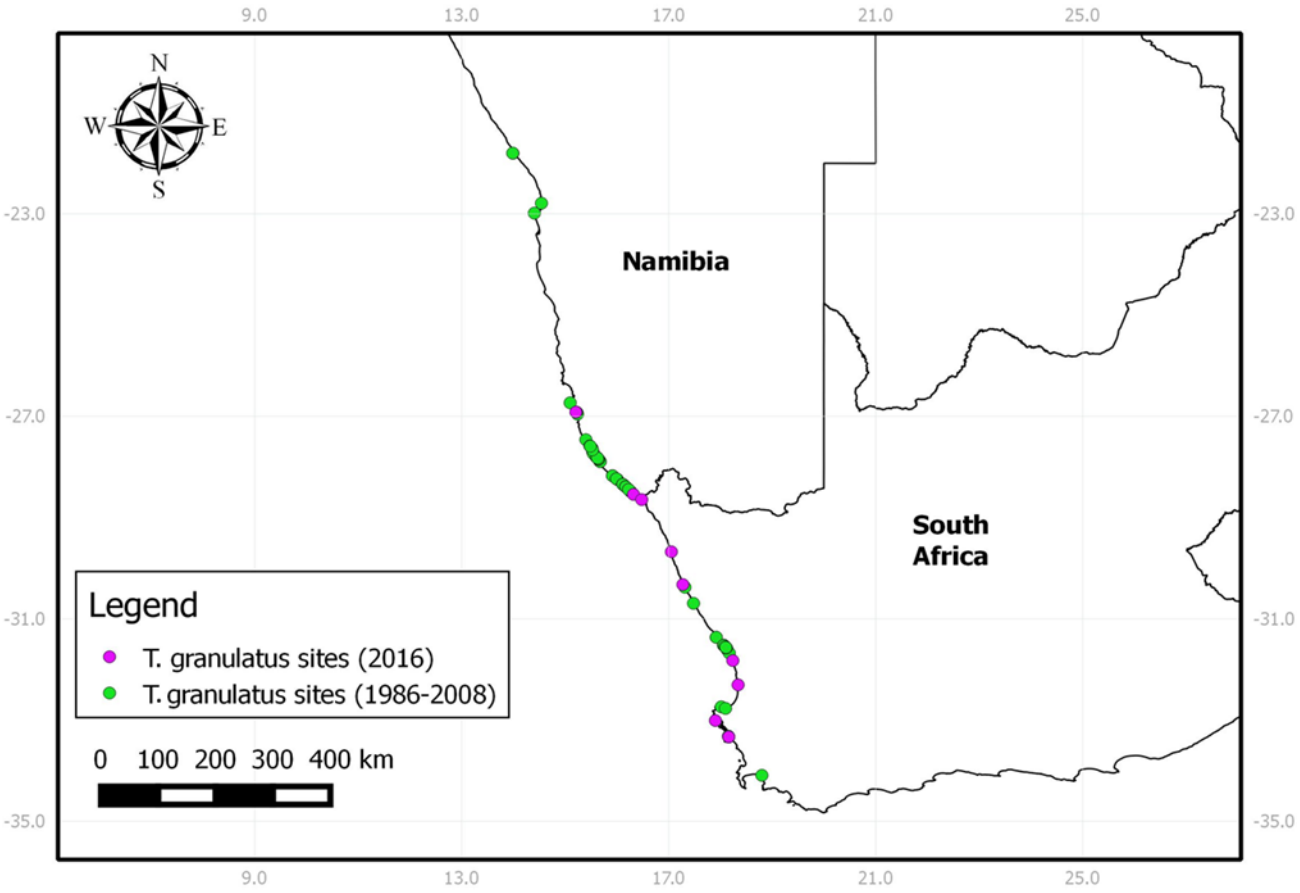

Figure 2.2: Map showing the distribution range of *Tylos granulatus* between 1986 to 2008 along the west coast of southern Africa. Locations covered for the purpose of this study are also shown to highlight difference between past and present day distribution patterns of *T. granulatus* along the west coast of southern Africa.

The combination of pressures of their environment, as well as their life-history characteristics makes *T. granulatus* a highly threatened species which should be ranked high in the International Union for Conservation of Nature (IUCN) Red Data listing as they are on the edge of extinction (Brown, 2000). However, this taxon has not yet been assessed for the IUCN Red List.

2.3 Influences of historical and contemporary processes on southern African marine populations

As much as the above section provides evidence of major threats to the abundance of *T. granulatus*, this raises a question to what extent historical and contemporary processes have played a role in driving population declines and structuring of *T. granulatus.* Current patterns of genetic diversity and population connectivity are driven by both past population processes and by historic and contemporary breaks in gene flow (Avise, 2000; Hemmer-Hansen et al. 2007). Additionally, historical climatic changes in the Pleistocene that brought about changes in ocean surface temperatures, sea level, ice sheet cover and oceanographic patterns have been connected to critical changes in demographic history (Janko et al. 2007), distribution of species (Grant & Bowen, 2006), genetic diversity (Lecomte et al. 2004), population structure (Bester-van der Merwe et al. 2011) and speciation (Avise et al. 1998; Shen et al. 2011).

Phylogeographic breaks are identified through levels of intra-specific population structure and genetic divergence (Rocha et al. 2005, 2007; Avise, 2009; von der Heyden et al. 2011; Hawkins, 2016). Although phylogeographic breaks are poorly understood in marine systems, several studies suggest that climate and oceanographic oscillations of the Pleistocene period had major impacts on biogeographic and phylogeographic patterns of marine species (Hewitt, 2000; Grant & Bowen, 2006; Janko et al. 2007; Teske et al. 2007; Muller et al. 2012; Toms et al. 2014; Muteveri et al. 2015). However, it is challenging to document impacts of sea level oscillations in areas that were not covered by ice during the glacial periods, such as southern Africa (Reynolds et al. 2014). Further, genetic divergence between marine populations is commonly defined by patterns of allopatric speciation. This divergence has shown to be strongly impacted by vicariant events (Toms et al. 2014), such as that of the formation and loss of the land bridge across the coast of Australia (Wares & Cunningham, 2001; York et al. 2008; Ayre et al, 2009). In the southern Africa region, there is a lack of evidence of such events (Teske et al. 2011) and only a few studies have successfully provided evidence of vicariance that has shaped phylogeographic patterns of South African marine populations (Toms et al. 2014; Reynolds et al. 2014; Henriques et al. 2014). For instance, Toms et al.'s (2014) study showed that the divergence of two lineages of the klipfish, *Clinus cottoides*, was linked to lowered sea levels that changed the topology and composition of the South African coastline. Specifically, there were large reductions in rocky shores and an increase in muddy or sandy shores, thus isolating populations of this obligate rocky shore fish.

Several studies from North America and Europe have created a foundation of research to understand the Expansion Contraction (EC) model of the Pleistocene period (Hewitt, 1999, 2000; Provan & Bennett 2008, Woodall et al. 2011) which explains changes in distribution range, genetic diversity and population structure of species in correspondence to historical glacial - interglacial (Marko et al. 2010; Zhang et al. 2014). The EC model explains the pole - ward shift of Northern Hemisphere species and their recent range expansion to higher altitudes after the LGM (Hewitt, 2004). In South Africa, several studies have provided evidence of species population expansion following the LGM (see Kirst et al. 1999; Marlow et al. 2000; Jahn et al. 2003; Neethling et al. 2008; Silva et al. 2010; von der Heyden et al. 2010; Teske et al. 2011; Muller et al. 2012; Henriques et al. 2014; Muteveri et al. 2015).

Processes that yield phylogeographic breaks within the South African region are poorly understood. However, a few studies have indicated climatic and oceanographic fluctuations as the main drivers of population structuring along the South African coast (Teske et al. 2007; Muller et al. 2012, Toms et al. 2014). Phylogeographic breaks may be a result of historical events; nonetheless, significant contemporary processes such as oceanographic currents, life-history traits, local adaptation and behavioral traits are essential to maintaining them (Neethling et al. 2008; Pelc et al. 2009; Teske et al. 2011; Wright et al. 2015). Oceanographic currents play a significant role in distribution patterns of coastal species through larval dispersion. However, some studies have shown that ocean currents can also act as barriers to population connectivity in marine populations and can even result in across-currents speciation events (Henriques et al. 2012, 2014). Species-specific requirements and life-history traits play a significant role in shaping population genetic structures (Santos et al. 2006), thus, genetic variability is highly influenced by dispersal (Silva et al. 2010). Gene flow between populations hinders genetic variability, but when gene flow is low and there is local adaptation, this creates genetic isolation of populations (Wei et al. 2012). One important life-history trait in close relation with dispersal and recruitment is pelagic larval duration (PLD) (Weersing & Toonen 2009; Reynold et al. 2014, Baco et al. 2016). Although PLD might not be the best to quantify genetic estimates for brooding and spawning species, life-history traits have proved to be extremely useful in assessing genetic structures in live - bearing species (Weersing & Toonen, 2009; Kelly & Palumbi, 2010; Selkoe & Toonen 2011; Wright et al. 2015, Baco et al. 2016). Larvae with high dispersal potential may migrate across habitats where barriers are absent and this generally results in genetically homogenous populations; however, some marine species have distinct genetic structure in spite of their high dispersal

potential. Lastly, another important process to take into account across phylogeographic breaks is local adaptation. Local adaptation across phylogeographic breaks is highly influenced by environmental factors and in South Africa, temperature gradients and sand inundation has been put forward as potential factors of adaptation (Teske et al. 2008, 2011).

With a plethora of natural and human induced threats to marine systems and sandy beaches in particular, there has been an increasing need to understand and include patterns of genetic diversity, population structure and expansion (Reed & Frankham, 2003) in conservation and management (Zhanng et al. 2014; von der Heyden et al. 2014, 2017). Given the changes to its habitat, *Tylos granulatus* is potentially an endangered species and a good candidate to invetigate phylogeographic and biogeographic patterns. Biological characteristics (no planktonic larval stage) of *T. granulatus* suggest limited gene flow between populations and for this reason and high levels of genetic differentiation between populations of *T. granulatus* along the west coast of southern African is expected. High allopatric divergence linked to biological characteristics of *Tylos* has been observed in several studies (see Hurtado et al. 2010, 2014; Niikura et al. 2015). Further, sessile and sedentary organisms that are mostly habitat specific are more prone to Pleistocene climatic oscillations considering their limited mobility (Marko, 2004).

This chapter makes use of a phylogeographic approach to determine levels of genetic structuring among populations the Giant Beach Pillbug *T. granulatus* to better understand possible impacts of oceanographic and climatic oscillations on southern African sandy beach species. The chapter focuses on the following questions: (i) Did historical oceanographic and climatic changes of the Pleistocene period play a significant role in shaping present day phylogeographic patterns in *T. granulatus*? (i) Is there a pattern of high levels of genetic differentiation for *T. granulatus*, as expected for a direct developer with habitat specialization, which has been observed for other coastal isopods, including *Tylos* species? (ii) And if so, is there a concordance between phylogeographic breaks in *T. granulatus* and phylogeographic breaks detected for other species and or biogeographic boundaries?

Materials and Methods

3.1 Specimen collection

A total of 214 specimens of *T. granulatus* were collected from nine evenly distributed localities along the west coast of South Africa and Namibia (Fig. 2.3 and Table 2.1). Sampling localities were chosen based on records of the species distribution range by Kensley (1978), Brown & Odendaal (1994). Distribution patterns of the Giant Beach Pillbug were further assessed by visiting locations that had known populations of *T. granulatus* using data from the year 1986 - 2008 (Appendix 1). Where typical burrows were found above the high tide mark, indicated the presence of *T. granulatus* on the field. Samples were collected by hand (digging) and preserved in 100% ethanol. Samples were obtained under permits issued by the Department of Agriculture, Forestry & Fisheries (RES2015/26, RES2017/40).

3.2 Examination of *T. granulatus* morphology

In the genus *Tylos*, species are distinguished based on the shape of fifth pleonite (Schultz & Johnson, 1984), see Fig. 2.4. This morphological trait was examined and photographed for all collected individuals of *T. granulatus* for correct identification (see Fig. 2.5) using an M125 microscope and the Leica Application Suite software. I further analysed the copulatory stylet on the 2nd pleopod (Fig. 2.6) at a higher magnification power using an AutoMontage microscope. This morphological characteristic has been useful to distinguish isopod species.

3.3 DNA extraction

Genomic DNA was isolated from 2 - 4 legs per specimen using the CTAB extraction protocol (Winnepenninckx et al. 1993). However, the CTAB protocol did not work for all the samples and in those cases, the NucleoSpin® Tissue kit (Machery - Nagel) was used to extract DNA according to the manufacturer's instructions. To determine the quantity (ng/ml) and quality of DNA obtained, each sample was analysed using a NanoDrop (ND-1000) Spectrophotometer.

Table 2.1: Information on *Tylos granulatus* sampling locations, GPS coordinates and sample size (*N*) together with the number of individuals sampled for both COI and 16S shown in brackets.

Location	*N* (COI, 16S)	Longitude	Latitude
Elizabeth Bay	12 (10, 1)	- 26.918333	15.207111
Oranjemund	21 (16, 2)	- 28.544417	16.320389
Alexander bay	6 (6, 2)	- 28.647639	16.479167
Kleinzee	15 (15, 1)	- 29.678139	17.052333
Hondeklip Bay	25 (21, 3)	- 30.326194	17.274722
Doringbaai	35 (22, 1)	- 31.828167	18.239361
Elands Bay	42 (35, 1)	- 32.307222	18.341444
Saldanha Bay	33 (30, 2)	- 33.012528	17.902806
Yzerfontein	25 (25, 2)	- 33.334056	18.160861

3.4 Molecular markers and DNA amplification

A Polymerase Chain Reaction (PCR) technique was used to amplify two mitochondrial genes fragments: cytochrome oxidase subunit I (COI) and 16S ribosomal RNA (primers and annealing temperatures in Appendix 2). Mitochondrial DNA (mtDNA) is one of the most commonly used genetic markers (Hare, 2001; Avise, 2009) and has been shown to be a sensitive indicator of speciation (Avise, 2009). The COI gene fragment was chosen because it is a highly conserved genetic marker within members of the same species, but is also variable enough to show genetic differentiation between closely related species of isopods (Edmands, 2001; Wetzer, 2001; Lee, 2012; Santamaria et al. 2013; Niikura et al. 2015). Further, its use in resolving large numbers of cryptic species and documenting biodiversity has been shown in initiatives such as the Barcode of Life (http://www.barcodeoflife.org/).

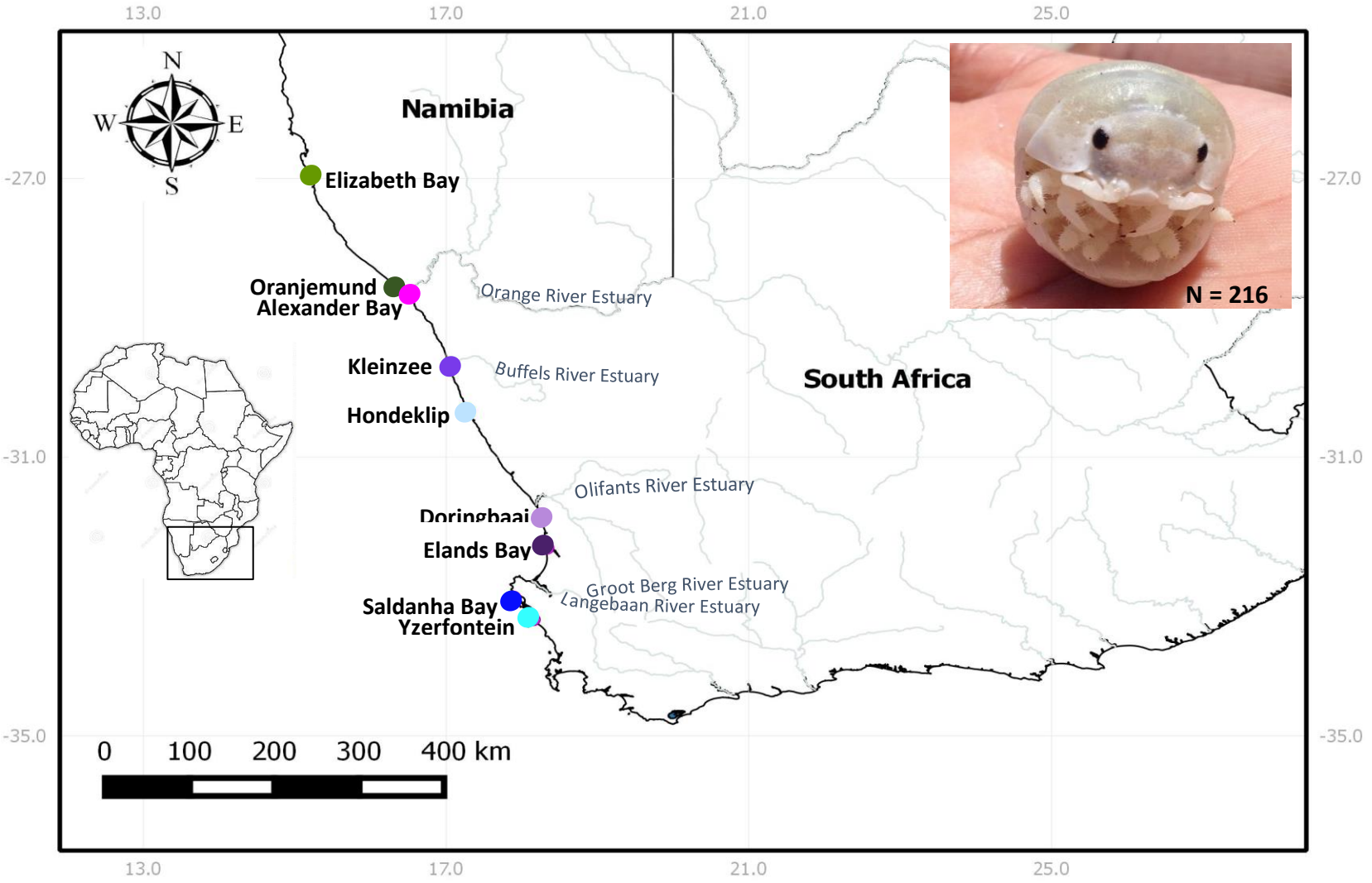

Figure 2.3: Sampling localities for *Tylos granulatus.*

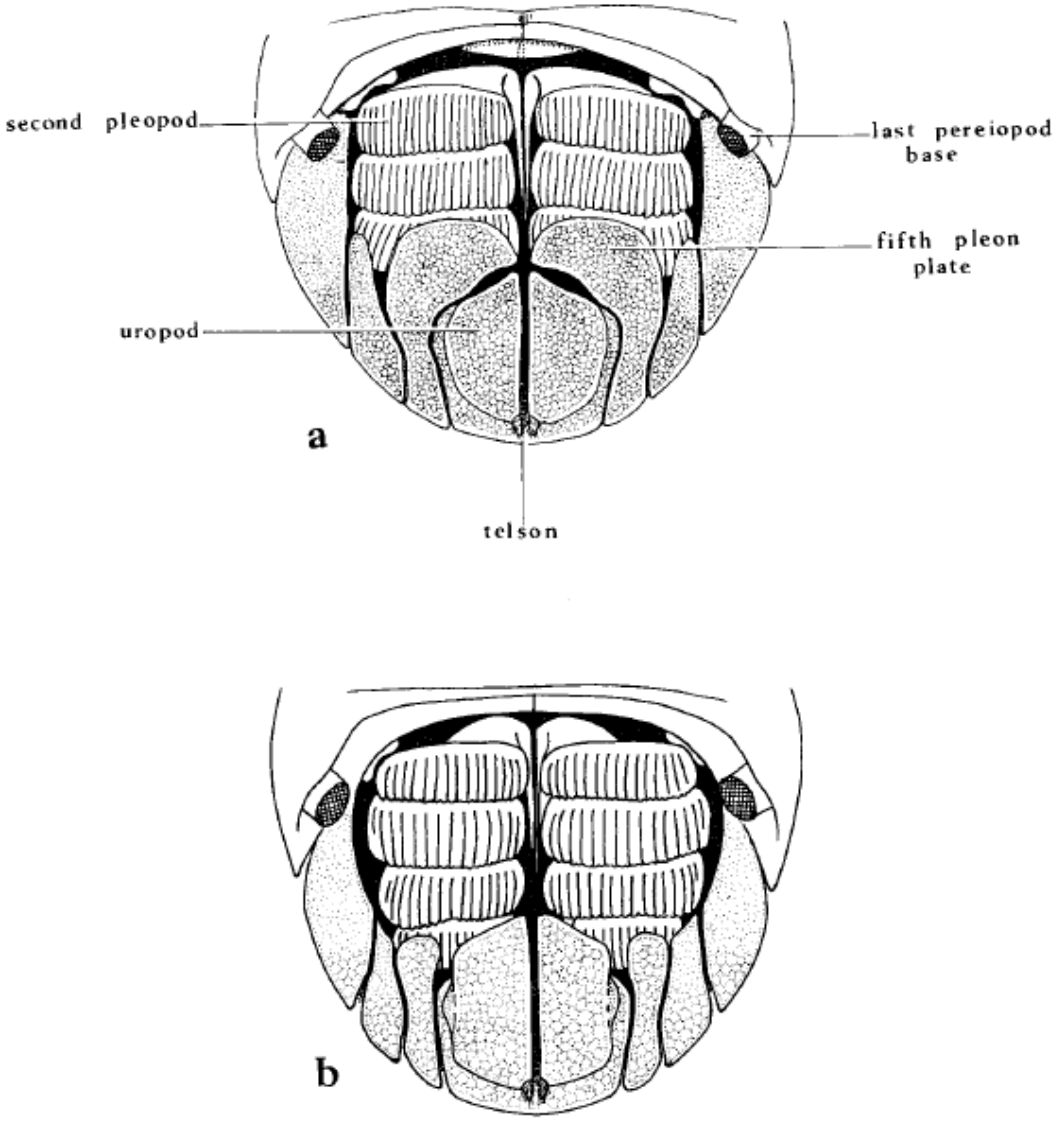

Figure 2.4: Ventral view of the pleon (a) *Tylos granulatus* (b) *Tylos capensis* (from Kensley, 1974).

