Genetic and morphometric variation of *Octopus vulgaris* in the Benguela Current Region

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Abstract

The Benguela Current is a cold eastern boundary current located on the south-western coast of the African continent. The establishment of its present day features approximately two million years ago has triggered allopatric events which have driven genetic and/or phenotypic differentiation in many of the warm-temperate organisms that previously had continuous distributions along the south and west coast of southern Africa. However, since many of these species have responded differently, despite similar isolation times, research in this region provides a unique opportunity to increase our understanding of evolutionary processes. The common octopus (*Octopus vulgaris,* Cuvier 1797) is a coastal, sedentary species, inhabiting coral reefs or rocky environments at depths of up to a 100m. It is considered to be one of the most extensively studied cephalopod species due to its worldwide distribution. However, very little research has been conducted on *O. vulgaris* in southern Africa. In order to gain a holistic understanding of the effects of the Benguela Current on population connectivity, genetic and phenotypic diversity, and evolutionary history of *O. vulgaris,* a comparative genetic and morphological study was conducted across the Benguela region.

A total of 168 specimens of *O. vulgaris* were collected from four different regions across the Benguela system. A small tissue sample was preserved in ethanol for molecular analysis, and the specimen was frozen whole for morphometric analysis in the laboratory.

Octopus vulgaris genetic population structure and evolutionary history was investigated using a 580bp fragment of the mitochondrial cytochrome b (*cytb*) gene for 76 individuals located within the Benguela region, yielding 10 different haplotypes. AMOVA and pairwise F_{ST} analyses revealed significant genetic differentiation suggesting a northern-southern Benguela divergence. Estimates of time since most recent common ancestor, based on biogeographical calibrators and coalescent analyses, indicated that isolation between the Angolan and South African population occurred between ~231 Ka and 1 Ma. Mismatch distribution analyses revealed a past population expansion within the South African *O. vulgaris* roughly 129.31 Ka, whilst Bayesian skyline plots were indicative of gradual demographic growth within the Angolan population in the last ~100 Ka. Observed *O. vulgaris* population structure and demographic history was likely the result of historical climate-induced change within the system. Reconstruction of phylogenetic relationships within the *Octopus* genus, using *cytb* and *COI* suggest that *O. vulgaris* is not a monophyletic group and a major systematic revision is required. Furthermore, unidentified individuals from South Africa were found to group with species from Indo-West Pacific Oceans and were therefore considered to have been translocated through ballast water from Asia.

While the molecular analysis indicated a significant northern-southern Benguela structure results from the principle component analysis (PCA) and discriminate function analysis (DFA) were unable to distinguish between *O. vulgaris* from different sampling localities throughout the Benguela Current region based on soft-parts, hard-parts and meristic characters. The lack of phenotypic variation, despite significant genetic divergence, highlights the importance of multi-method approaches in gaining a holistic understanding of the taxonomy and biogeography of species.

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CHAPTER 1

General Introduction

A comprehensive understanding of speciation, population structure and connectivity, and the historical and contemporary mechanisms driving such processes, is critical to the effective management and conservation of species and populations around the globe (Coyle 1997). Furthermore, past and current patterns of population divergence have the potential to inform scientists of the outcome of historical interactions between a species and its surrounding physical, chemical and biological environmental conditions (Grosberg & Cunningham 2001). Inferences regarding future responses to ongoing climate change may therefore be developed based on the evolutionary history of a species.

Population divergence arises as a consequence of the intricate contemporary and historical interactions between ecological, evolutionary and physical processes (Grosberg & Cunningham 2001). Contemporary acting evolutionary mechanisms, which may potentially drive local adaptation and subsequently population structure within marine organisms, include asynchronous spawning events or the use of differential spawning grounds, the presence of oceanographic features such as currents, frontal zones and upwelling cells, as well as the existence of environmental gradients and increased physical distances (Palumbi 1994, Hemmer-Hansen et al. 2007, Grillo 2011). Historical processes such as large scale climatic events and geological changes, can also strongly influence the evolution of a species (Wilson 2006, Hemmer-Hansen et al. 2007, Pardiñas et al. 2010). A prime example of such historical mechanisms is the rise of the Isthmus of Panama, 3 Million years ago (Mya), at the end of the Pliocene, resulting in the separation of the Pacific and Atlantic Oceans. The formation of the land bridge, coupled with the development of unique environmental conditions on either side, subsequently led to the separation and isolation of several marine taxa, such as the Echinoid genus *Echinometra*, the snapping shrimp (*Alpheus* spp.) and the Haemulid fishes of the genus *Anisotremus*, resulting in multiple speciation events (Knowlton & Weigt 1998, McCartney et al. 2000, Bernardi & Lape 2005, Lessios 2008). In addition, the formation of the Isthmus of Panama and the events surrounding this significant geological transformation resulted in major changes in climate patterns, current dynamics, temperature and biological productivity in both oceanic basins, and was the driver of the Pliocene-Pleistocene transition (Coates & Obando 1996, Lessios 2008).