The 16S gene has been applied in a number of studies on phylogenetic patterns of the sandy beach isopod *Tylos* (Hurtado et al. 2010, 2013, 2014). Applying this gene would allow a comparison between southern African *Tylos* species to international *Tylos* sequences available on GenBank (http://www.ncbi.nlm.nih.gov/genbank). Due to time as a limiting factor, only 15 sequences were obtained from the 16S gene. These were only included in a phylogenetic tree, no further analyses were performed for the 16S marker. All other results will be reported based on the COI gene.

Each 25 µl PCR reaction contained 1 µl of ~90 - 150 ng/µl of the template DNA, 2.5 µl of the 1 x reaction buffer, 2.5 µl of 2mM $MgCl_2$, 2.5 µl of 0.1 mM dNTP, 1.25 µl of 0.5 pmol of each primer, 0.1 µl of 0.5 u Super-Therm BioTaq DNA polymerase (Super-Therm, JMR Holdings, London, United Kingdom), 2 µl of 10 mg ml^{-1} Bovine Serum Albumin (BSA) solution and distilled water. PCR products were separated and visualised on 1% agarose gels in TBE buffer containing ethidium bromide (0.5µL/mL). PCR products were loaded and run alongside a 1kB DNA ladder at 100 volts for 60 minutes using a BioRad Electrophoretic apparatus and then photographed. Correct fragment sizes from all the PCR products were carefully extracted from the gels using Biospin Gel Extraction Kits following the manufacturer's instructions. Gel purified products were sent for sequencing at the Central Analytic Facility (CAF) at the Stellenbosch University using an ABI 3730 Capillary sequencer with Big Dye terminator chemistry (Applied Biosystems).

3.5 Sequence datasets and alignments for phylogenetic analysis

Sequences were manually aligned, edited and trimmed in Geneious v7.1.9 (http://www.geneious.com, Kearse et al. 2012). All sequences were referenced to the chosen taxonomic group *T. granulatus* for mitochondrial COI and 16S rRNA in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov) to determine which species they matched to, and to obtain outgroups to root the trees. Additional sequences for comparing South African and Namibian *T. granulatus* to other *Tylos* sequences were obtained from GenBank (see Appendix 3 for the downloaded sequences and GenBank accession numbers). A few sequences of *T. capensis* were kindly provided by Karien Bezuidenhout, Zoology Department, Nelson Mandela Metropolitan University (NMMU), but these have not yet been published (see Appendix 4 for *T. capensis* information). To ensure all the sequences were in the correct reading frame and to check that no stop codons were present in the protein - coding gene COI, all nucleic acid sequences were translated to peptide sequences using the software Geneious v7.1.9. No stop codons were present in any of the protein coding sequences.

3.6 Genetic diversity

DNA - sp version 4.90.1 (Rozas & Rozas, 1999) was used to generate a haplotype data file. Genetic variability within populations was quantified as the number of haplotypes (*H*), haplotype diversity (*h*) and nucleotide diversity (π). These indices were calculated for each location using Arlequin 3.5.1.2 (Excoffier, 2004; Excoffier et al. 2010).

3.7 Phylogeography and demographic history

3.7.1 Analysis of genetic structure

An AMOVA analysis was used to determine variance within and between groups (populations). Population pairwise fixation indices (Φ_{st}) were also calculated between the nine populations; their significances was tested with a nonparametric approach with 10,000 permutations. To test the hypothesis of correlation between genetic and geographic distance among populations of *T. granulatus*, estimates of Isolation by distance (IBD) were verified using the Mantel test (Mantel, 1967). The analysis was carried out using Φ_{st} / (1 - Φ_{st}) as executed in GENEPOP 4.6 (Raymond & Rousset 1995; Rousset, 2008). Geographic distances between sampling localities (in kilometres) were obtained from Google Earth Pro (https://www.google.com/earth/). The significance of correlation between genetic and geographic distances was estimated in XLSTAT 2017 (https://www.xlstat.com/en/) using the Mantel *Z*-test (Mantel, 1967) with 10,000 permutations. Because analyses showed two distinct lineages and because the signal of IBD can be inflated by strong population genetic structure (Meirmans et al. 2012), patterns of IBD for the two lineages were analysed separately.

In order to visualise the connections between individual *T. granulatus* sampled from different areas, a parsimony-based haplotype networks of the COI region was constructed using NETWORK 5 (http://www.fluxus - engineering.com/sharenet.htm, Bandelt et al. 1999).

3.7.2 Demographic history

To test whether populations of *T. granulatus* are at equilibrium, Fu′s Fs (Fu, 1997) test was performed in Arlequin with 10,000 permutations. A negative and statistically significant F value indicates an excess number of low frequency haplotypes, signifying recent population expansion (Fu, 1997). A positive F value indicates a lack of alleles, as would be expected

from populations that went through genetic bottlenecks. Fu's Fs were considered significant when the P-values < 0.05. To further explore the hypothesis of a recent population expansion in *T. granulatus*, demographic expansion parameters were estimated using the mismatch distribution analysis in Arlequin (Ramos-Onsins & Rozas, 2002). These include the raggedness index (*r*) and the sum of squared deviations (SSD) (Rogers & Harpending, 1992). Low and non-significant *r* and SSD supported the hypothesis of demographic expansion. Since the analyses showed two distinct lineages, to avoid over exaggeration of the genetic pattern, the two lineages were analysed separately.

3.8 Phylogenetic analyses

3.8.1 Data preparation

After preliminary analysis, data suggested deep genetic divergence among mitochondrial lineages in *T. granulatus*; thus, in order to place the evolutionary history of *T. granulatus* into context, I added sequences generated from my study into a phylogenetic tree with other *Tylos* species (see Fig. 2.10 and Appendix 3). The aim was to work out the level of divergence between the two lineages, specifically to see whether they correspond to population or species - level divergences. Between one to three sequences were selected from the two lineages to represent all the sampling sites. Best - fit evolution models were determined by using the Akaike information criterion in MrModeltest v2.3 (Nylander, 2004). Both the COI and 16S were analysed independently. As preliminary results from both the COI and 16S trees indicated deep genetic divergence between lineages in *T. granulatus*, they were concatenated for a Bayesian analysis to estimate evolutionary relationships (Huelsenbeck & Ronquist, 2003; Zhang et al. 2012).

3.8.2 Phylogenetic tree constructions and sequence divergence

3.8.2.1 Bayesian analysis

Sequences were selected for Bayesian analysis of phylogeny in Mr Bayes version 3.2.6 (Huelsenbeck & Ronquist, 2003). For both concatenated and individual trees, the analyses were run between five and ten million generations. The trees were set to be sampled every 1000 generation with two runs. By default, the number of chains was set to four, meaning

that MrBayes used three heated chains and one "cold" chain (Ronquist et al. 2011). The burnin value was set to 25% of the number of generations, meaning that the first 25% of the trees were discarded. Convergence in MrBayes was determined by ensuring that the standard deviation of split frequencies was below 0.01 (Ronquist et al. 2011). Alternatively, convergence was also determined by the software Tracer v1.6 (Rambaut & Drummond, 2007) using the Effective Sample Size (ESS > 200). Trees were visualised and edited in FigTree v1.4.2 (Rambaut, 2012).

3.8.2.2 Sequence divergence estimates

Estimates of sequence divergence were performed in the software MEGA 7.0.20 using the uncorrected pairwise method with the P-distance model (Kumar et al. 2016). Since genetic divergence occurs at different molecular rates in various taxa, there is a need to establish an interspecific 'standard' for species differentiation (Havermans et al. 2013). This was achieved by looking into differences between intra and interspecies P-distances of *T. granulatus* and other *Tylos* species.

3.9 Time since population divergence

To retrieve extra information on the genetic history of *Tylos*, estimates of the time of divergence were taken into account. Molecular clocks have been used to date certain events such as historical climatic oscillations or geological processes that influence phylogeographic patterns. Although useful, molecular clocks can be problematic when calibration points are missing or unknwon. Since calibration points (fossils or vicariant events) and mutation rates in *Tylos* are unknown, substitution rates to estimate divergence times were borrowed from distant isopods: *Stenasellus* and *Orthometopon*. In order to test the influence of historical events on the divergence of populations of *T. granulatus*, different COI mutation rates were used (varying between 1.25 - 2.6%/Ma; see Wares & Cunningham, 2001; Spooner & Lessios, 2009; Markow & Pfeiler, 2010) to provide estimates of divergence. As no obvious calibration point of sequence divergence is available for *T. granulatus*, three substitution rates were used to date the divergence between major lineages of *T. granulatus*. A rate of 1.25%/Ma estimated for *Stenasellus* isopods by Ketmaier et al. (2003) was used. Secondly, Poulakakis

& Sfenthourakis' (2008) evolutionary rates of 1.56 - 1.72%/Ma estimated for *Orthometopon* isopods were used to estimate time of divergence among *T. granulatus* major lineages. These evolutionary rates were implemented in BEAST 1.8.3 (Drummond et al. 2011) to estimate divergence time to the most recent common ancestor (TMRCA) between major lineages within *T. granulatus*. The BEAST package was used to run an analysis for 30 million steps of the MCMC sampled every 1000 generation using the HKY model. The data was partitioned by codon position following a strict clock theory (Drummond et al. 2006). After testing several models that best fit the data, the Yule model was used to analyse lineages detected by the phylogenetic analyses (Drummond & Rambaut, 2007). Convergence was calculated with ESS values, where an ESS > 200 was used as an indicator of convergence.

Results

4.1 *T. granulatus* distribution

For some of the locations that were visited between 2015 and 2016 that had known populations of *T. granulatus*, they were not found (Fig. 2.2, Appendix 1). These findings indicate local extinction of *T. granulatus* in some South African beaches.

4.2 Examination of *T. granulatus* morphology

As illustrated in Fig. 2.5, no evident morphological differences were found in the shape of the ventral plates of the fifth pleonite in individuals of *T. granulatus* belonging to the genetically differentiated lineages (as indicated by the phylogenetic results, Fig. 2.10). In addition, no differences were found in the copulatory stylets of *T. granulatus* (Fig. 2.6).

4.3 Genetic diversity

A partial fragment of the COI gene was obtained for 180 sequences; after trimming 617 base pairs were used for analyses. 15 sequences, each with 467 base pairs were selected and included in the phylogenetic analysis.

Sequences from the 180 COI sequences yielded 44 haplotypes, only four haplotypes were shared between 2 - 4 localities and 40 (91%) were singletons (see Table 2.2). Overall, results indicated high haplotype diversity (h = 0.25 - 1.00) and low nucleotide diversity (π = 0.00 - 0.13, Table 2.3). Notably, individuals collected from the northernmost localities, Elizabeth Bay, Oranjemund, Alexander Bay and Kleinzee exhibited higher levels of haplotype diversity (Table 2.3) than Hondeklip Bay, Doringbaai, Elands Bay, Saldanha Bay and Yzerfontein (Table 2.3).

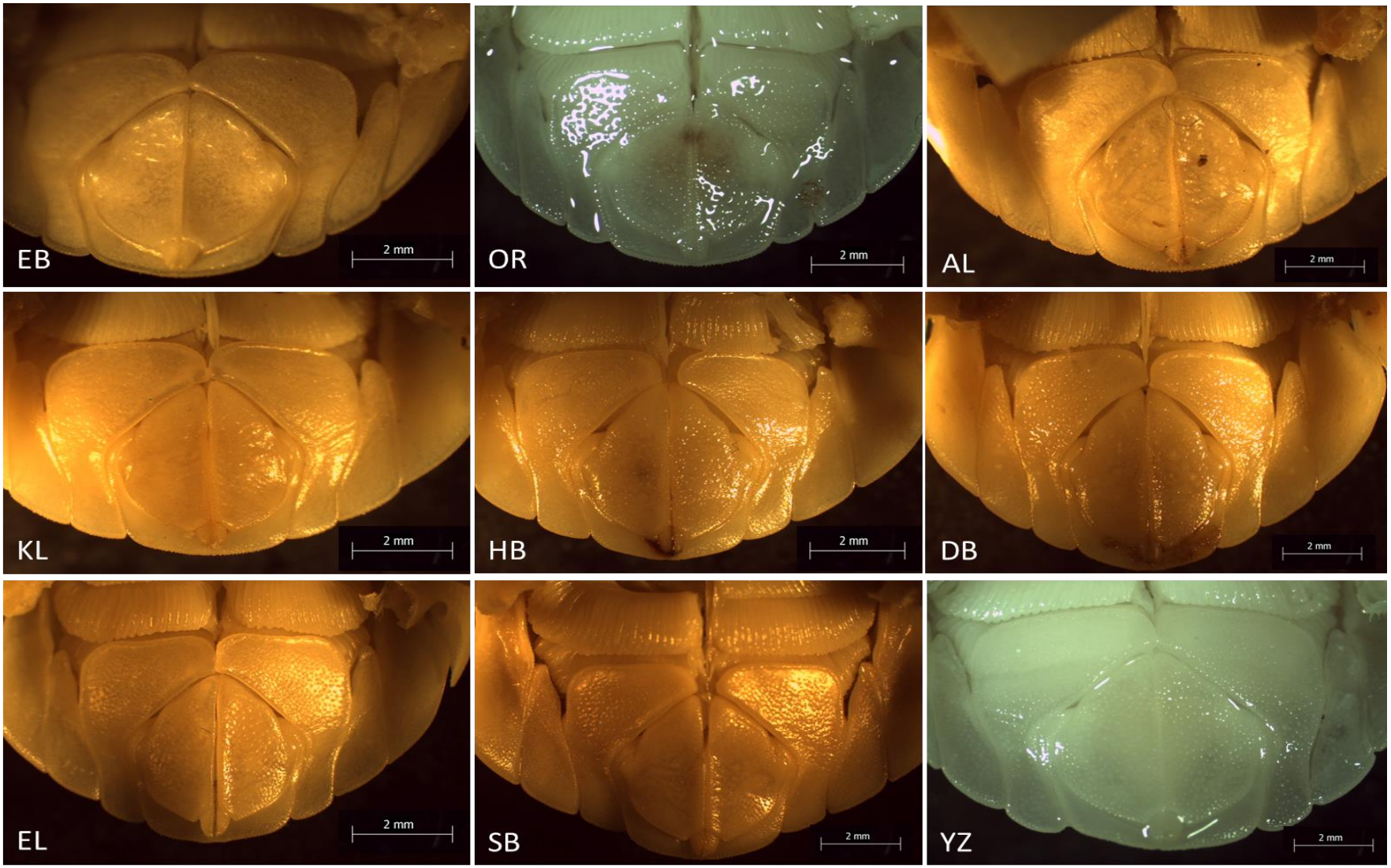

Figure 2.5: Pleon ventral shape of *Tylos granulatus* from Elizabeth Bay (EB), Oranjemund (OR), Alexander Bay (AB), Kleinzee (KL), Hondeklip Bay (HB), Doringbaai (DB), Elands Bay (EL), Saldanha (SB) and Yzerfontein (YZ).

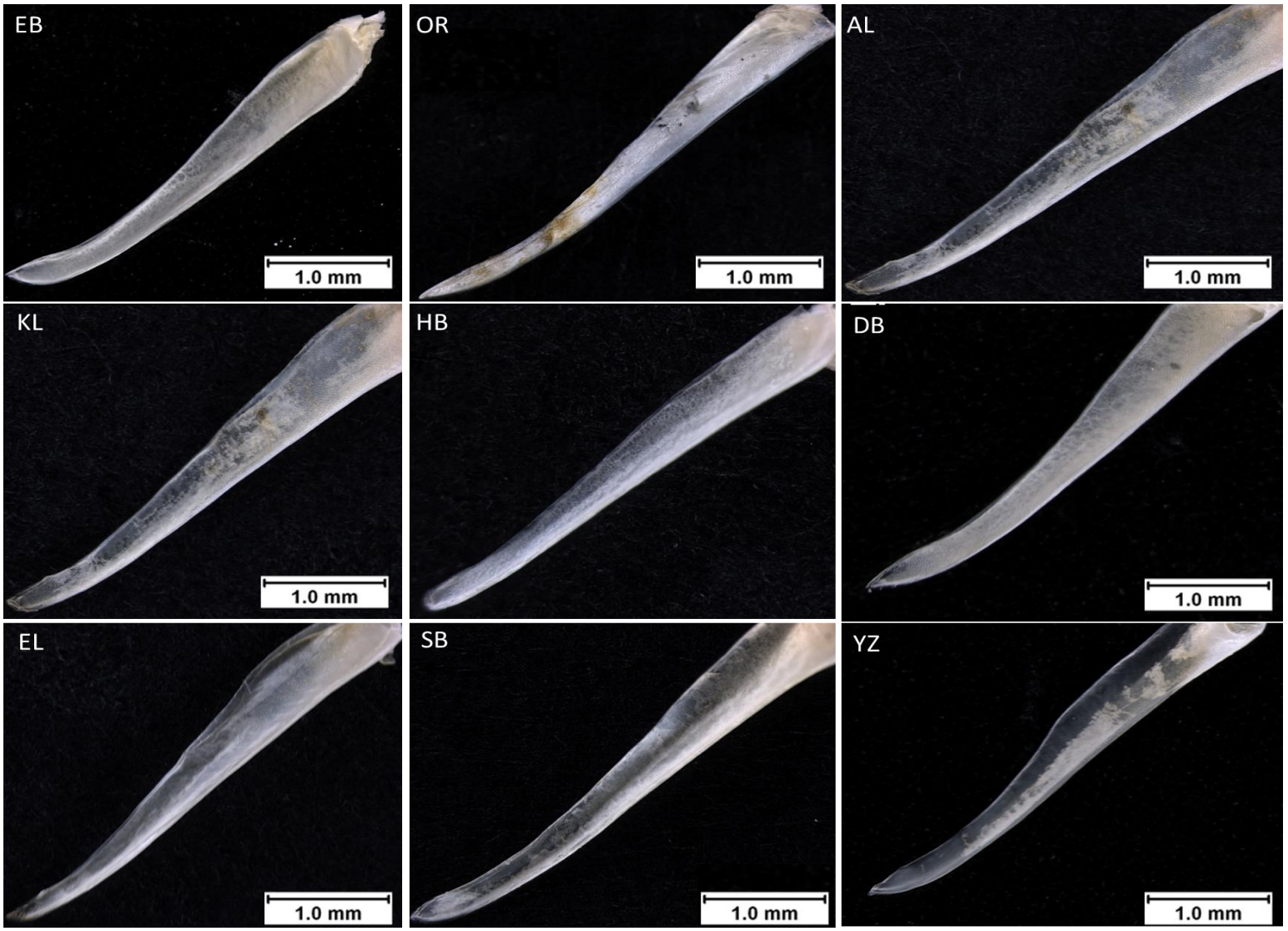

Figure 2.6: Images of male copulatory stylet in *Tylos granulatus.*

Table 2.2: Haplotype frequencies for the nine localities of *T. granulatus* along the west coast of South Africa and Namibia. Sample size (*N*) for each locality is also shown. Shared haplotypes are indicated in grey.

	N	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44
EB	10		1	1			1			1	1	1	1		1	1							1																					
OR	16		7		1	2		1	1															1		1		1	1														
AB	6	1	1																						3		1																	
KL	15													1			6	1	1	1	1	4																							
HB	21																														13												7	1	
DB	22																													19									2		1				
EL	35																													19			9		1		1		2	1	1	1			
SB	30																													26				1		1		1						1	
YZ	25																													1		24													

Table 2.3: Diversity indices for all nine sampling localities of *Tylos granulatus*.

	EB	OR	AB	KL	HB	DB	EL	SB	YZ
N	10	16	6	15	21	22	35	30	35
H	10	9	4	7	3	3	8	5	8
h	1.00 (± 0.04)	0.82 (±0.10)	0.80 (±0.17)	0.80 (±0.08)	0.53 (±0.08)	0.26 (±0.12)	0.65 (±0.07)	0.25 (±0.10)	0.65 (±0.07)
π	0.08 (±0.04)	0.13 (±0.07)	0.23 (±0.14)	0.03 (±0.02)	0.01 (±0.01)	0.00 (±0.00)	0.01 (±0.01)	0.00 (±0.00)	0.01 (±0.01)

Sample size (*N*), number of haplotypes (*H*), haplotype diversity (*h*) and nucleotide diversity (*π*). Standard deviations are shown in bracket

4.4 Population structure

The AMOVA analysis indicated that most of the genetic diversity is due to variability among populations of *T. granulatus* (89.75%) and within populations (10.25%, Table 2.4). The Global Φ_{st} across all populations was very high (Φ_{st} = 0.90, P = 0.00) (Table 2.4). Further analyses based on the pairwise comparisons (Φ_{st}) showed significant differences in eight populations of *T. granulatus*, ranging from 0.01 to 0.98 ($P < 0.05$) (Table 2.5). There was only one pairwise population comparison that was not significant; this was Oranjemund and Elizabeth Bay. Notably, significant pairwise Φ_{st} values were still detected between populations that were close to each other (~73 - km). For example, the Φ_{st} value between Yzerfontein and Saldanha Bay was 0.81 (P = 0.00).

The haplotype network (Fig. 2.7) obtained from the COI gene supports the findings of high levels of population genetic structuring. Genetic variation is mainly explained by the existence of two deeply divergent evolutionary lineages that are separated by 53 mutation steps (Fig. 2.7). *Tylos granulatus* Lineage I includes the North West coast populations (Kleinzee, Alexander Bay, Oranjemund and Elizabeth Bay) and Lineage II represents populations sampled in the southern range (Yzerfontein, Saldanha Bay, Elands Bay, Doringbaai and Hondeklip Bay). These results show a strong phylogeographic break between *T. granulatus* Lineage I and *T. granulatus* Lineage II; where Lineage I exhibits higher Φ_{st} values (ranging from 0.01 to 0.96) than Lineage II (0.06 to 0.63). This break is located somewhere between Kleinzee and Hondeklip Bay. A total of 28 haplotypes occurred in 47 individuals from the North West coast populations (*T. granulatus* Lineage I) and only 16 from the 133 samples of the southern range (*T. granulatus* Lineage II). No haplotypes were shared between the two lineages. *Tylos granulatus* Lineage I is characterized by higher levels of genetic structuring, with lower frequencies of haplotypes but more mutational steps (Fig. 2.7, Table 2.2). Genetic variation within the lineage (within populations) is explained by 64.48%. In the southern lineage, there is one very dominant (possibly ancestral) haplotype that is found in differing frequencies throughout all populations in addition to some single step mutations to other haplotypes (Fig. 2.7 and Table 2.2). There was a positive correlation between pairwise Φst and geographic distances in *T. granulatus* Lineage I, providing evidence for isolation by distance (see Fig. 2.8; Mantel's test r = 0.63, P = 0.05). The matrices were not correlated in *T. granulatus* Lineage II, see Fig. 2.9; Mantel's test r = 0.14, P = 0.70.

Table 2.4: Analysis of the molecular variance (AMOVA) for the COI gene from populations of *T. granulatus*.

Source of variation	d.f.	Sum of Squares	Variance components	% Variation	Fixation index
All populations					
Among populations	8	2104.09	13.40	89.75	Fst : 0.90
Within populations	171	261.54	1.53	10.25	
***T. granulatus* Lineage I**					
Among populations	3	114.47	2.91	35.42	Fst : 0.35
Within populations	43	227.25		64.48	
***T. granulatus* Lineage II**					
Among populations	4	79.46	0.74	73.53	Fst : 0.74
Within populations	128	34.28	0.27	26.47	

Table 2.5: Pairwise Φ_{st} values of mitochondrial COI among nine *T. granulatus* sampling sites.

	EB	OR	AB	KL	HB	DB	EL	SB
OR	**0.06**							
AB	0.44**	0.27**						
KL	0.43**	0.39**	0.63**					
HB	0.95**	0.90**	0.90**	0.97**				
DB	0.95**	0.90**	0.91**	0.98**	0.83**			
EL	0.96**	0.92**	0.93**	0.98**	0.78**	0.09**		
SB	0.96**	0.92**	0.93**	0.98**	0.84**	0.15**	0.11**	
YZ	0.96**	0.91**	0.93**	0.98**	0.89**	0.85**	0.65**	0.81**

Signification at $P < 0.05$*; Significant at $P < 0.01$**; Bold Φ_{st} values are non-significant with $P > 0.05$

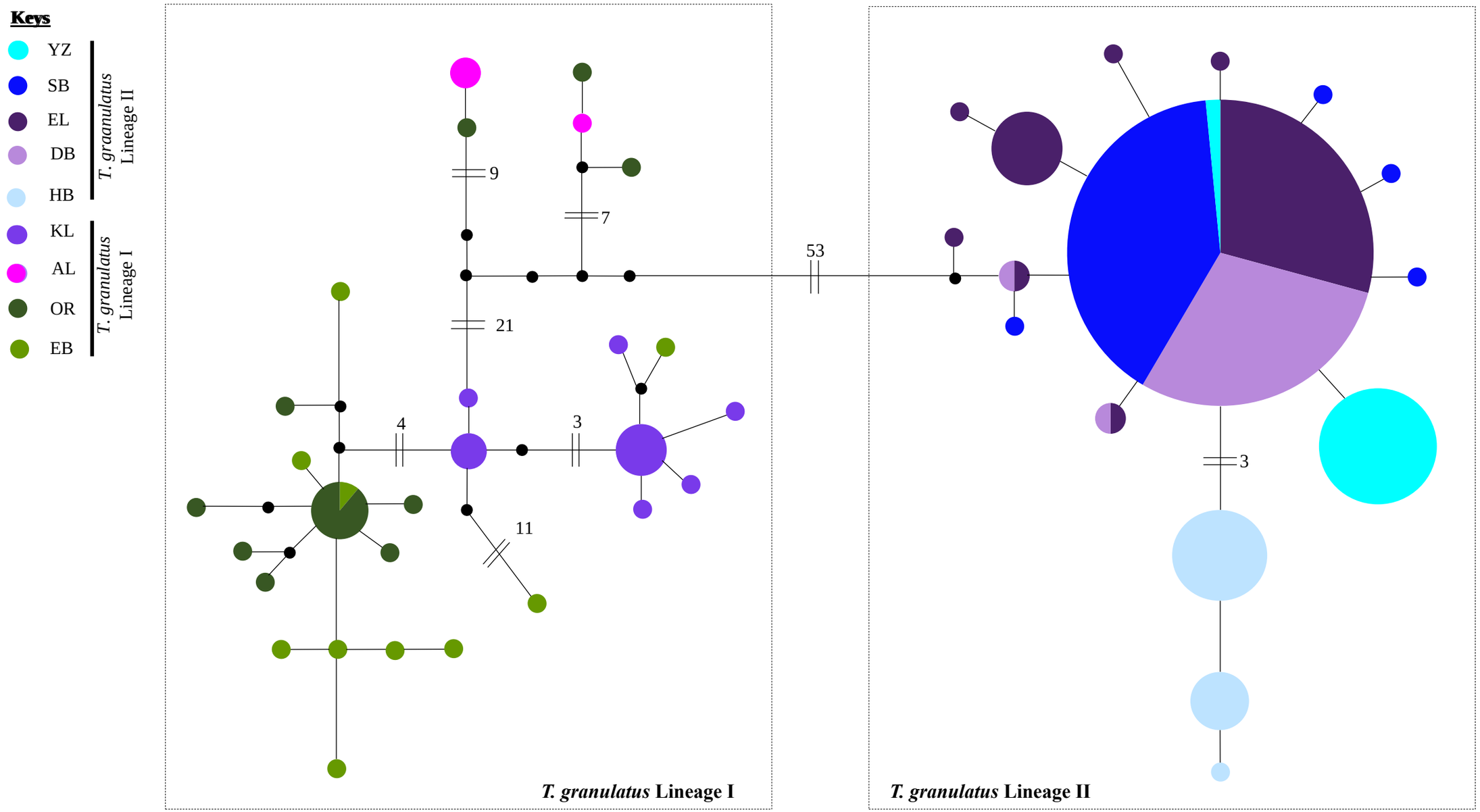

Figure 2.7: Parsimony-based haplotype network of the COI gene for *Tylos granulatus*. Different colours distinguish haplotypes and their sampling localities, colours are consistent with those used in Fig. 2.3. Circle size is comparative to the frequency of individuals in each haplotype. The partitions inside the circles represent the proportion of each population within each haplotype. A branch represent one mutational step, black dots represent missing, unsampled haplotype or extinct haplotypes.

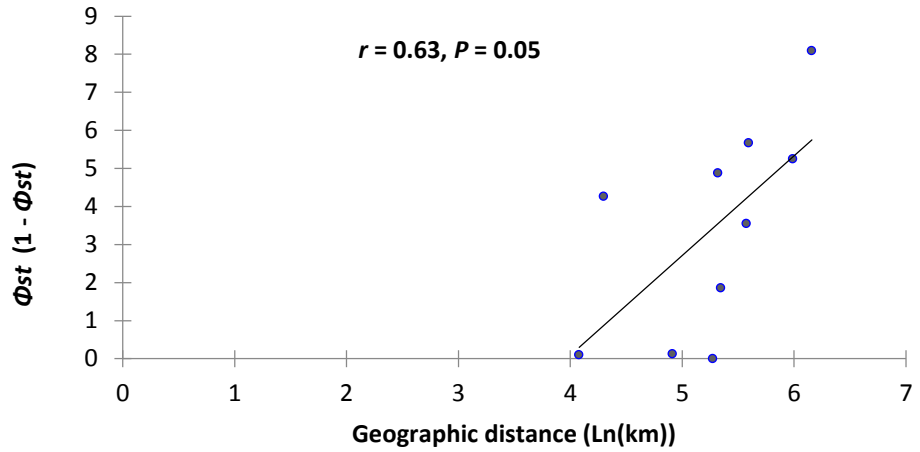

Figure 2.8: Isolation by distance in *Tylos granulatus* Lineage I, including samples from Elizabeth Bay, Oranjemund, Alexander Bay and Kleinzee. Geographic distances are plotted against genetic divergence estimates (Φ_{st} / (1 - Φ_{st}) between pairs of populations.

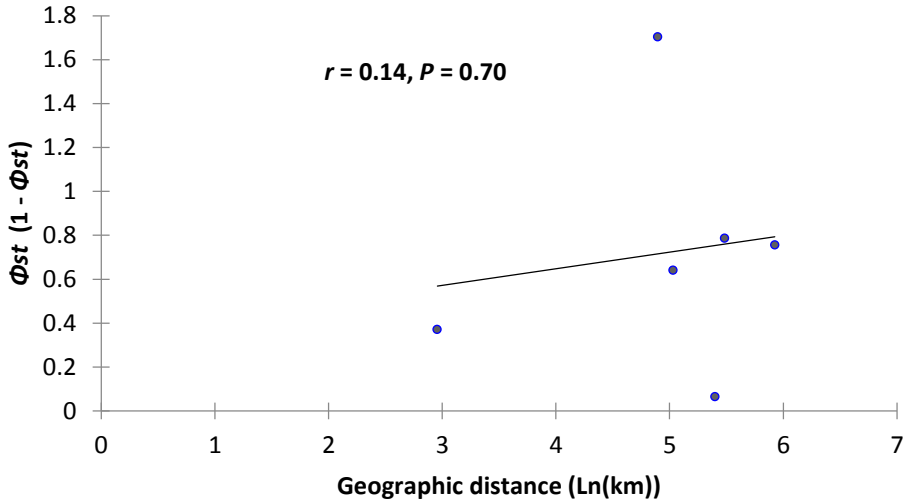

Figure 2.9: Isolation by distance in *Tylos granulatus* Lineage II, including samples Hondeklip Bay, Doringbaai, Elands Bay, Saldanha Bay and Yzerfontein. Geographic distances are plotted against genetic divergence estimates (Φ_{st} / (1 - Φ_{st}) between pairs of populations.

4.5 Demographic history

Fu′s Fs were negative and significant for Elands Bay, Doringbaai, Elands Bay, Saldanha Bay and Yzerfontein indicating an excess number of low frequency haplotypes, supporting a recent demographic expansion in the northern and southernmost populations (Table 2.6 and 7). *Tylos granulatus* populations exhibited a unimodal mismatch distribution pattern, which was indicated mostly by low and non-significant *r* and SSD values for most populations (Table 2.6 and 7).

Table 2.6: Genetic demographic history of *T. granulatus* Lineage I from four localities along the west coast of southern Africa. Neutrality test (Fu's Fs), mismatch distribution parameters θ_o and θ_1 = Pre - expansion and post - expansion populations size, τ = time in number of generations passed since the sudden expansion period, Sum of squared deviations (SSD) and the Raggedness index (*r*) are listed. P-values are also shown.

Mismatch distribution parameters	EB	OR	AB	KL
θo	8.68	2.58	0.00	0.00
θ1	99.00	4.76	145.31	5.18
τ	1.73	1.14	38.30	5.06
SSD	0.01	0.04	0.14	0.03
SSD p - value	0.91	0.39	0.03	0.54
r	0.02	0.05	0.28	0.07
r p - value	0.93	0.78	0.21	0.61
Fs	- 3.87	2.42	4.74	- 0.87
Fs p - value	0.02	0.85	0.98	0.31

Fu's Fs, mismatch analysis parameters (*r* and SSD) are significant at $P < 0.05$

Table 2.7: Genetic demographic history of *T. granulatus* Lineage II from five localities along the west coast of southern Africa. Neutrality test (Fu's Fs), mismatch distribution parameters θo and θ1 = Pre - expansion and post - expansion populations size, τ = time in number of generations passed since the sudden expansion period, Sum of squared deviations (SSD) and the Raggedness index (r) are listed. P-values are also shown.

Mismatch distribution parameters	HB	DB	EL	SB	YZ
θo	0.00	0.00	0.00	0.00	0.00
θ1	1.63	0.36	99.00	0.34	0.09
τ	2.79	3.00	0.99	3.00	3.00
SSD	0.13	0.00	0.01	0.00	0.00
SSD p - value	0.19	0.46	0.14	0.57	0.27
r	0.49	0.30	0.12	0.34	0.71
r p - value	0.11	0.57	0.15	0.57	0.84
Fs	1.47	- 1.31	- 4.24	- 3.70	- 1.60
Fs p - value	0.78	0.04	0.00	0.00	0.06

Fu's Fs, mismatch analysis parameters (*r* and SSD) are significant at $P < 0.05$

4.6 Phylogenetic analysis and sequence divergence estimates

4.6.1 Bayesian analysis

When 15 individuals of *T. granulatus* were selected from each of the two lineages and included into a tree of other *Tylos* species, *T. granulatus* showed two monophyletic lineages which were significantly different from all other *Tylos* species from Hurtado et al. (2014) (see Fig. 2.10). Separation between the two monophyletic lineages of *T. granulatus* was supported by a posterior probability (PP) of 1; the monophyly of the two lineages was also supported by

PP of > 0.96. The tree also confirmed that all populations of *T. granulatus* were distinct from *T. capensis*.

4.6.2 Sequence divergence estimates

Average genetic distances within the studied *T. granulatus* populations based on the COI sequences were relatively low ranging between 0 - 4%, whereas the mean genetic distances among populations were high (0 - 11%, see Appendix 5).

There was a clear separation between Intra and interspecies P-distances among *T. granulatus* lineages, the highest intra - species distance being 1.50% and the highest interspecies distance 10%. For other *Tylos* species, highest intra - species distance was 6.40% and the highest interspecies distance was 14.90% (Table 2.8).

Table 2.8: Intra and inter-specific COI divergence (P-distances (%)) determined for *T. granulatus* Lineage I and II and for other *Tylos* species. Sequences were selected from Fig. 2.10.