Large-scale climatic changes, such as those observed during the Pleistocene, are considered to have greatly influenced the evolutionary history of several extant marine taxa (Liu et al. 2007, Thum & Harrison 2008, Larmuseau et al. 2009). Fluctuations in the global climate system, during this geological period led to recurrent variations in sea surface temperatures, average sea levels, oceanic circulatory patterns and biological productivity (Hemmer-Hansen et al. 2007). The effects of such dynamic climatic oscillations thus had major impacts on population dynamics and connectivity of marine species. Therefore, in order to gain a comprehensive understanding of the processes governing the observed population sub-structuring in the marine environment, the "nature, efficacy and establishment" of barriers to gene flow need to be determined (Lessios et al. 2003).

The invisible nature of oceanic biogeographical barriers, coupled with the potential for high dispersal capabilities of marine organisms, particularly those with planktonic larval stages, introduces difficulties into the study of speciation and population connectivity within the marine realm and leads to the assumption that most marine fauna would have panmictic populations (McCartney et al. 2000, Lessios et al. 2001, Riginos & Nachman 2001). The concept of oceanic continuity, and subsequently the hypothesis of panmixia, was further supported by morphologically conservative, yet widely distributed species (Rocha-Olivares et al. 2000). Despite this, marine biogeographical barriers have been identified to influence patterns of population structure by limiting or restricting dispersal between regions, and are therefore considered to be of utmost importance in the study of the evolutionary history of marine fauna (Teske et al. 2006, Gaither et al. 2010). However, the efficacy of oceanic barriers is largely determined by the complex interaction between the environmental and physical attributes of the barrier and the biological characteristics of the species that occur in the region.

The Benguela Current is a cold water oceanographic system situated along the south-west African coast and is often referred to as the Benguela barrier (Rocha et al. 2007). The establishment of the Benguela upwelling system is thought to have occurred ~12 Mya, however the contemporary oceanographic attributes of the region are documented to have developed ~2 Mya (Diester-Haass 1988, Krammer et al. 2006). The intensification of the upwelling system, during the Pleistocene, is considered to have played a fundamental role in initiating the differentiation and speciation of biota between the western Atlantic and the Indian Ocean (Floeter et al. 2008). However, a series of comparative systematic studies conducted across the Benguela region on multiple species, have revealed contrasting evolutionary responses to the establishment of this eastern boundary coastal upwelling regime (Henriques 2012, Richardson 2011). For example, the blacktail seabream (*Diplodus capensis* Smith, 1844) exhibits significant genetic and morphological differentiation between the northern and southern Benguela sub-systems (Henriques 2012, Richardson 2011). In contrast, there was little morphological differentiation in the geelbeck croaker (*Atractoscion aequidens* Cuvier, 1830) (Kruger, unpublished data), and in leervis (*Lichia amia* Linnaeus, 1758), in spite of observed significant molecular divergence between the two sub-systems (Henriques 2012). Interestingly, amongst the cephalopoda a comparative morphological investigation on *Loligo reynaudii* (d'Orbigny, 1845) revealed three distinct stocks in the region (Angola, the South African west coast and south coast - van der Vyver, submitted), although molecular analyses failed to detect the presence of population sub-structuring along the South African coastline (Shaw et al. 2010).

The observed variability in evolutionary responses highlights the differentiation in the efficacy of the cold water Benguela barrier between species. Therefore what may be considered a 'soft' barrier, limiting but not completely restricting dispersal in one species due to either physiological, morphological, ecological or behavioural characteristics or a combination of such traits, may be considered a 'hard' barrier to another (Rocha-Olivares et al. 2000, Luiz et al. 2012). Research within the Benguela region therefore provides a unique opportunity to increase the understanding of evolutionary processes in the marine environment across a wide range of species.