	Taxa	P-distance (%)
INTRA-SPECIFIC		
T. granulatus Lineage I	DB1 vs HB12	0.50
T. granulatus Lineage II	AB2 vs KL13	1.50
Other *Tylos* species	*T. marcuzzii* (Cuba) vs *T. marcuzzii* (Cuba)	3.10
	T. europaeus (Libya) vs *T. europaeus* (Italy)	6.40
INTER-SPECIFIC		
T. granulatus Lineage I & II	DB1 vs AB2	10.00
T. granulatus Lineage I & II	HB12 vs KL13	10.00
Other *Tylos* species	*T. sp* (Cuba) vs *T. niveus* (Puerto Rico)	14.30
	T. poncticus (Libya) vs *T. europaeus* (Italy)	14.90

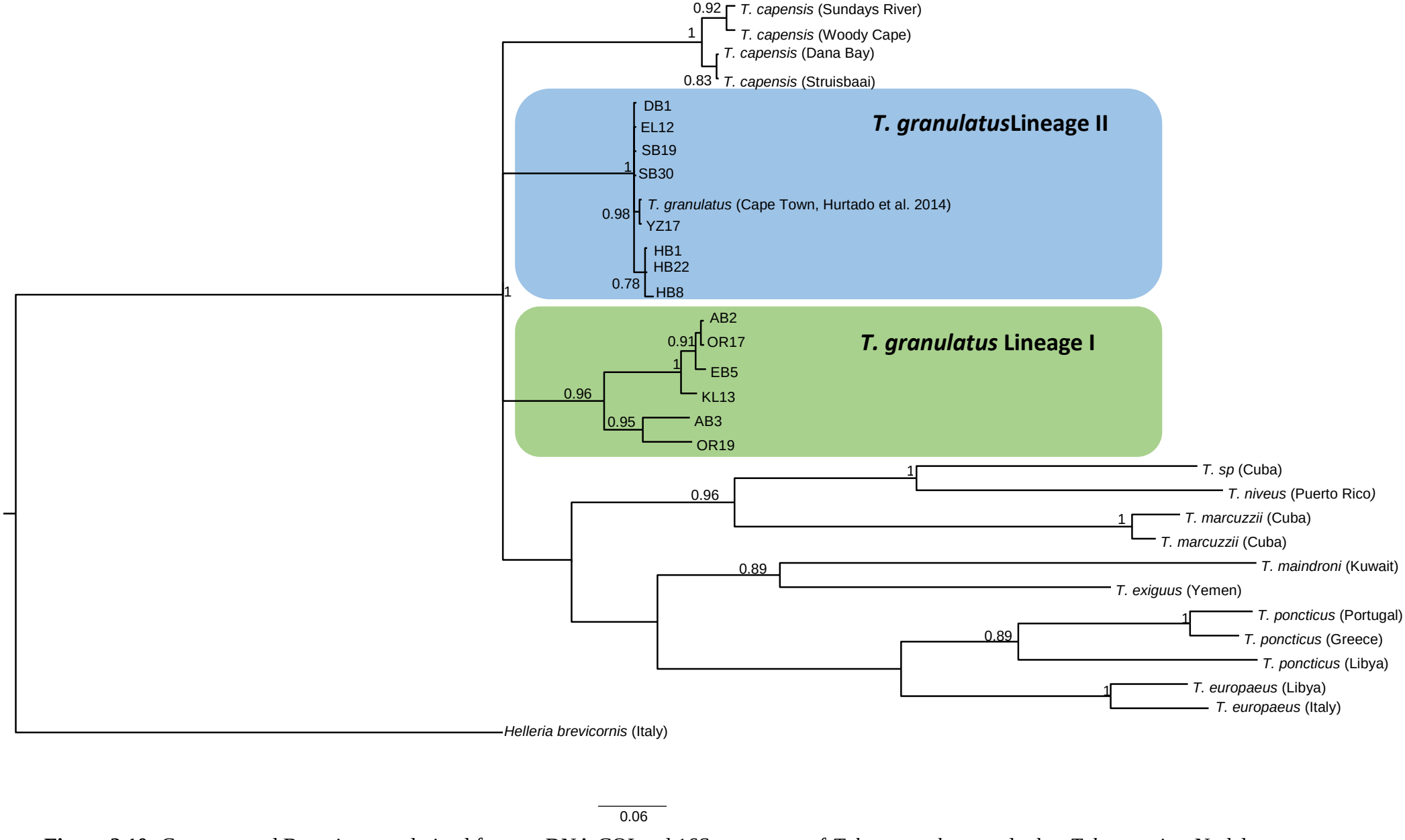

Figure 2.10: Concatenated Bayesian tree derived from mtDNA COI and 16S sequences of *Tylos granulatus* and other *Tylos* species. Nodal support is given as Bayesian posterior probabilities (BPPs). BPP values smaller than 0.70 are omitted.

4.7Time since population divergence

Estimates of time to the most recent common ancestor (TMRCA) obtained with the mutation rate 1.25%/Ma indicated that the divergence of the two *T. granulatus* lineages occurred at least 2.21 million years ago (Ma). When the mutation rate 1.56%/Ma was applied in the BEAST analysis, it suggested that the two genetic lineages diverged about 2.62 Ma. At 1.72%/Ma, *T. granulatus* lineages divergence occurred 2.84 Ma.

Discussion

5.1 Evolutionary history of two divergent *T. granulatus* lineages

The estimates of the divergence (2.21 - 2.84 Ma) suggest a strong link with the Plio-Pleistocene transition, with a complete loss of gene flow between the two lineages, as evidenced by a 53 step mutational difference. Further, both lineages are characterised by high levels of population genetic structuring, particularly in the northern part of the range. Both the mtDNA COI and 16S gene trees (data not shown), as well as the combined COI and 16S tree indicated two monophyletic lineages of *T. granulatus* (Fig. 2.10), a split that was also recovered with the mtDNA COI haplotype network (Fig. 2.7). The timing of this divergence, estimated using the mtDNA COI gene falls well within the Plio-Pleistocene transition. During this interval, the world's oceans became cooler and more stratified (Hill et al. 2017) and resulted in increased upwelling within the Benguela Current system (Avise et al. 1998; Marlow et al. 2000; Filippelli & Flores, 2009). The Plio-Pleistocene glaciation was characterized by low ocean temperatures and rapid climate and oceanographic oscillations which had major impacts on biogeographic and phylogeographic patterns of marine species (Avise, 1998; Hewitt, 2000). Henriques et al. (2014) showed that intensification of upwelling cells during the Plio-Pleistocene transition resulted in the isolation of the coastal fish, *Atractoscion aequidens*. It is highly possible that the movement of these upwelling cells could also have influenced the genetic structure of *T. granulatus* along the west coast of southern Africa. The Hondeklip Bay upwelling cell is located exactly where there was a sharp increase in genetic differentiation between the southern and northern lineage, our study found a phylogeographic break located between this region and Kleinzee (Fig. 2.7 and Fig. 2.10). Pairwise comparisons between Hondeklip Bay and Kleinzee were significantly high (Φ_{st} = 0.97, P < 0.05). Interestingly, Hondeklip Bay and Kleinzee are not that far from each other ~ 77 km, which means the phylogeographic break between the two locations, is a strong barrier to gene flow. These results indicate that the Hondeklip Bay region prevents transportation of the juveniles towards the south direction. This is consistent with results found by Muller et al. (2012), where the Hondeklip Bay upwelling cell was invoked as a barrier to gene flow between populations of the sea urchin *Parechinus angulosus* along the west coast of South Africa.

Theoretically, we expected a divergence across this upwelling cell or even freshwater trails such as that of the Orange River which has been cited as a barrier to gene flow for some taxonomic groups (see Matthee & Flemming, 2002; von der Heyden et al. 2007; Portik & Bauer, 2012). This was not the case for this study, *T. granulatus* populations found on either side of the river (Oranjemund and Alexander Bay), were genetically similar (Φ_{st} = 0.06 - 0.98, $P < 0.05$) and presented a low genetic distance (4.06%, Appendix 5) based on COI sequences. As much as the northern lineage indicates a stronger genetic structure than the one observed in the southern lineage (Fig. 2.7), the Benguela Current may facilitate some gene flow between the northern populations, as there is no significant difference between Elizabeth Bay and Oranjemund. Furthermore, two South African populations, Alexander Bay and Kleinzee, grouped with Oranjemund and Elizabeth Bay to form the northern lineage. This means the Orange River is not a phylogeographic barrier between South African and Namibian populations of *T. granulatus*. However, the Orange River may represent a barrier between Alexander and Oranjemund. On a finer scale, it would be interesting and ideal to sample both north and south of the phylogeographic break between Hondeklip Bay and Kleinzee; this could shed some light on its cogency as a barrier to gene flow. However, it is difficult to obtain samples from this region, the Namaqua bioregion is a highly restricted area due to diamond mining safety measures.

Upwelling and increased productivity might have influenced phylogeographic patterns of *T. granulatus*, but cannot explain it all from a terrestrial perspective. Isopods within the genus *Tylos* are characterized by low vagility (Schultz, 1970; Kensley, 1974; Brown & Odendaal, 1994). Furthermore, the biology of this isopod is also marked with limited dispersal capabilities (Brown & Odendaal, 1994). These traits can explain both inland and overwater population structures, as observed in *T. granulatus* and other semi-terrestrial isopods such as *Ligia* (see Taiti et al. 2003; Hurtado et al. 2010, 2013; Santamaria et al. 2013, Yin et al. 2013; Raupach et al. 2014; Santamaria et al 2014, 2017). During the Plio-Pleistocene transition, the Northern Hemisphere became cooler; the southern regions were warmer and characterized by aridification (Bonnefille, 1985; van Zinderen Bakker, 1986). The southern regions became a stable refugia, supporting a widespread of flora and faunal lineages (Rambau et al 2003; Daniels et al. 2007). Perhaps, there was an expansion of sandy beaches during this time that allowed for the spread of *T. granulatus* from Namibia to South Africa. Sandy beach subtrates could have facilitated the migration process (Brown & Odendaaal, 1994), but because of intense upwelling during this period, the Hondeklip Bay upwelling cell split *T. granulatus*

into two lineages: *T. granulatus* Lineage I and *T. granulatus* Lineage II. This genetic pattern may well be maintained by the Benguela Current, life-history traits (lack of a planktonic larval stage) and possibly even local adaptation (Neethling et al. 2008; Pelc et al. 2009; Teske et al. 2011; Wright et al. 2015).

5.2 Distinct genetic patterns of the two lineages

5.2.1 The northen range: T. granulatus Lineage I

Based on the COI gene, the overall patterns of genetic structure and divergence differ between the northern and southern lineages. A total of 28 haplotypes occurred in 47 individuals from the northern populations (*T. granulatus* Lineage I), in contrast to only 16 from the 133 samples from the southern range. There is more pronounced genetic structure in the northern lineage, with lower frequencies of haplotypes but these are separated by more mutational steps than those in the southern lineage (Fig. 2.7). The pattern of diversity for the *T. gtanulatus* Lineage I, suggests more population stability. Further, this provides evidence that the lineages separated a long time ago and thus a lot of differentiation has accumulated. The northern lineage must have been connected to the southern lineage at some stage, either we did not sample intermediary populations or these have gone extinct.

5.2.2 The southern range: *T. granulatus* Lineage II

Most notably, the haplotype network for the southern lineage suggests a pattern typical of populations that have experienced a recent expansion (Fig. 2.7). *Tylos granulatus* Lineage II represents a star - like distribution pattern that is characterized by one dominant (ancestral) haplotype that is found in various frequencies in almost all the populations. There are also lower frequency haplotypes connected by single step mutations connecting. As shown in Fig. 2.7, the distribution of the central ancestral haplotype is from EL to YZ, consistent with a founder effect in this region that was later followed by expansions (Mayr, 2001; Evans et al. 2004). Further, star - like pattern, high haplotype diversity (h = 0.25 - 0.65), low nucleotide diversity (π = 0.00 to 0.01) and negative Fu's Fs for the YZ, SB, EL and DB populations, can be seen as additional signatures of expanding populations towards the southern direction. The pre-expansion and post-expansion population sizes (θ_o and θ_1, Table 2.7) support the hypothesis of expansions in this region.

5.3 Are the two lineages species or just deeply divergent populations?

Results from this study provide evidence that *T. granulatus* found on the west coast of South Africa and Namibia is composed of two deeply divergent lineages, with a phylogeographic break located within South Africa. To link whether the two distinct lineages represent different species or just deeply divergent lineages, intra and interspecific sequence divergence of *T. granulatus* and other *Tylos* species were taken into account. Results indicated that intra-specific sequence divergence between the two distinct lineages of *T. granulatus* range between 0.5 - 1.5%. These values were slightly lower when compared to intra-specific sequence divergence of other *Tylos* species (3.1 - 6.4%, Table 2.8). Inter-specific sequence divergence between the two lineages of *T. granulatus* was 10%, this value was also lower compared to inter-specific sequence divergence of other *Tylos* species with a range of 14.3 - 14.9%. With no universal threshold for any molecular marker to delineate species (Lefébure et al. 2006; Havermans et al. 2013), it is difficult to conclude whether *T. granulatus* is composed of species or deeply divergent lineages along the west coast of South Africa and Namibia. There is a clear distance-based separation between the two lineages of *T. granulatus* and this corresponds to distance data reported for other *Tylos* species. The divergence between lineage I and II is comparable to species level divergence among *Tylos* species. Even if morphological difference were not detected for the morphological characters examined, this does not mean they do not correspond to different species (see Santamaria et al. 2016). The presence of possible cryptic *Tylos* species in southern Africa is consistent with recent studies of *Ligia*, a semi-terrestial rocky shore isopod. Greenam et al (2017) reported the presence of two distinct and well supported clusters: the 'Western' cluster (Namibia to Cape Agulhas) and the 'Eastern' cluster (Knysna to KwaZulu-Natal). This is similar to the two major clades observed in this study, where *T. granulatus* is represented by two deeply divergent lineages. Similarly, Hurtado et al (2010) found seven major lineages in the regions of Central California to Central Mexico for the isopod *Ligia occidentalis*. These studies are comparable to the findings of this study. This was expected as *Tylos* and *Ligia* show similar geographic distributions, similar life-istory traits (direct developers) and are expected to have been impacted by the same historical events (Hurtado et al. 2013).

From a management perspective, the strong population structure and significant differences across populations of *T. granulatus* highlight the importance of recognising each population as a separate Management Unit (MU) (Moritz, 1994; Palsbøll et al. 2007; Funk et al. 2012).

These findings have a large and important implication in the conservation and management of southern Africa sandy beaches.

Conclusion

This study showed a strong genetic structure defined by two distinct lineages of *T. granulatus*. Furthermore, no evidence of morphological differences was found between the two lineages, which puts into perspective that *T. granulatus* consists of deeply divergent evolutionary lineages that likely do not represent distinct species. This is supported by high Φ_{st} values that indicated that almost every sandy beach was unique from the others. High levels of uniqueness found on the west coast of southern Africa, should increase the conservation priority level of this particular region (Bickford et al. 2007). This study, amongst a few others (Grant & da Silva-Tatley, 1997; Laudien et al. 2003, Bezuidenhout et al. 2014; Muteveri et al. 2015; Hawkins, 2016), has shown that molecular data could reveal genetic diversities that were overlooked, especially in sandy beach science.

This study is the first to show a sandy beach species that was differently affected by historical processes. The really fascinating part of the main findings is that the two lineages show (i) stability (*T. granulatus* Lineage I) and (ii) expansions (*T. granulatus* Lineage II). Such findings highlight the ability of molecular approaches to identify historical process that shape contemporary genetic structures of marine species. With escalating pressures along the southern African coast, this expanding species is at great risk of local extinction. When taking into account the narrow range of *T. granulatus* along the west coast of South Africa and Namibia, and that it is endemic to this region but shows complex phylogeographic patterns, this raises awareness that sandy beach diversity should not be underestimated and their conservation should not be overlooked.

From a molecular perspective, results presented in this study are fascinating. We can draw a conclusion that historical oceanographic and climatic changes of the Plio-Pleistocene glaciation period played a significant role in shaping present day phylogeographic patterns in *T. granulatus*. We found no obvious phylogeographic and biogeographic overlap. *Tylos granulatus* is separated by various phylogeographic breaks along the west coast of southern Africa. This shows that *T. granulatus* is composed of MUs, thus, each beach should be

protected perhaps as a single entity. It is important to note that most of the results in this study are reported based on a single marker (COI). A phylogeny concluded from a single marker could lead to interpretation problems, because other molecular markers could reveal different evolutionary rates or even a completely different genetic history (Patwardhan et al. 2014). Although the 16S gene was included in the phylogenetic tree, multiple genes should be considered in order to make convincing conclusions on the evolutionary history of *T. granulatus* along the southern African coast. Likewise, there are no mutation rates available for *Tylos* (Hurtado et al. 2013, 2014, 2016, 2017).

The west coast of southern Africa is shown in this study as an important area of genetic interest. However, this region, especially the Namaqua area, has been off limit to biologist due to diamond mining security measures. For this reason, the west coast is poorly protected by Marine Protected Areas (MPAs), yet it is known as to be the most threatened region in the South African coastline (Harris, 2012; Sink et al. 2012), with pressures in this region including diamond/mineral mining, reduced freshwater flow, coastal development, kelp/seaweed harvesting and coastal squeeze (Harris, 2012). In a review by von der Heyden (2009), the west coast was identified as an area of genetic interest in terms of genetic diversity; however, this region is poorly covered in the South African MPA network (Nielsen et al. 2017).

This work adds to the growing field of sandy beach science. It also contributes to the few studies on biogeographic and phylogeographic patterns of southern African sandy beach specie that have been published (Grant & da Silva-Tatley, 1997; Laudien et al. 2003; Bezuidenhout et al. 2014; Muteveri et al. 2015). In conclusion, this research provides a scope of work to build a better understanding of the significance, vulnerability and complexity of sandy beach ecosystems for conservation and management aims.

CHAPTER III

Comparative phylogeography of two *Excirolana* species

6.1 Introduction

This chapter follows the same pattern of analyses as for *Tylos granulatus*, but will be more focused on defining and comparing phylogeographic patterns of two sandy beach isopods, *Excirolana natalensis* and *Excirolana latipes*, that are characterised by similar life histories and sampled from the same regions.

The genus *Excirolana* (Richardson, 1912) belongs to the family Cirolanidae (Dana, 1852). *Excirolana* isopods have a global distribution (Poore & Bruce, 2012) composed of 18 recognized species listed in the World Register of Marine Species (WoRMS) (http://www.marinespecies.org). Of the 18 species in the genus, *Excirolana natalensis*, commonly known as the Natal beach louse Vanhoeffen, 1914 and *Excirolana latipes* (invalid synonym: *Pontogeloides latipe*) (Barnard, 1914) are endemic to southern African sandy beaches (Bruce & Soares, 1996; Harris, 2012). Both *E. latipes* and *E. natalensis* occupy all recognized bioregions of South Africa, which makes them ideal species for a phylogeographic study.

Both species dominate and coexist in the littoral zone (Harris, 2012), although they do not always occur in equal densities and in some regions one species may dominate the other. As all peracarids, *E. latipes* and *E. natalensis* are direct developers (Hurtado et al. 2016). Recent papers on phylogeographic patterns of *Excirolana* refer to their dispersal limiting characteristics (direct development and habitat specificity) and expectations of allopatric divergence (Hurtado et al. 2016, 2017). Unlike *T. granulatus*, *Excirolana* can swin and may also be transported in the water. For this reason, genetic differentiation may also be less than in *Tylos* that does not swim, cannot survive long periods within the water column, and avoids immersing in the sea, and is constrained to the drier upper portions of the sandy beach.

Molecular studies of cirolanid isopods are very limited (Hurtado et al. 2017); nonetheless, phylogeographic and biogeographic patterns of the beach isopod *Excirolana braziliensisi* have been well documented (Spooner & Lessios, 2009; Varela & Haye, 2012; Tourinho et al.

2016; Hurtado et al. 2017). *Excirolana braziliensisi* has been reported to comprise several cryptic lineages. For example, Sponer & Lessios (2009) conducted a study in three regions of Panama to investigate phylogeographic patterns of *E. braziliensisi* using fragments of 12S and COI genes. Genetic and moprphological data revealed the presence of three deeply divergent lineages suggested to represent three species each from the regions covered in the study. Correspondingly, Varela & Haye (2012) revealed three distinct lineages of *E. braziliensis* with 14 to 19% of genetic divergence along the coast of Chile. Later, Tourinho et al (2016) found distinct lineages of *E. braziliensisi* across Uruguay which was separate fron the ones reported by Sponer & Lessios (2009) and Varela & Haye (2012). As mentioned previously, such studies are fundamental in management and conservations planning as they disclose areas of high diversity and/or uniqueness (Bickford et al. 2007), as well as adding vital biodiversity knowledge.

This chapter utilises a phylogeographic approach to determine levels of genetic structuring among populations the *E. natalensis* and *E. latipes* to better understand the processes contributing towards shaping the evolution of sandy beach species along the southern African coast. This chapter is a comparative study based on phylogeographic patterns of *E. latipes* and *E. natalensis*. This will be achieved by addressing the following questions: (i) Did historical oceanographic and climatic changes of the Pleistocene period play a significant role in shaping present day phylogeographic patterns in both *E. latipes* and *E. natalensis*? (ii) Is there a pattern of high levels of genetic differentiation for both *Excirolana* species, as expected for a direct developer with habitat specialization, which has been observed in *T. granulatus*? (iii) And if so, is there a concordance between phylogeographic breaks in *E. latipes* and *E. natalensis* and phylogeographic breaks detected for other species and or biogeographic boundaries?

Materials and Methods

7.1Specimen collection

A total of 140 specimens of *E. latipes* were recovered from eight sandy beaches along the South African coast and two locations in Namibia (Fig. 3.1 and Table 3.1). For *E. natalensis*, a total of 171 specimens were collected from eight stations along the South African coast and one location in Namibia (Fig. 3.1 and Table 3.1). Sampling localities were chosen based on records of the species distribution range by Kensley (1978), Brown & Odendaal (1994) and Branch et al. (2002). Samples were sieved through a 1 mm sieve bag and preserved in 100% ethanol.

Table 3.1: Information on *Excirolana latipes* and *Excirolana natalensis* sampling locations, GPS coordinates and sample size (*N*) together with the number of individuals sampled for COI shown in brackets.

Location	Longitude	Latitude	*E. latipes* (*N*)	*E. natalensis* (*N*)
Elizabeth Bay (EB)	- 26.918333	15.207111	3 (3)	
Oranjemund (OR)	- 28.544417	16.320389	10 (8)	21 (14)
Alexander Bay (AB)	- 28.647639	16.479167		18 (17)
Port Nolloth (PN)	- 29.244583	16.864722	24 (24)	
Hondeklip Bay (HB)	- 30.326194	17.274722		23 (17)
Doringbaai (DB)	- 31.828167	18.239361		7 (7)
Lambert's Bay (LB)	- 32.088972	18.309889	10 (10)	27 (16)
Elands Bay (EL)	- 32.307222	18.341444	21 (16)	30 (25)
Yzerfontein (YZ)	- 33.334056	18.160861	15 (15)	14 (14)
Simon's Town (ST)	- 34.159833	18.431806	9 (5)	8 (5)
Struisbaai (St)	- 34.796389	20.050417	20 (13)	
Knysna (KN)	- 34.072472	23.002917	21 (21)	
Port Elizabeth (PE)	- 33.987528	25.673139	7 (6)	
Port Alfred (PA)	- 33.610111	26.890583		21 (21)

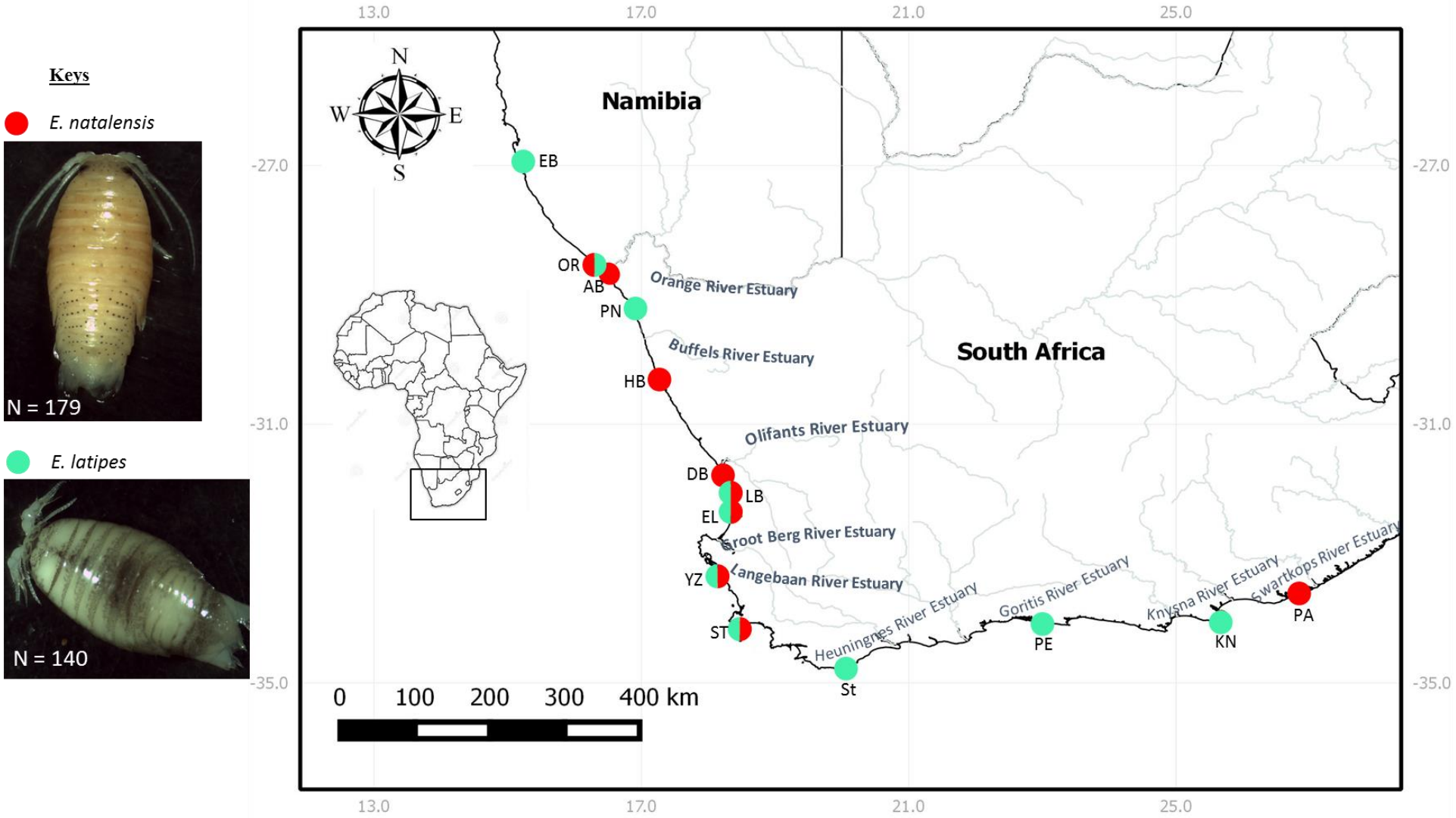

Figure 3.1: Sampling localities for both *Excirolana natalensis* and *Excirolana latipes.*

7.2 Examination of *Excirolana* morphology

The morphologies of the antennules and antennae are commonly used to distinguish species of *Excirolana*. These (among other morphological characteristics) were carefully examined and photographed for all individuals of *E. natalensis* and *E. latipes* using an M125 microscope and the Leica Application Suite software. In *E. natalensis*, the antennule has a long flagellum which is almost the same length as the antenna (Fig. 3.2A). In *E. latipes*, the antennule is defined by a shorter flagellum; both the antennule and the antenna are almost the same length (Fig. 3.2B).

7.3 DNA extraction

Genomic DNA was isolated from 2 - 4 legs per specimen using the NucleoSpin® Tissue kit (Machery - Nagel) according to the manufacturer's instructions. If the specimen was too small, the whole organism was used. To determine the quantity (ng/ml) and quality of DNA obtained, each sample was analysed using a NanoDrop (ND-1000) Spectrophotometer.

7.4 Molecular markers and DNA amplification

A Polymerase Chain Reaction (PCR) technique was used to amplify one mitochondrial gene fragment: cytochrome oxidase subunit I (COI) (primers and annealing temperatures in Appendix 2). Mitochondrial DNA (mtDNA) is one of the most commonly used genetic markers (Hare, 2001; Avise, 2009) and has been shown to be a sensitive indicator of speciation (Avise, 2009). Further, it is generally variable enough to delineate cryptic species of isopods (Edmands, 2001; Wetzer, 2001; Lee, 2012; Santamaria et al. 2014; Niikura et al. 2015).

Each 25 µl PCR reaction contained 1 µl of ~90 - 150 ng/µl of the template DNA, 2.5 µl of the 1 x reaction buffer, 2.5 µl of 2mM $MgCl_2$, 2.5 µl of 0.1 mM dNTP, 1.25 µl of 0.5 pmol of each primer, 0.1 µl of 0.5 u Super-Therm BioTaq DNA polymerase (Super-Therm, JMR Holdings, London, United Kingdom), 2 µl of 10 mg ml - 1 Bovine Serum Albumin (BSA) solution and distilled water. PCR products were separated and visualised on 1% agarose gels in TBE buffer containing ethidium bromide (0.5µL/mL). PCR products were loaded and run

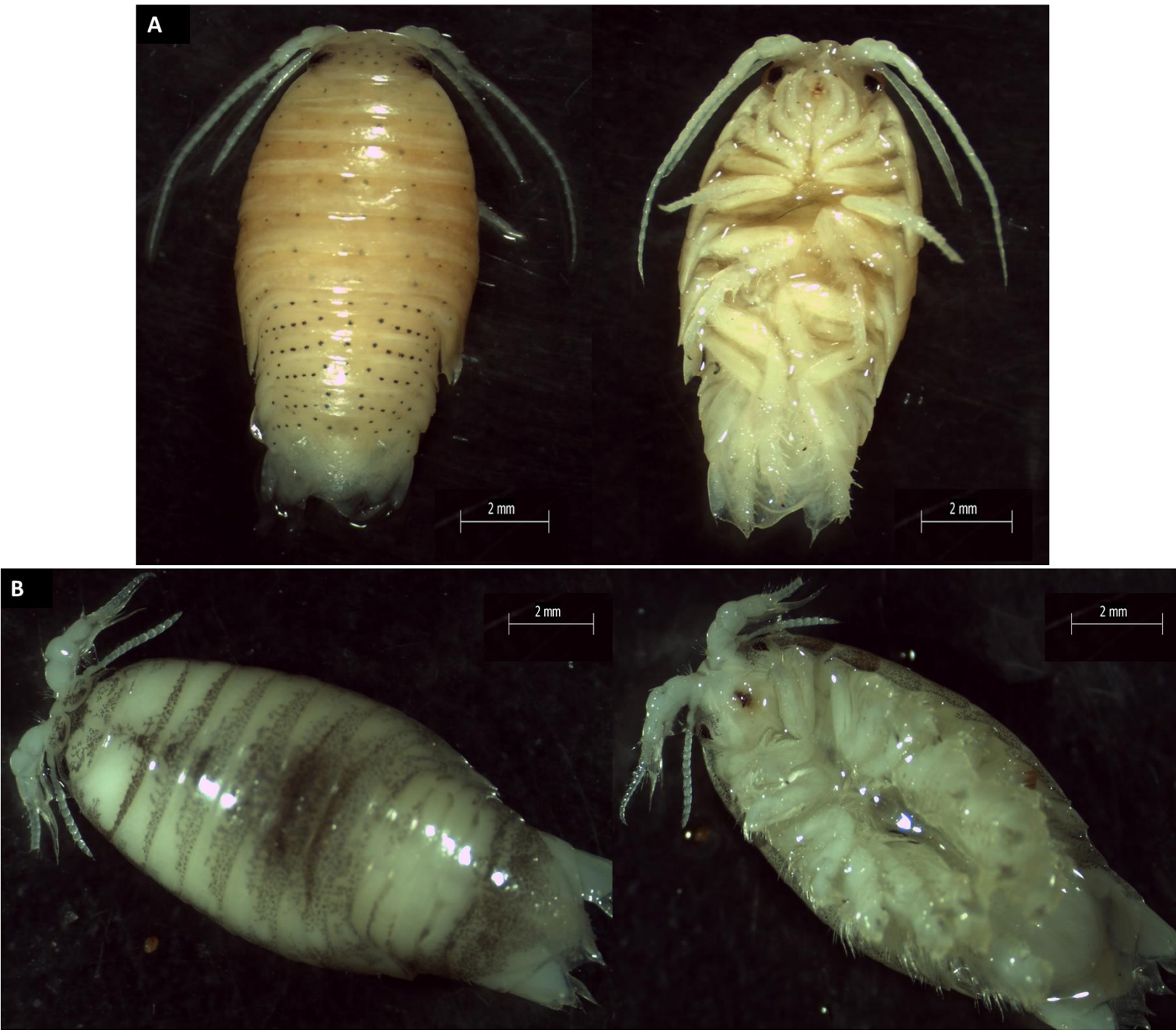

Figure 3.2: Dorsal and ventral view of (A) *E. natalensis* with longer pairs antennules and antennae and (B) *E. latipes* with shorter pairs of antennules and antennae. *Excirolana natalensis* was collected from Simon's Town and *E. latipes* is from Oramjemund.

alongside a 1kB DNA ladder at 100 volts for 60 minutes using a BioRad Electrophoretic apparatus and then photographed.Correct fragment sizes from all the PCR products were carefully extracted from the gels using Biospin Gel Extraction Kits following the manufacturer's instructions. Gel purified products were sent for sequencing at the Central Analytic Facility (CAF) at the Stellenbosch University using an ABI 3730 Capillary sequencer with Big Dye terminator chemistry (Applied Biosystems).

8. Sequence datasets and alignments for phylogenetic analysis

Sequences were manually aligned, edited and trimmed in Geneious v7.1.9 (http://www.geneious.com, Kearse et al. 2012). All sequences were referenced to the chosen taxonomic groups, *E. natalensis* and *E. latipes* for mitochondrial COI in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov) to determine which species they matched to, and to obtain outgroups to root the trees. Additional sequences for comparing *E. natalensis and E. latipes* to other *Excirolana* sequences were obtained from GenBank (see Appendix 6 for the downloaded sequences and GenBank accession numbers).

To ensure all the sequences were in the correct reading frame and to check that no stop codons were present in the protein - coding gene COI, all nucleic acid sequences were translated to peptide sequences using the software Geneious v7.1.9. No stop codons were present in any of the protein coding sequences. All datasets for both *E. latipes* and *E. natalensis* were analysed independently, but followed the same analytical steps.

8.1 Genetic diversity

DNA - sp version 4.90.1 (Rozas & Rozas, 1999) was used to generate a haplotype data file. Genetic variability within populations was quantified as the number of haplotypes (*H*), haplotype diversity *(h*) and nucleotide diversity (π). These indices were calculated for each location using Arlequin 3.5.1.2 (Excoffier, 2004; Excoffier et al. 2010).

9. Phylogeography and demographic history

9.1 Analysis of genetics structure

An AMOVA analysis was used to determine variance within and between groups (populations). Population pairwise fixation indices (Φ_{st}) were also calculated between populations of *E. natalensis* and *E. latipes*; their significances were tested with a nonparametric permutations approach with 10,000. To test the hypothesis of correlation between genetic and geographic distance among populations of *Excirolana*, estimates of Isolation by distance (IBD) were verified using the Mantel test (Mantel, 1967). The analysis was carried out using Φ_{st} / (1 - Φst) as executed in GENEPOP 4.6 (Raymond & Rousset, 1995; Rousset, 2008). Geographic distances between sampling localities (in kilometres) were obtained from Google Earth Pro (https://www.google.com/earth/). The significance of correlation between genetic and geographic distances was estimated in XLSTAT 2017 (https://www.xlstat.com/en/) using the Mantel Z-test (Mantel, 1967) with 10,000 permutations. Because analyses for each species resolved two divergent lineages and because the signal of IBD can be inflated by populations structure (Meirmans et al. 2012), the lineages were analysed separately. I did not report IBD for *E. natalensis* Lineage II because of missing data (sampling points) between populations.

In order to visualise the connections between populations of *E. natalensis* and *E. latipes*, parsimony-based haplotype networks of the COI region were constructed in NETWORK 5 (http://www.fluxus - engineering.com/sharenet.htm, Bandelt et al. 1999).

10. Demographic history

To test whether populations of *E. natalensis* and *E. latipes* were at equilibrium, Fu′s Fs (Fu, 1997) test was performed in Arlequin with 10,000 permutations. A negative and statistically significant F value indicated an excess number of low frequency haplotypes, signifying recent population expansion (Fu, 1997). A positive F value indicated a lack of alleles, as would be expected from populations that went through genetic bottlenecks. Fu's Fs were considered significant when the P-values < 0.02. To further explore the hypothesis of a recent population expansion in the genus *Excirolana*, demographic expansion parameters were estimated using the mismatch distribution analysis in Arlequin (Ramos-Onsins & Rozas, 2002). These include the raggedness index (*r*) and the sum of squared deviations (SSD) (Rogers & Harpending, 1992). Low and non-significant *r* and SSD supported the hypothesis of demographic expansion. Lineages were analysed separately. After premilinary results, Port

Elizabeth was excluded from the analysis because the variance of the mismatch distribution was too small, thus, no demographic parameters could be estimated for this population.

11.Phylogenetic analyses

11.1 Data preparation

After preliminary analysis, data suggested genetic divergence among mitochondrial lineages of both *E. latipes* and *E. natalensis*. A phylogenetic tree with both South African *Excirolana* and other species was constructed to explore their evolutionary relationships (see Fig. 3.8). To construct the tree, one to three sequences were selected from each of the lineages to represent all the sampling sites. Best - fit evolution models were determined by using the Akaike information criterion in MrModeltest v2.3 (Nylander, 2004).

11.2 Phylogenetic tree constructions and sequence divergence

11.2.1 Bayesian analysis

Sequences were selected for Bayesian analysis of phylogeny in Mr Bayes version 3.2.6 (Huelsenbeck & Ronquist, 2003). The analyses were run for one million generations. The trees were set to be sampled every 100 generation with two runs. By default, the number of chains was set to four, meaning that MrBayes used three heated chains and one "cold" chain (Ronquist et al. 2011). The burnin value was set to 25% of the number of generations, meaning that the first 25% of the trees were discarded. Convergence in MrBayes was determined by ensuring that the standard deviation of split frequencies was below 0.01 (Ronquist et al. 2011). Alternatively, convergence was also determined by the software Tracer v1.6 (Rambaut & Drummond, 2007) using the Effective Sample Size (ESS > 200). Trees were visualised and edited in FigTree v1.4.2 (Rambaut, 2012).

12. Sequence divergence estimates

Estimates of sequence divergence of *E. latipes* and *E. natalensis* were performed in the software MEGA 7.0.20 using the uncorrected pairwise method with the P-distance model (Kumar et al. 2016). Since genetic divergence takes occurs at different molecular rates in various taxa, there is a need to establish an interspecific 'standard' for species differentiation

(Havermans et al. 2013). This was achieved by looking into differences between Intra and interspecies P-distances of *E. latipes*, *E. natalensis* and other *Excirolana* species.

13. Time since population divergence

In order to test the influence of historical events on the divergence of populations of *E. latipes* and *E. natalensis*, different COI mutation rates (varying between 1.25 - 2.6%/Ma; see Wares & Cunningham, 2001; Sponer & Lessios, 2009; Markow & Pfeiler, 2010) were used to provide estimates of divergence. See previous chapter for problems associated with the use of molecular closks. As no obvious calibration point of sequence divergence is available for *Excirolana* isopods, three substitution rates were used to date the divergence between major lineages formed. A rate of 1.25%/Ma estimated for *Stenasellus* isopods by Ketmaier et al. (2003) was used. Secondly, Poulakakis & Sfenthourakis' (2008) evolutionary rates of 1.56 - 1.72%/Ma estimated for *Orthometopon* isopods were used to estimate time of divergence among major lineages. These evolutionary rates were implemented into BEAST 1.8.3 (Drummond et al., 2011) to estimate divergence time to the most recent common ancestor (TMRCA) between major lineages within *E. latipes*. The BEAST package was used to run an analysis for 30 million steps of the MCMC sampled every 1000 generation using the HKY model. The data was partitioned by codon position following a strict clock theory (Drummond et al. 2006). After testing several models that best fit the data, the Yule model was used to analyse lineages detected by the phylogenetic analyses (Drummond & Rambaut, 2007). Convergence was calculated with ESS values, whereas ESS > 200 was used as an indicator of convergence.

Results

14. Genetic diversity

Although *E. latipes* and *E. natalensis* are sympatric, I could not collect both species from all sites (Table 3.1, Fig. 3.1). Some of the smaller samples did not have enough tissue to extract DNA, so from a total of 140 samples of *E. latipes*, 121 sequences each comprising of 635 base pairs after trimming, were obtained from the COI gene. Sequences from the COI gene yielded 21 haplotypes, only four haplotypes were shared between 2 - 6 localities and 17 (81%) were singletons (see Table 3.2). Overall, results indicated high haplotype diversity (h = 0.26 - 0.73) and low nucleotide diversity (π = 0.01 - 0.05, Table 3.4). Notably, PE revealed extremely low haplotype and nucleotide diversities (h = 0.00, π = 0.00).

A total of 136 sequences (597 bp after trimming) were obtained from 169 samples of *E. natalensis*. The sequences resulted in 12 haplotypes, five haplotypes shared between several locations and 7 (58%) singletons (Table 3.3). Haplotype diversity was high (h = 0.18 - 76) and low nucleotide diversity (π = 0.01 - 0.04, Table 3.5). Haplotype and nucleotide diversities were the lowest in ST (h = 0.00, π = 0.00).

Table 3.2: Haplotype frequencies for the ten localities of *E. latipes* along southern African coast. Sample size (*N*) for each locality is also shown. Shared haplotypes are indicated by in grey.

	N	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
EB	3	2										1										
OR	8	5			1		1	1														
PN	24	3		1								20										
LB	10	4								1		4	1									
EL	16	11				1					1	3										
YZ	15	13	1						1													
ST	5																		3	2		
Stb	13													2						8	1	2
KN	21													13	1	1	5	1				
PE	6													6								

Table 3.3: Haplotype frequencies for the nine localities of *E. natalensis* along southern African coast. Sample size (*N*) for each locality is also shown. shared haplotypes are indicated by in grey.

	N	1	2	3	4	5	6	7	8	9	10	11	12
OR	14		9	1	1		3						
AB	17		14				3				1		
HB	17	1	13				2	1					
DB	7	3	2				2						
LB	16	6	9					1					
EL	25	11	11			1	2						
YZ	14	1	7				4		1	1			
ST	5					5							
PA	21											19	2

Table 3.4: Diversity indices for all ten sampling localities of *Excirolana latipes.*

	EB	OR	PN	LB	EL	YZ	ST	Stb	KN	PE
N	3	8	24	10	16	15	5	13	21	6
H	2	4	3	4	4	3	2	4	5	1
h	0.67 (±0.31)	0.64 (±0.18)	0.30 (±0.11)	0.73 (±0.10)	0.52 (±0.13)	0.26 (±0.14)	0.60 (±0.18)	0.62 (±0.14)	0.58 (±0.10)	0.00 (±0.00)
π	0.02 (0.02)	0.02 (±±0.02)	0.01 (±0.01)	0.02 (±0.02)	0.02 (±0.01)	0.01 (±0.01)	0.03 (±0.03)	0.03 (±0.02)	0.05 (±0.03)	0.00 (±0.00)

Table 3.5: Diversity indices for all nine sampling localities of *E. natalensis*.

	OR	AB	HB	DB	LB	EL	YZ	ST	PA
N	14	17	17	7	16	25	14	5	21
H	3	3	4	3	3	4	5	1	2
h	0.57 (±0.13)	0.39 (±0.13)	0.43 (±0.14)	0.76 (±0.11)	0.58 (±0.08)	0.63 (±0.06)	0.70 (±0.10)	0.00 (±0.00)	0.18 (±0.10)
π	0.02 (±0.02)	0.02 (±±0.02)	0.01 (±0.01)	0.04 (±0.03)	0.02 (±0.02)	0.03 (±0.02)	0.04 (±0.03)	0.00 (±0.00)	0.01 (±0.01)

15. Population structure

The AMOVA analysis indicated that most of the genetic diversity is due to variability among populations of *E. latipes* (95.81%, $P = 0.00$) and *E. natalensis* (91.91%, $P = 0.00$), with two divergent lineages recovered within both species (Table 3.6). Genetic variability within populations of *E. natalensis* was lower (8.09%) than in *E. latipes* (10.25%). The Global Φ_{st} across all populations was very high, *E. latipes* ($\Phi_{st} = 0.95$, $P = 0.00$) and *E. natalensis* ($\Phi_{st} = 0.92$, $P = 0.00$) (Table 3.6). Results indicate that *E. latipes* Lineage II had higher genetic diversity withinin populations (68.65%) than *E. latipes* Lineage I (62.84%). *Excirolana natalensis* Lineage I had higher genetic variability within populations (67.23%). I could not run the analysis for *E. natalensis* Lineage II as it only included one population, sampled only from Port Alfred.

Further analyses based on the pairwise comparisons (Φ_{st}) showed significant differences between the ten populations of *E. latipes*, ranging from 0 to 0.99 ($P < 0.05$) (Table 3.7). Most west coast populations of *E. latipes* (Oranjemund, Port Nolloth, Lambert's Bay, Elands Bay and Yzerfontein) exhibited lower and non-significant Φ_{st} values (Table 3.5). The southern populations (Simon's Town, Struisbaai, Knysna and Port Elizabeth) were defined by higher and significant Φ_{st} values (Table 3.5). Similarly, *E. natalensis* west coast populations (Alexander Bay, Hondeklip Bay and Doringbaai) were defined by lower and non-significant Φ_{st} values (Table 3.8), whereas the southern range populations (Simon's Town and Port Alfred) showed higher and significant Φ_{st} values (Table 3.8).

The haplotype network (Fig. 3.3) obtained from the mtDNA COI gene clearly shows strong levels of population genetic structuring of *E. latipes*. Genetic variation is mainly explained by the existence of two distinct evolutionary lineages that are separated by 105 mutation steps (Fig. 3.2). *Excirolana latipes* Lineage I includes the west coast populations (Elizabeth Bay, Oranjemund, Port Nolloth, Lambert's Bay, Elands Bay and Yzerfontein) and *E. latipes* Lineage II represents populations sampled in the southern range (Simon's Town, Struisbaai, Knysna and Port Elizabeth). There is a strong phylogeographic break between the two lineages located across Cape Point. A total of 12 haplotypes occurred in 76 individuals from the west coast populations (*E. latipes* Lineage I) and 9 from 45 samples from the southern range (*E. latipes* Lineage II). No haplotypes were shared between the two lineages. More structure is in *E. latipes* Lineage II, with lower frequencies of haplotypes but more mutational

steps (Fig. 3.3, Table 3.3). In *E. latipes* Lineage II, there are two dominant haplotype that are found in various frequencies in all populations and then some single step mutations to other haplotypes (Fig. 3.3 and Table 3.3).

Table 3.6: Analysis of the molecular variance (AMOVA) for both *E. latipes* and *E. natalensis*. Results are based on the COI gene.

Source of variation	d.f.	Sum of Squares	Variance components	% Variation	Fixation index
All populations (*E. latipes*)					
Among populations	9	905.84	8.57	95.19	Fst : 0.95
Within populations	171	261.54	1.53	10.25	
All populations (*E.natalensis*)					
Among populations	8	577.31	4.80	91.91	0.92
Within populations	128	54.13	0.42	8.09	
***E. latipes* Lineage I**					
Among populations	4	8.48	0.16	37.16	Fst : 0.37
Within populations	55	15.07	0.27	62.84	
***E. latipes* Lineage II**					
Among populations	3	12.66	0.47	31.05	Fst : 0.31
Within populations	29	30.56	1.06	68.65	
***E. natalensis* Lineage I**					
Among populations	7	25.86	0.23	32.77	0.33
Within populations	108	50.51	0.47	67.23	

Table 3.7: Pairwise Φ_{st} values of mitochondrial COI among ten *E. latipes* sampling sites.

	EB	OR	PN	LB	EL	YZ	ST	Stb	KN
OR	**0.01**								
PN	**0.31**	0.60**							
LB	**0**	0.21*	**0.15**						
EL	**0**	0.11*	0.40**	**0.04**					
YZ	**0.15**	**0.10**	0.67**	0.30**	**0.10**				
ST	0.97*	0.97**	0.98*	0.97**	0.98**	0.99**			
Stb	0.97**	0.97**	0.98**	0.97**	0.98**	0.98**	0.31*		
KN	0.94**	0.95**	0.96**	0.95**	0.96**	0.96**	0.34*	0.41**	
PE	0.99**	0.98**	0.99**	0.98**	0.98**	0.99**	0.73*	0.66**	**0.10**

Signification at $P < 0.05$*; Significant at $P < 0.01$**; Bold Φ_{st} values are non-significant with $P > 0.05$

Table 3.8: Pairwise Φ_{st} values of mitochondrial COI among nine *E. natalensis* sampling sites.

	OR	AB	HB	DB	LB	EL	YZ	ST
AB	**0**							
HB	**0**	**0.00**						
DB	**0.06**	**0.12**	**0.10**					
LB	0.19**	0.20**	0.12*	**0.08**				
EL	0.14**	0.15**	0.10*	**0**	**0**			
YZ	**0**	**0.02**	**0.04**	**0**	0.17**	**0.11**		
ST	0.84**	0.88**	0.88**	0.82**	0.89**	0.79**	0.78**	
PA	0.98**	0.98**	0.98**	0.98**	0.98**	0.98**	0.97**	0.99**

Signification at $P < 0.05$*; Significant at $P < 0.01$**; Bold Φ_{st} values are non-significant with $P > 0.05$

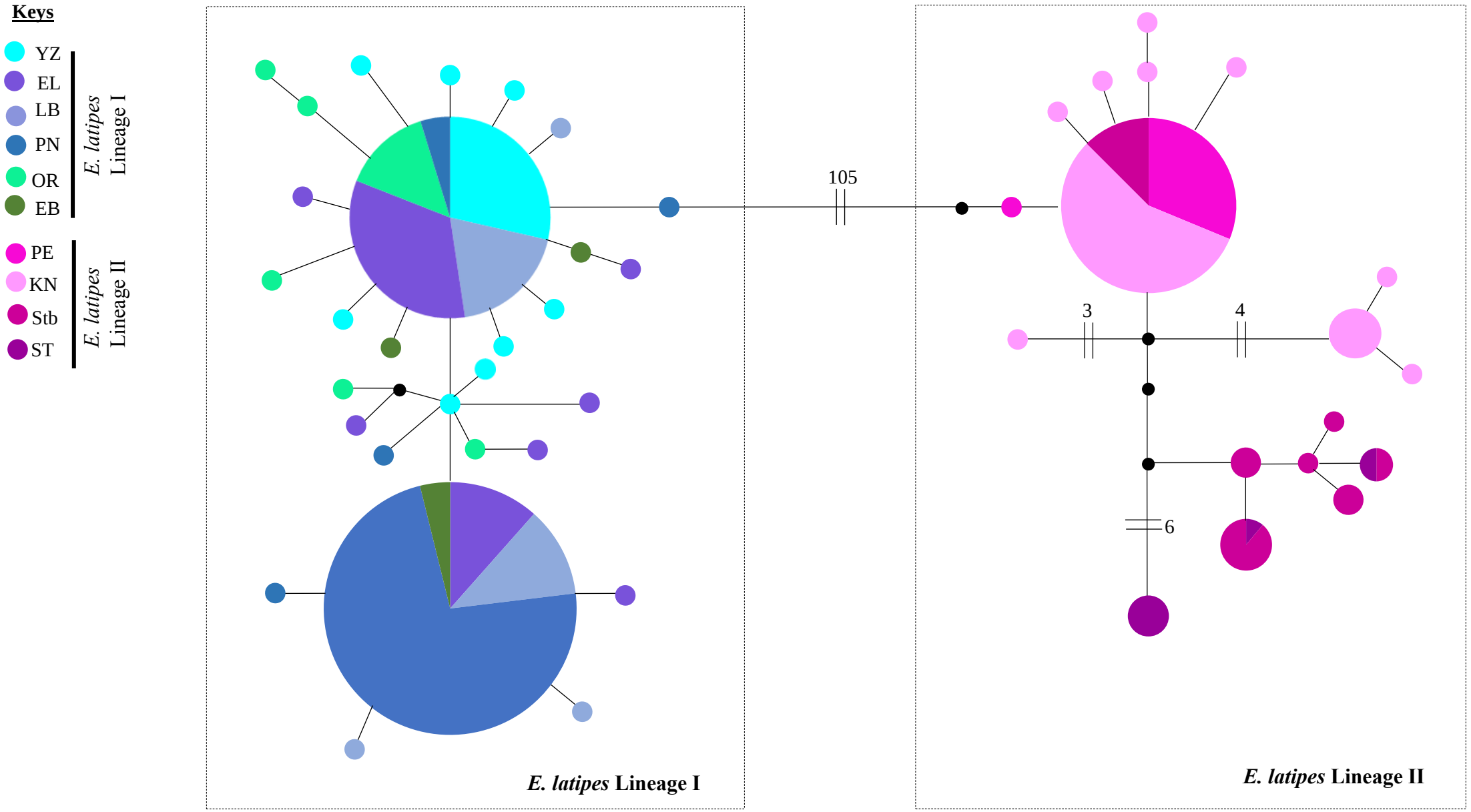

Figure 3.3: Parsimony-based haplotype network of the COI gene for *E. latipes*. Different colours distinguish haplotypes and their sampling localities. Circle size is comparative to the frequency of individuals in each haplotype. The partitions inside the circles represent the proportion of each population within each haplotype. A branch represent one mutational step, black dots represent missing, unsampled haplotype or extinct sequences.

For *E. natalensis*, the haplotype network (Fig. 3.4) obtained from the mtDNA COI gene revealed two lineages that are separated by 91 mutation steps. *Excirolana natalensis* Lineage I includes the west coast populations (Oranjemund, Alexander Bay, Hondeklip Bay, Doringbaai, Lambert's Bay, Elands Bay, Yzerfontein and Simon's Town) and *E. natalensis* Lineage II represents the southern range (Port Alfred). *Excirolana natalensis* Lineage I exhibits lower Φ_{st} values (ranging from 0.04 to 0.89) than *E. natalensis* Lineage II (0.97 to 0.99). A total of 10 haplotypes occurred in 115 individuals from the west coast populations (*E. natalensis* Lineage I) and 2 from 21 samples from the southern range (*E. natalensis* Lineage II). No haplotypes were shared between the two lineages. The results clearly demonstrate shallow genetic structure within the lineages, but potentially a positive correlation between pairwise Φ_{st} and geographic distances. However, we did not calculate IBD for *E. natalensis* due to missing sampling locaties between the two major lineages.

We found no evidence of IBD in *E. latipes* Lineage I (Fig. 3.5; r = - 0.08, P = 0.66). In *E. latipes* Lineage II, there was a positive correlation between pairwise Φ_{st} and geographic distances providing evidence for IBD (see Fig. 3.6; Mantel's test r = 0.83, P = 0.04). *E. natalensis* Lineage I (Fig. 3.7; r = 0.60, P = 0.00) shows a positive correlation between genetic and geographic distances.

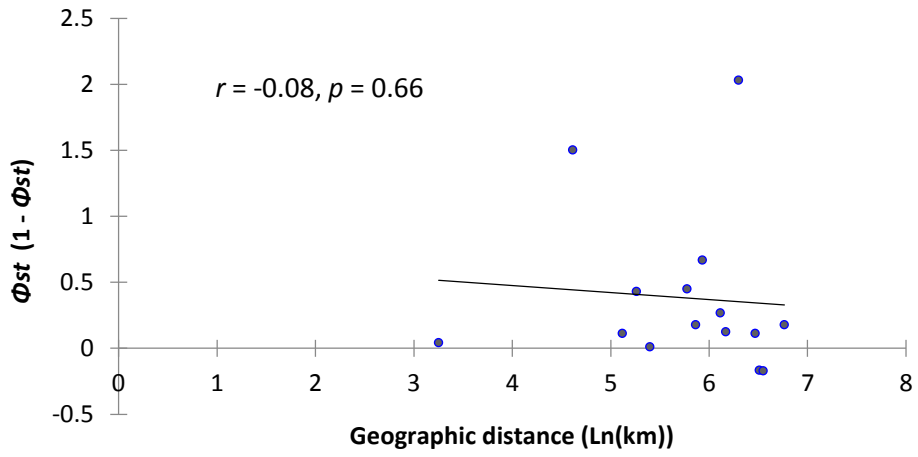

Figure 3.5: Isolation by distance in *E. latipes* Lineage I samples from the west of South Africa and Namibia. Geographic distances are plotted against genetic divergence estimates (Φ_{st} / (1 - Φ_{st}) between pairs of populations.

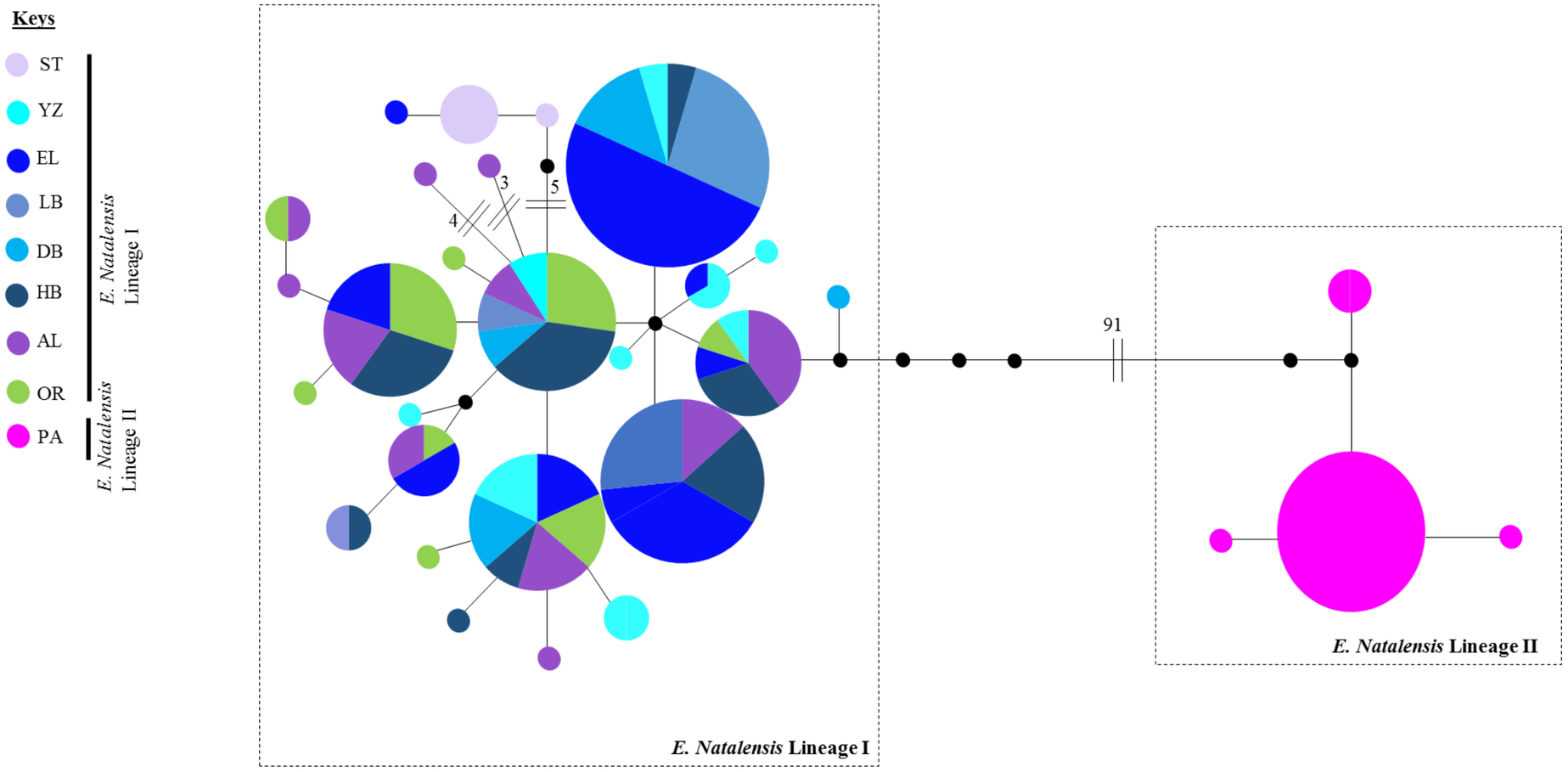

Figure 3.4: Parsimony-based haplotype network of the COI gene for *E.natalensis*. Different colours distinguish haplotypes and their sampling localities. Circle size is comparative to the frequency of individuals in each haplotype. The partitions inside the circles represent the proportion of each population within each haplotype. A branch represent one mutational step, black dots represent missing, unsampled haplotype or extinct sequences.

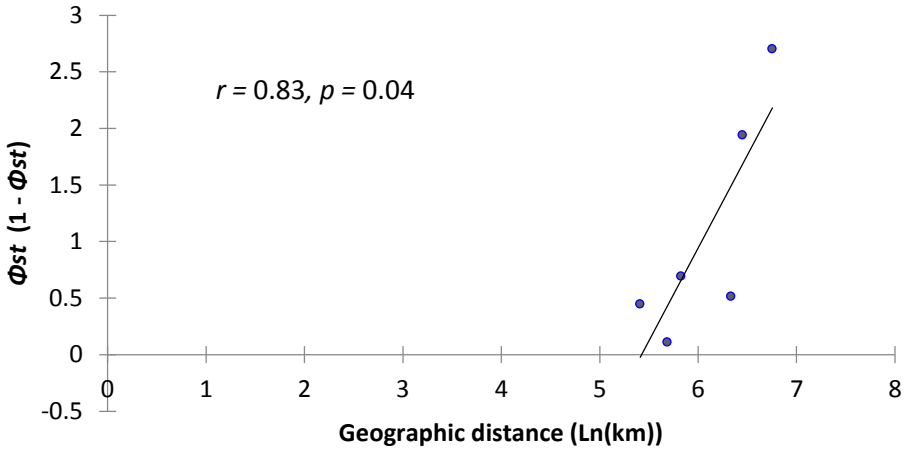

Figure 3.6: Isolation by distance in *E. latipes* Lineage II samples from the south coast of South Africa. Geographic distances are plotted against genetic divergence estimates (Φ_{st} / (1 - Φ_{st}) between pairs of populations.

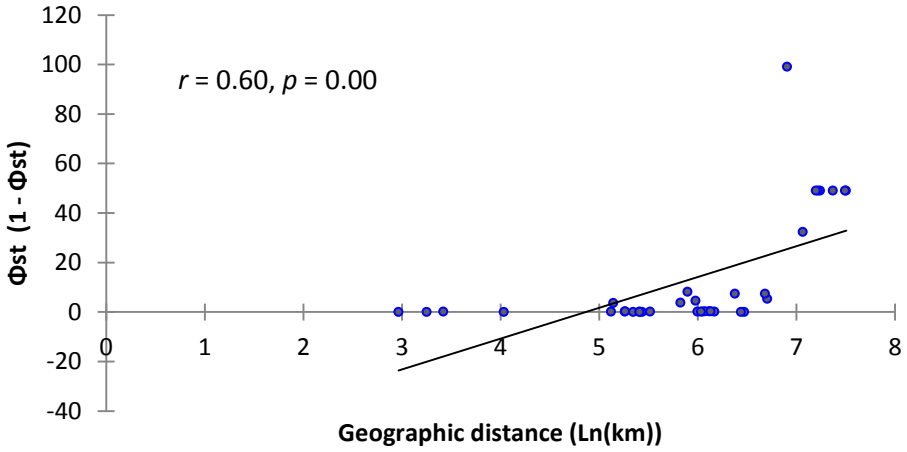

Figure 3.7: Isolation by distance in *E. natalensis* Lineage I samples from the south coast of South Africa. Geographic distances are plotted against genetic divergence estimates (Φ_{st} / (1 - Φ_{st}) between pairs of populations.

16. Demographic history

The hypothesis of recent demographic expansion was not supported in *E. latipes* as the majority of Fu's Fs were positive and not statistically supported (P > 0.05, Table 3.9 and 18). Fu's Fs were only significant in two populations, LB and YZ. Results also indicated no evidence of expansion in *E. natalensis*, as Fu's Fs in both lineages of this isopod were all non-significant (results not shown).

Table 3.9: Genetic demographic history of *E. latipes* Lineage I from five localities along the west coast of southern Africa. Neutrality test (Fu's Fs), mismatch distribution parameters θ_o and θ_1 = Pre - expansion and post - expansion populations size, τ = time in number of generations passed since the sudden expansion period, Sum of squared deviations (SSD) and the Raggedness index (*r*) are listed. P-values are also shown.

Mismatch distribution parameters	OR	PN	LB	EL	YZ
θo	0.00	0.00	0.00	0.00	0.01
θ1	2.08	0.37	99.99	99.99	99.99
τ	1.72	3.00	1.31	0.63	0.38
SSD	0.00	0.00	0.04	0.00	0.00
SSD p - value	0.70	0.60	0.40	0.85	0.64
r	0.06	0.32	0.23	0.10	0.23
r p - value	1.00	0.51	0.36	0.77	0.57
Fs	- 0.19	- 0.82	- 1.22	- 0.21	- 1.32
Fs p - value	0.24	0.19	0.06	0.32	0.02

Fu's Fs, mismatch analysis parameters (*r* and SSD) are significant at $P < 0.05$

Table 3.10: Genetic demographic history of *E. latipes* Lineage II from three localities along the west coast of southern Africa. Neutrality test (Fu's Fs), mismatch distribution parameters θ_o and θ_1 = Pre - expansion and post - expansion populations size, τ = time in number of generations passed since the sudden expansion period, Sum of squared deviations (SSD) and the Raggedness index (*r*) are listed. P-values are also shown.

Mismatch distribution parameters	ST	Stb	KN
θo	0.00	0.00	0.00
θ1	0.00	99.99	3.30
τ	0.00	2.11	5.27
SSD	0.00	0.01	0.10
SSD p - value	0.00	0.55	0.11
r	0.00	0.08	0.21
r p - value	0.00	0.00	0.00
Fs	1.61	- 0.79	0.97
Fs p - value	0.46	0.22	0.71

Fu's Fs, mismatchanalysis parameters (*r* and SSD) are significant at $P < 0.05$

17. Phylogenetic analysis and sequence divergence estimates

17.1 Bayesian analysis

When sequences of *E. latipes* and *E. natalensis* were included in a phylogenetic tree with other *Excirolana* species, the tree indicated that *E. latipes* and *E. natalensis* are sister species with strong support (PP = 1). Both *E. latipes* and *E. natalensis* are monophyletic and significantly different from all other *Excirolana* species (see Fig. 3.8).

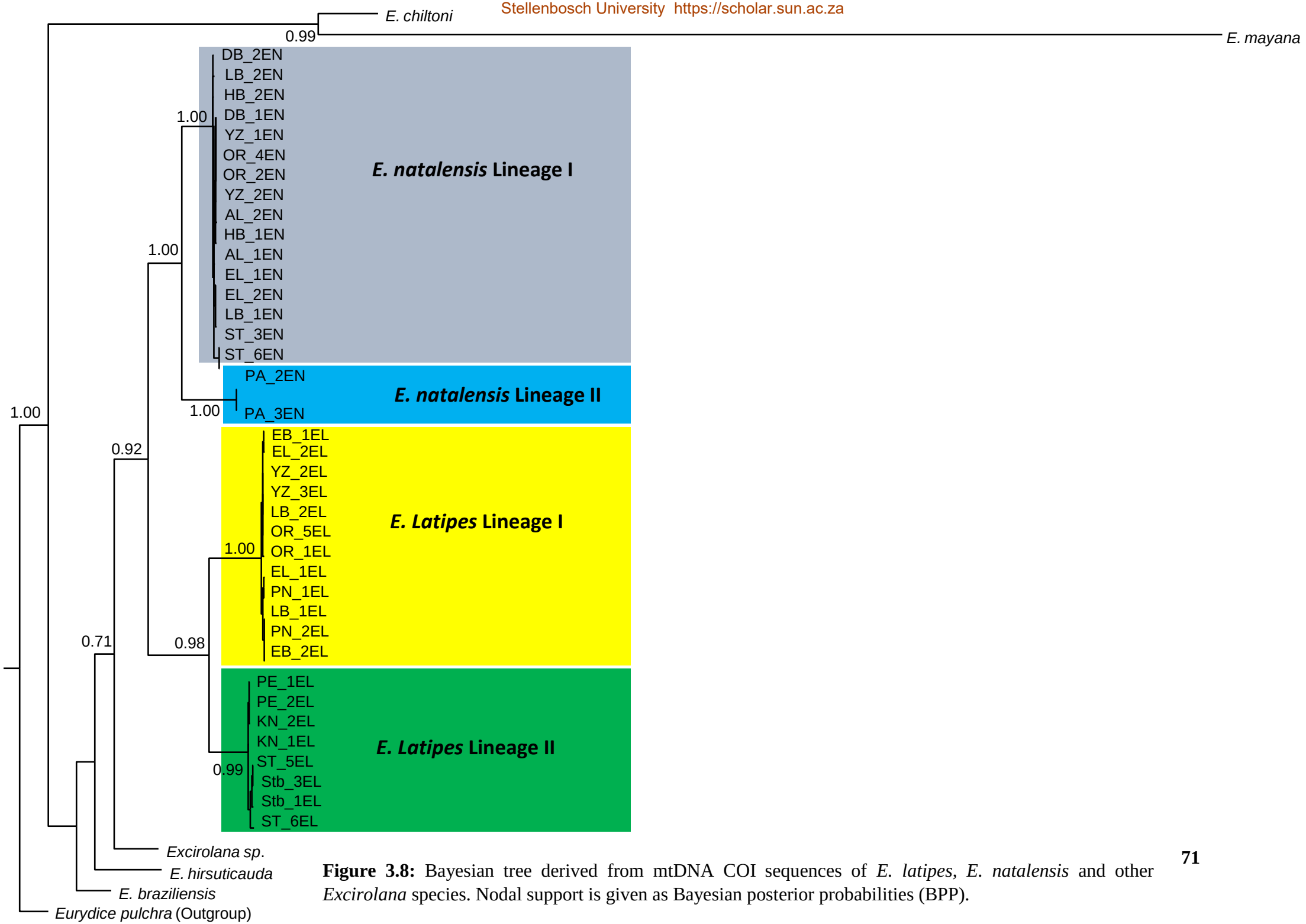

Figure 3.8: Bayesian tree derived from mtDNA COI sequences of *E. latipes*, *E. natalensis* and other *Excirolana* species. Nodal support is given as Bayesian posterior probabilities (BPP).

17.2 Sequence divergence estimates

Estimates of sequence divergence between lineages of *E. latipes* and *E. natalensis* indicated that the lowest genetic distance detected using the COI gene was 1% and the highest value was 20% (results not shown).

There was a clear separation between Intra and inter - species/lineage P-distances among *E. latipes* lineages, the highest intra - species distance being 2.00% and the highest interspecies distance 18.00%. In *E. natalensis* lineages (Table 3.12), highest intra - species distance was 1.70% and the highest interspecies distance 17.00%. For other *Excirolana* species, the highest interspecies distance was 20.00% (Table 3.11), but I was not able to calculate intra - species distances, because of a lack of available sequences.

Table 3.11: Intra and inter-specific COI divergence (P-distances (%)) determined for *E. latipes* Lineage I and II and for other *Excirolana* species. Sequences were selected from Fig. 3.8.

	Taxa	**P-distance (%)**
Intra-specific		
E. latipes Lineage I	EB1 vs PN2	1.00
E. latipes Lineage II	PE1 vs ST6	2.00
Inter-specific		
E. latipes Lineage I and II	EL2 vs Stb1	18.00
E. latipes Lineage I and II	KN2 vs YZ2	17.00
Other *Excirolana* species	*Excirolana. sp* vs *E. hirsuticauda*	20.00
	Excirolana. sp vs *E. braziliensis*	18.00

Table 3.12: Intra and inter-specific COI divergence (P-distances (%)) determined for *E. natalensis* Lineage I and II and for other *Excirolana* species. Sequences were selected from Fig. 3.8.

	Taxa	P-distance (%)
Intra-specific		
E. natalensis Lineage I	DB2 vs ST6	1.70
E. natalensis Lineage II	PA2 vs PA3	0.00
Inter-specific		
E. natalensis Lineage I and II	LB2 vs PA3	17
E. natalensis Lineage I and II	ST3 vs PA2	17

18. Time since population divergence

Estimates of time to the most recent common ancestor (TMRCA) obtained with the mutation rate 1.25%/Ma indicated that the divergence of the two *E. latipes* two lineages occurred 150 000 years ago. When the mutation rate 1.56%/Ma was applied in the BEAST analysis, it suggested that the two genetic lineages diverged about 980 000 Ma. At 1.72%/Ma, *E. latipes* lineages divergence occurred 1.23 Ma.

Excirolana natalensis indicated similar TMRCA to *E. latipes*. At mutation rate 1.25%/Ma, *E. natalensis* lineage divergence occurred 140 000 years ago. When the mutation rate 1.56%/Ma was applied in the BEAST analysis, it suggested that the two genetic lineages diverged about 730 000 Ma. At 1.72%/Ma, *E. latipes* lineages divergence occurred 1.17 Ma.

Discussion

19. Evolutionary history of *Excirolana* latipes

Results from this study revealed that *E. latipes* is comprised of two genetically distinct lineages (Fig. 3.3) that are separated by a strong phylogeographic break located between Yzerfontein and Simon's Town. The estimates of the divergence within *E. latipes* (150 000 - 1.23 Ma) suggest a strong link with the Pleistocene period, with a complete loss of gene flow between the two lineages, as evidenced by a 105 step mutational difference. This suggests that *E. latipes* is potentially structured around the Cape Point region. Several studies have demonstrated gene flow discontinuities around the Cape Point region, for examples see Teske et al. 2006 (estuarine crustaceans); von der Heyden et al. 2008 (clinid fish, *Clinus cottoides*); Muller et al. 2012 (*P. angulosus*) and Reynolds et al. 2014 (*T. serrata*). Reviews by Teske et al. (2011) and von der Heyden (2009) also identified this region as one of the major biogeographic breaks in southern Africa. This area is characterized by different temperature regimes on either sides of the Cape Point (Reynolds et al. 2014), which may influence larval dispersal and adaptation patterns of coastal species along this region and thus maintain patterns of genetic differentiation.

What really stands out from these findings is that the previous chapter identified a phylogeographic break for *T. granulatus* between Hondeklip Bay and Kleinzee; other studies have also identified the Orange River as a barrier to gene flow, and both phylogeographic breaks do not correlate with phylogeographic patterns of *E. latipes*. This highlights that not all species show the same biogeographical and phylogeographical patterns (Harrison, 2002; Teske et al. 2009; von der Heyden, 2009). We found no obvious overlap between phylogeographic and biogeographic patterns of *E. latipes*.

Taking into account the time of divergence between *E. latipes* Lineage I and II (150 000 - 1.23 Ma), climatic and oceanographic oscillations of the Pleistocene period had major impacts on biogeographic and phylogeographic patterns of *E. latipes*. As discussed in the previous chapter, the Pleistocene epoch possibly led to an expansion of sandy beaches that allowed for population connectivity. However, upwelling intensification (Henriques et al. 2014) around the south-western Cape during the Pleistocene period could have resulted in the separation of *E. latipes* into two lineages. Because of swimming abilities in *Excirolana* species, *E. latipes* has probably adapted to the two different marine environments on either

side of the genetic break. Notably, the southern lineage (*E. latipes* Lineage I) is more structured than the western lineage (*E. latipes* Lineage II). The observed genetic pattern could be influenced by the Agulhas Current and its upwelling proceedings nearby Port Elizabeth (Lutjeharms et al. 2000). Retroflections of the Agulhas Current in Port Elizabeth potentially reduce gene flow among populations of *E. latipes* in this region, hence, the higher and significant *Φst* values (ranging from 0.31 to 0.99, $P < 0.05$).

20. Evolutionary history of *E. natalensis*

Results from this study revealed that the Natal beach louse, *E. natalensis*, is also composed of two distinct lineages (Fig. 3.4). *Excirolana natalensis* Lineage I is characterized by low and non-significant Φ_{st} values, indicating only very shallow genetic structuring across this region. This pattern is similar to the one observed in the western Lineage of *E. latipes* (*E. latipes* Lineage I). The most interesting finding is that, unlike *E. latipes*, the Cape Point biogeographic transition is not a barrier to gene flow in *E. natalensis*, as both phylogeographic and phylogenetic analyses did not detect a barrier to gene flow between Simon's Town and Yzerfontein. Further, the genetic break described in chapter II for *T. granulatus* located between Kleinzee and Hondeklip Bay, does not influence the phylogeographic patterns of *E. natalensis* (Fig. 3.4).

The estimates of the divergence among *E. natalensis* Lineages (140 000 - 1.17 Ma) are similar to *E. latipes*, suggest a strong link with the Pleistocene period, with a complete loss of gene flow between the two lineages, as evidenced by a 91 step mutational difference. I could not determinine where the genetic break is located on the south coast, given the sampling gap, which requires further fieldwork and sampling.

21. Evidence for cryptic species or distinct lineages?

Results from this chapter indicate that *E. latipes* and *E. natalensis* are sister species (Fig. 3.8). The two species are sympatric and have similar life-history patterns but show different phylogeographic patterns. These findings correspond to Hurtado's et al (2007) paper on a different group of intertidal invertebrates where two sympatric *Nertia* species also showed different phylogeographic patterns, as observed in *Excirolana*. Genetic connectivity in two

sympatric species of marine snails, *Nerita scabricosta* and *Nerita funiculate*, was assessed with the COI gene. Hurtado et al (2007) found no evidence of genetic differentiation throughout the Gulf of California and Baja peninsula for either species. However, *N. scabricosta* showed a significant population structure when omparisons between Gulf of California/Baja and Panama populations were taken into account. This was not the case for *N. funiculate*. The genetic differences observed in *Nerita* were explained by ecological and behavioral differences, which could be the same for *Excirolana* in this study. The ability to swim allows both *Excirolana* species to respond differently to environmental gradients along the southern African coast through local adaptation.

The results imply that inter-specific sequence divergence between both *E. latipes* and *E. natalensis* ranges between 17.00 - 18.00%. This range falls within the same array as that of other *Excirolana* species with a range of 18.00 - 20.00% (Table 3.11 and 20). I was unable to compare intra-specific sequence divergence of *E. latipes* and *E. natalensis* to other *Excirolana* species. In conclusion, both species are composed of distinct evolutionary lineages that should be regarded as management units. These MUs should be considered separately in conservation and management aims of sandy beaches.

Conclusion

To my knowledge, no studies in South Africa have attempted to document genetic patterns of sandy beach species using taxa with similar life histories, sampled from the same areas. Thus, this study is the first to successfully define and compare phylogeographic patterns of two sympatric sandy beach isopods. Mitochondrial COI revealed patterns of genetic divergence among two distinct evolutionary lineages of *E. latipes*. With no morphological differences found between these, it can be concluded that *E. latipes* should be treated as distinct MUs separated by a phylogeographic located in Cape Point. For *E. natalensis*, although there is a gap of missing data points between Simon's Town and Port Alfred, mtCOI still detected two divergent lineages. These are strong signals of cryptic diversity that should be further investigated by nuclear markers and increased sampling efforts in areas such as Cape Agulhas or Struisbaai, Port Elizabeth, Knysna and East London. For future studies, sampling efforts in phylogeographic studies should cover the whole species distribution range. This could help to reveal unknown diversities or lineages to better understanding genetic patterns of coastal isopod species. This is well illustrated in Hurtado et al (2016), where they investigated phylogeographic patterns of *E. braziliensis* using various molecular markers and a set of data from previous studies by Sponer & Lessios (2009), Verela & Haye (2012) and Tourinho et al (2016). The large dataset from this study revealed new divergent lineages of *E. braziliensis*. Hurtado et al (2016) represents the most comprehensive phylogeographic analyses of a nominal *Excirolana* species to date in terms of the geographic range. This study highlights the importance of intense sampling efforts in phylogeographic studies of broadly distributed species with high cryptic diversity such as *Excirolana*.

It is fascinating that both *E. latipes* and *E. natalensis* did not correspond to previously identified phylogeographic breaks (the Orange River and the phylogeographic break located between Kleinzee and Hondeklip Bay). *Excirolana latipes* and *E. natalensis* are however structured differently across the Cape Point biogeographic break. Since we are dealing with sympatric species, it is highly likely that competition to mutual ecological resources might have influenced distribution patterns of both *Excirolana* species along the southern African coast. According to Teske et al. (2007), it is possible that coastal South African species with large distribution ranges are composed of two or more distinct evolutionary lineages, which are influenced by competition for similar resources. This is in line with the findings of this

study, but it also means that this study could be improved by (i) further morphological analyses in case there were previous unnoticed differences, (ii) the use of nuclear markers and (iii) running experiments to test whether temperature regimes and other environmental factors such as salinity, have any impact on distribution patterns of both *E. latipes* and *E. natalensis*.

Both *Excirolana* species showed signals of cryptic diversity, indicating that sandy beaches are more diverse than currently described. This, as for *T. granulatus* has important implications for the conservation and management of sandy beaches in South Africa.

CHAPTER IV

Every beach an island

The results from my thesis provide support for strong phylogeographic structuring of widely disitrbuted sandy beach isopod species in southern African. Results from both chapter II and III have shown that sandy beach genetic divergence or diversity is twice the amount we thought it was. Both chapters revealed unexpected strong genetic patterns that potentially indicate higher levels of cryptic speciation. Conclusions that can be drawn from chapter II is that *T. granulatus* is a highly threatened species that is on the edge of extinction and needs to be ranked high in the IUCN Red Data list. When taking into account the narrow range of *T. granulatus* along the west coast of South Africa and Namibia and that it is endemic to this region but shows complex phylogeographic patterns, this raises awareness that sandy beach diversity should not be underestimated and sandy beach conservation should not be overlooked. In chapter III, I showed strong signals of cryptic diversity within two sympatric species of *Excirolana* (Fig. 4.1). These results indicate that southern African sandy beaches are composed of MUs that should be considered separately for conservation and management purposes.

The overall aim of this thesis was to provide a scope of work that builds towards a better understanding of the complexity, vulnerability and significance of southern African sandy beaches and to provide insinghts for conservation and management of these systems. As mentioned in the introductory chapter (chapter I), sandy beach science is a new and emerging field that is mostly neglected in conservation aims. With escalating pressures on marine ecosystems, there is a growing need to understand species distribution patterns and my study contributes significantly towards highlighting the unknown biodiversity component, with large implications for the conservation and management of sandy shores.

Most importantly, this study covered a large area of the west and south coasts (Fig. 4.1), with the former particularly regarded as one of the most threatened regions in South Africa due to mining, coastal developments, kelp harvesting and coastal squeeze. Results from this study showed that the west coast region is an area of interest regarding genetic diversity and thus the enforcement of MPAs in this region should be emphasised.

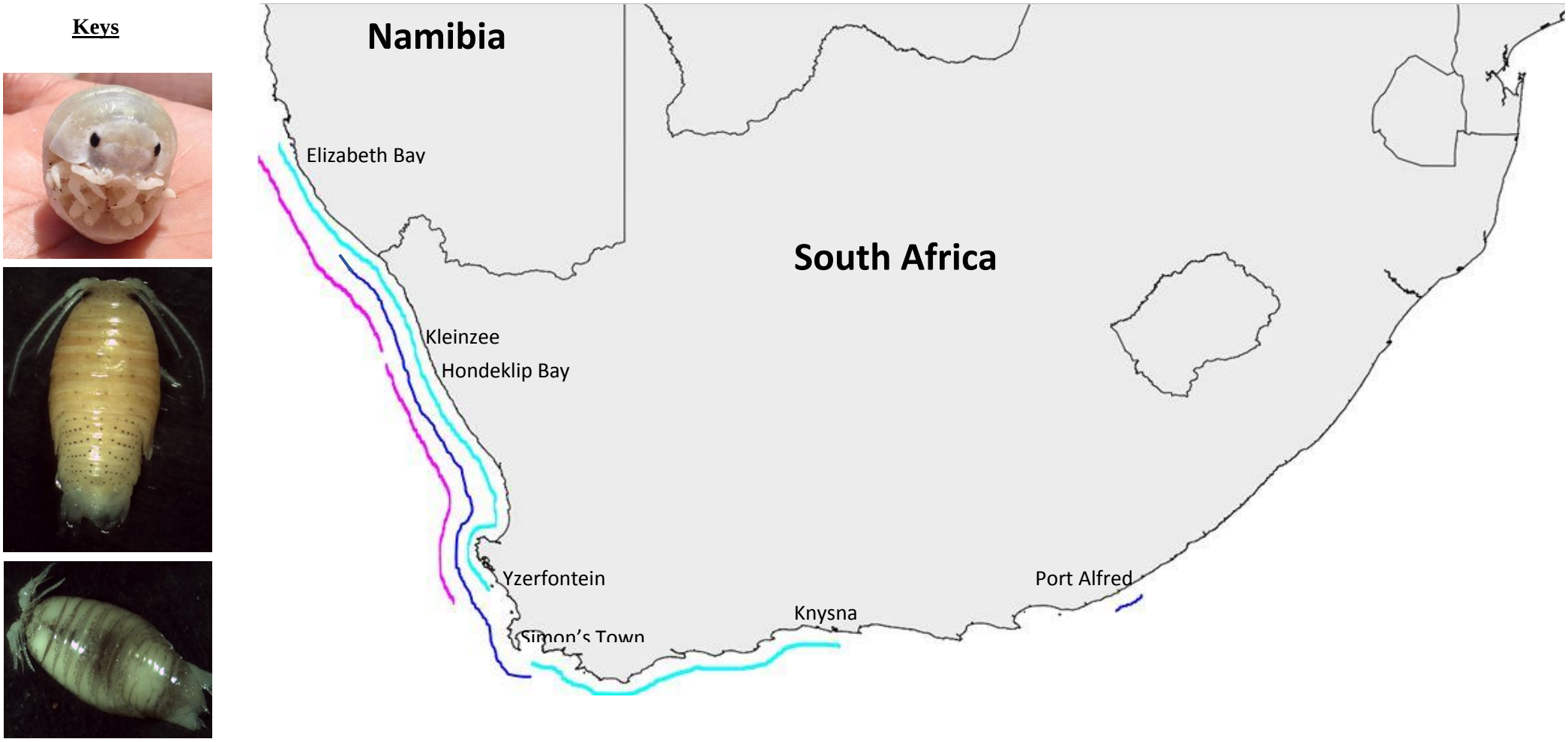

Figure 4.1: Distribution patterns of *T. granulatus*, *E. natalensis* and *E. latipes* lineages along the southern African coastline.

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Appendix 1: Sampling records of *Tylos granulatus* along the west coast of South Africa and Namibia between 1986 to 2008. Data was received from Karien Bezuidenhout, Zoology Department, Nelson Mandela Metropolitan University (NMMU). Beaches that were visited and we did not find *T. granulatus* are indicated in grey.

Transect	Latitude	Longitude	Description	Year	Indiv. Per transect
Bogenfels	- 27.46336300	15.39940500	Bogenfels	1993	62.5
Britannia Bay	- 32.74161700	18.01567500	Brittania	1992	123.0
C1	- 31.52623333	18.06358333	North - central geelwal	2002	9354.5
C2	- 31.36753333	17.91948333	Stompneus	2002	2034.8
CC I	- 21.80526600	13.98916900	Cape Cross I	1986	45.0
CC II	- 21.80200500	13.98583300	Cape Cross II	1986	45.0
CI1	- 31.57550000	18.11101667	Chipbaai	2002	48.5
CI2	- 31.60863333	18.12780000	Bethel	2002	183.3
COTTON	- 34.09508700	18.80148700	Cotton Beach	1998	19.5
DBs1	- 27.70716667	15.53583333	Dernburg Bay	1998	77.3
DBs2	- 27.69566667	15.53116667	Dernburg Bay	1998	351.5
DERN	- 27.73658600	15.54328500	Dernburg Bay	1997	326.7
EBayV05	- 26.92568600	15.22508700	Elizabeth Bay	1994	210.0
Elizabeth Bay1	- 26.95891000	15.23760900	Elizabeth Bay1	1993	100.5
Elizabeth Bay2	- 26.92012400	15.21732000	Elizabeth Bay2	1993	34.7
FI1	- 31.52425000	18.06140000	Duiwegat	2002	7466.7
FI2	- 31.56528333	18.10316667	Rooidiun	2002	8700.0
GBuchtI05	- 26.73516300	15.09620300	Grossebucht 1	1994	390.0

Groenrivier	- 30.69700100	17.47980900	Groenrivier	1992	2003.0
HI1	- 31.55105000	18.08908333	Dirkie Lutz	2002	4400.0
HI2	- 31.58090000	18.11526667	Geelwal	2002	466.7
K149N	- 28.17500000	15.91333333	Diamond area 1 (Sberrgebiet)	2001	139.4
K149S	- 28.17666667	15.91500000	Diamond area 1 (Sberrgebiet)	2001	181.4
K35N	- 28.24500000	16.00500000	Diamond area 1 (Sberrgebiet)	2001	125.2
K35S	- 28.24666667	16.00500000	Diamond area 1 (Sberrgebiet)	2001	42.9
K60N	- 28.23450000	15.98900000	Diamond area 1 (Sberrgebiet)	2001	54.5
K60S	- 28.23633333	15.99100000	Diamond area 1 (Sberrgebiet)	2001	43.2
Langstrand	- 22.79186600	14.54038000	Langstrand	1986	1.0
M35N	- 28.36833333	16.14000000	Diamond area 1 (Sberrgebiet)	2001	158.3
M35S	- 28.37000000	16.14166667	Diamond area 1 (Sberrgebiet)	2001	11.2
M75N	- 28.34183333	16.10983333	Diamond area 1 (Sberrgebiet)	2001	639.8
M75S	- 28.34516667	16.11316667	Diamond area 1 (Sberrgebiet)	2001	137.3
Paaltjies	- 22.98755000	14.40360500	Paaltjies	1986	1.0
PB1N	- 27.88883333	15.66866667	Pocket Beach 1 sample 2 (PB1N)	2008	432.0
PB1S	- 27.89716667	15.67633333	Pocket Beach 1, sample 1 (PB1S)	2008	168.0
PB1s1	- 27.88883333	15.66866667	Pocket Beach 1 sample 1	1998	84.8
PB1s2	- 27.89716667	15.67633333	Pocket Beach 1 sample 2	1998	289.1
PB2N	- 27.84283333	15.63866667	Pocket Beach 2 sample 2 (PB2N)	2008	58.5
PB2S	- 27.85216667	15.64533333	Pocket Beach 2 sample 1 (PB2S)	2007	45.0
PB2s1	- 27.85216667	15.64533333	Pocket Beach 2 sample 1	1998	116.7
PB2s2	- 27.84283333	15.63866667	Pocket Beach 2 sample 2 (PB2N)	1998	62.1

PB3/4N	- 27.80416667	15.60133333	Pocket Beach 3/4 sample 2 (PB3/4N)	2008	120.0
PB3/4S	- 27.81983333	15.61933333	Pocket Beach 3/4 sample 1 (PB3/4S)	2008	93.3
PB3s1	- 27.81983333	15.61933333	Pocket Beach 3/4 sample 1 (PB3/4S)	1998	68.2
PB3s2	- 27.80416667	15.60133333	Pocket Beach 3/4 sample 2 (PB3/4N)	1998	106.7
PB4	- 27.82615000	15.62429700	Pocket Beach 4 sample	1997	87.3
PB8N	- 27.63366667	15.51750000	Pocket Beach 8 sample 2 (PB8N)	2008	324.0
PB8S	- 27.66383333	15.52616667	Pocket Beach 8 sample 1 (PB8S)	2008	315.0
PB9N	- 27.58533333	15.47683333	Pocket Beach 9 sample 2 (PB9N)	2004	108.0
PB9S	- 27.59300000	15.48533333	Pocket Beach 9 sample 1 (PB9S)	2007	45.0
Slipper Bay	- 32.77283200	18.09891600	Slipper Bay	1992	18.0
Spoegrivier	- 30.37964400	17.31332600	Spoegrivier	1992	603.0
Strandfontein	- 31.68028900	18.17175200	Strandfontein	1992	494.0
TBS02	- 31.52425000	18.06140000	Duiwegat	2003	4890.0
TBS03	- 31.52623333	18.06358333	Geelwal N	2003	4418.2
TBS04	- 31.56528333	18.10316667	Rooiduin N	2003	2648.5
TBS05	- 31.55105000	18.08908333	Lutzbaai	2003	3908.7
TBS06	- 31.57550000	18.11101667	Chipbaai	2003	25.5
TBS09	- 31.56836667	18.10566667	Rooiduin S	2003	473.9
TH1	- 31.36753333	17.91948333	Stompneus	2004	1221.8
TH2	- 31.52425000	18.06140000	Duiwegat	2004	291.5
TH3	- 31.52623333	18.06358333	Geelwal N	2004	604.5
TH4	- 31.56528333	18.10316667	Rooiduin N	2004	163.6
TH5	- 31.55105000	18.08908333	Lutzbaai	2004	261.8

TH6	- 31.57550000	18.11101667	Chipbaai	2004	38.2
TH7	- 31.58088333	18.11526667	Scratchpatch	2004	190.9
TH9	- 31.56836667	18.10566667	Rooiduin S	2004	750.0
U180N	- 28.39166667	16.16666667	Diamond area 1 (Sberrgebiet)	2001	166.7
U180S	- 28.39500000	16.17000000	Diamond area 1 (Sberrgebiet)	2001	114.5
U40N	- 28.48783333	16.26466667	Diamond area 1 (Sberrgebiet)	2001	26.4
U40S	- 28.48983333	16.26666667	Diamond area 1 (Sberrgebiet)	2001	13.0
U90N	- 28.44616667	16.22450000	Diamond area 1 (Sberrgebiet)	2001	55.6
U90S	- 28.44766667	16.22600000	Diamond area 1 (Sberrgebiet)	2001	45.0
YZC	- 33.33972600	18.16006700	Yzerfontein Central	1998	1433.3
YZN	- 33.32034900	18.15532100	Yzerfontein North	1998	3878.8

Appendix 2: Polymerase chain reaction (PCR) primer information and annealing temperatures (Tm).

Gene	Name	Primer sequences	Tm	References
COI	jgHCO - 2198	5’ - TAIACYTCIGGRTGICCRAARAAYCA - 3’	54	Geller et al. 2013
	jgLCO - 1490	5’ - TITCIACIAAYCAYAARGAYATTGG - 3’		
16S	16s SAR	5’ - CGCCTGTTTATCAAAAACAT - 3’	49	Palumbi 1996
	16s SBR	5’ - CCGGTCTGAACTCAGATCACGT - 3’		

Appendix 3: Information on *Tylos* sequences that were used in the phylogenetic analysis of both COI and 16S, these sequences were downloaded from GenBank (Hurtado et al. 2014).

Species	Locality	Country	Sequence ID	GenBank Accession Nos. (16S rDNA, COI)	Voucher ID
T. sp[1]	Yaguanabo, Cienfuegos	Cuba	CU	KF007549, KF007724	
T. niveus[3]	Aguada	Puerto Rico	28	KJ468181, KJ468120	
T. marcuzzii[2]	Maria La Gorda, Pinar del Rio	Cuba	Tmar2	KJ468178, KJ468118	MZUF 8660
T. marcuzzii[2]	Ciego de Avila, Cayo Coco	Cuba	Tmar1	KJ468177, KJ468117	MZUF 8659
T. wegeneri[4]	Golfo Nicoya	Costa Rica	#08	KJ468188, KJ468126	LACM 25 July 1985 J.A.Vargas
T. spinulosus[2]	Atacama	Chile	Ts1	KJ468187, KJ468125	MZUF 1096
T. chilensis[2]	Los Vilos, Punta Tablas	Chile	Tch1	KJ468168, KJ468109	MZUF 279
T. ponticus[5]	Unknown	Portugal	428 - 1	KJ468185, KJ468123	
T. ponticus[2]	Preveli Beach, Crete	Greece	27	KJ468184, KJ468122	MZUF 8398
T. ponticus[2]	Susah, Cyrenaica	Libya	Tp1	KJ468186, KJ468124	MZUF 9447
T. europaeus[2]	Sabratah	Libya	Te1	KJ468170, KJ468111	MZUF 9445
T. europaeus[2]	Burano, Tuscany	Italy	25	KJ468169, KJ468110	MZUF 2295
T. granulatus[2]	Cape Town, Rondeberg	South Africa	Tg1	KJ468172	MZUF 219
T. capensis[2]	Knysna	South Africa	Tc1	KJ468167	MZUF 220
T. maindroni[2]	Wafra	Kuwait	Tma1	KJ468176, KJ468116	MZUF 2156
T. exiguus[2]	Qalansiyah, Socotra Island	Yemen	Tex1	KJ468171, KJ468112	MZUF 8687
T. minor[2]	Aldabra Island, Grande Terre	Seychelles	Tm1	KJ468179	MZUF 1249

T. albidus[2]	Felidu Atoll	Maldives	Ta1	KJ468166	MZUF 9104
T. granuliferus[6]	AnDeok, Jeju island	South Korea	CY	KJ468173, KJ468113	
T. granuliferus[7]	Toyooka, Hyogo	Japan	HYO	KJ468174, KJ468114	
T. opercularis[2]	Palu, Sulawesi	Indonesia	To1	KJ468182	MZUF 3244
T. opercularis[2]	Cape Tribulation, Queensland	Australia	To2	KJ468183, KJ468121	MZUF 9425
T. neozelanicus[2]	Piha Beach, North Island	New Zealand	Tnz1	KJ468180, KJ468119	MZUF 8869
H. brevicornis[2]	Burcei, Sardinia	Italy	Hb	KJ468175, KJ468115	MZUF 9448

[1] Sequences from Hurtado et al. (2013)

[2] Museo di Storia Naturale "La Specola", Zoological section, in Florence, Italy

[3] Dr. Luis Hurtado (Texas A&M University, U.S.A)

[4] Natural History Museum of Los Angeles County

[5] Dr. Jonathan Wright (Pomona College)

[6] Dr. Do Heon Kwon (Inje University, South Korea)

[7] Miyuki Niikura (University of Tsukuba, Japan)

Appendix 4: Information on COI *Tylos capensis* sequences that were used in the phylogenetic analysis.

Species	Location	Reference
T. capensis	Woody Cape	Karien Bezuidenhout, Zoology Department NMMU)
T. capensis	Sundays River	Karien Bezuidenhout, Zoology Department NMMU
T. capensis	Dana Bay	Karien Bezuidenhout, Zoology Department NMMU
T. capensis	Struisbaai	Karien Bezuidenhout, Zoology Department NMMU

Appendix 5: Average genetic distances between the studied *T. granulatus* populations based on the COI sequences.

	AB	EB	OR	KL	BD	EL	SB	HB
EB	4.47%							
OR	4.06%	2.01%						
KL	4.44%	1.55%	2.27%					
BD	10.30%	10.27%	10.43%	9.66%				
EL	10.32%	10.30%	10.45%	9.69%	0.10%			
SB	10.30%	10.26%	10.42%	9.65%	0.05%	0.11%		
HB	10.64%	10.71%	10.84%	10.10%	0.64%	0.70%	0.64%	
YZ	10.45%	10.41%	10.57%	9.80%	0.18%	0.24%	0.18%	0.77%

Appendix 6: Information on *Excirolana* sequences that were used in the phylogenetic analysis of COI, these sequences were downloaded from GenBank.

Species	GenBank Accession Nos. (COI)	Voucher ID
E. chiltoni	AF260841.1	
Excirolana sp.	KJ592750.1	ES120301
E. braziliensis	KT870130.1	
E. hirsuticauda	FJ532097.1	
E. mayana	KP172617.1	CMPC138
Eurydice pulchra (Outgroup)	KT209446.1	MT03145

Appendix 7: Average genetic distances between the studied *E. latipes* populations based on the COI sequences.

	EL	OR	PN	EB	YZ	LB	KN	PE	Stb
OR	2%								
PN	2%	1%							
EB	1%	0%	0%						
YZ	1%	0%	1%	0%					
LB	2%	1%	0%	0%	0%				
KN	17%	18%	18%	18%	18%	18%			
PE	17%	18%	17%	18%	18%	18%	1%		
Stb	18%	19%	19%	19%	19%	19%	2%	1%	
ST	19%	20%	20%	20%	20%	20%	5%	5%	5%

Appendix 8: Average genetic distances between the studied *E. natalensis* populations based on the COI sequences.

	LB	OR	AL	HB	ST	EL	YZ	DB
OR	1%							
AL	0%	0%						
HB	0%	0%	0%					
ST	2%	2%	2%	2%				
EL	0%	1%	1%	1%	2%			
YZ	1%	1%	1%	1%	2%	1%		
DB	1%	1%	1%	1%	3%	1%	1%	
PA	16%	16%	16%	16%	17%	16%	16%	16%