Cephalopods are of increasing importance as a resource within the Benguela region, however, cephalopod systematics is often regarded as substandard in comparison to that of other marine fauna (Roeleveld 1998). This is a major concern as a complete understanding of the systematics of any organism is fundamental to the effective management of fisheries and the conservation of biodiversity globally (Roeleveld 1998, Baron & Re 2002). However, systematics is only of value if rigorous, and if the accuracy is built on a foundation of comprehensive morphological and molecular research (Rosen & DeBach 1973). Comparative morphometric and genetic studies are thus critical in determining taxonomic units, distinguishing between species, as well as identifying populations and management units (Baron & Re 2002). The lack of extensive systematic resolution within the class Cephalopoda is often attributed to difficulties associated with the soft bodied nature of these marine invertebrates (Roeleveld 1998), and the relatively poor fossil record of the Cephalopoda (Strugnell et al. 2005).

Study species

Octopus vulgaris Cuvier, 1797 belongs to the family Octopodidae (Mollusca: Cephalopoda), which consists of approximately 23 genera of benthic octopus (Norman & Lu 2000). The species, also referred to as the common octopus, is a coastal cephalopod, inhabiting coral reefs and rocky environments of depths up to 200m (Katsanevakis & Verriopoulos 2004a). The abundance of the species has been shown to be negatively correlated with increasing depths (Quetglas et al. 2000), with the majority of individuals being associated with the continental shelf and most abundant in waters of up to 100m deep (Katsanevakis & Verriopoulos 2004b).

Although *O. vulgaris* is the most studied octopus species, its geographic distributional patterns are yet to be well defined (Warnke et al. 2004). Roper et al. (1984) described *O. vulgaris* as a cosmopolitan species with undefined distributional ranges. However, this global distribution pattern has been questioned by several authors, with some indicating that the true species may be restricted to the waters of the Mediterranean Sea and the Eastern Atlantic Ocean (Mangold & Hochberg 1991). In addition, Norman (2000) attributed the confusion in the species' range to the occurrence of a cryptic species complex, stipulating that multiple closely related, but isolated, tropical and temperate species had been classified as *O. vulgaris*. The restricted geographical range outlined by Mangold & Hochberg (1991) was later disputed by Warnke et al. (2004), who employed molecular data and demonstrated that *O. vulgaris* occurs in the Mediterranean Sea, the western and eastern Atlantic Ocean, as well as the north-western Pacific Ocean. This genetic-based geographical distribution was later extended to include specimens found in the Indian Ocean (Guerra et al. 2010).

Within the southern extremities of the African continent *O. vulgaris* has been documented to occur from Lüderitz, in southern Namibia, to Durban, on the east coast of South Africa (Oosthuizen & Smale 2003). Despite its commercial importance, research on *O. vulgaris* within southern Africa has predominantly focused on the biology and diet of the species and on specimens occurring in South African waters (Smale & Buchan 1981). There is, therefore, a severe lack of knowledge on the population sub-structuring and connectivity across the Benguela Current region for this species.

Octopus vulgaris has a semelparous life history strategy, in which all reproductive resources are allocated to a single spawning event (Rodríguez-Rúa et al. 2005). Male octopus die soon after mating and females once hatching has occurred (Hernández-García et al. 2002, Rodríguez-Rúa et al. 2005). During mating, the male transfers spermatophores into the mantle cavity of the female through the use of a hectocotylised arm (Mather 1998). Once within the oviducts, the spermatophores are stored within the oviducal glands for future fertilisation and spawning (Mather 1998). Female octopus lay a single egg mass containing approximately 42 000-790 000 eggs in its den (Oosthuizen & Smale 2003). However, female fecundity has been found to be relatively variable between geographically disjoint populations (Oosthuizen & Smale 2003). The eggs, which are laid in strings and attached to the substrate of the den, are cleaned and oxygenated by the brooding female during the incubation period (Hernández-García et al. 2002). Upon hatching, the female expels the planktonic hatchlings into the water column, where they remain for approximately 33-60 days depending on the ambient water temperature (Oosthuizen 2003, Pecl et al. 2004). This early life history stage is characterised by exponential growth for approximately 2-4 months, after which growth continues at a slower rate (Jackson 2004, Pecl et al. 2004, Semmens et al. 2004). While this pattern holds true for most populations, studies have reported high levels of intra-specific growth variability within the species (Smale & Buchan 1981, Pecl et al. 2004). Results from laboratory and field studies suggest that temperature, food availability and quality are the key environmental factors influencing growth in *O. vulgaris*, specifically during their early life history phase (Smale & Buchan 1981, Giménez & García 2002, Pecl et al. 2004, Semmens et al. 2004).

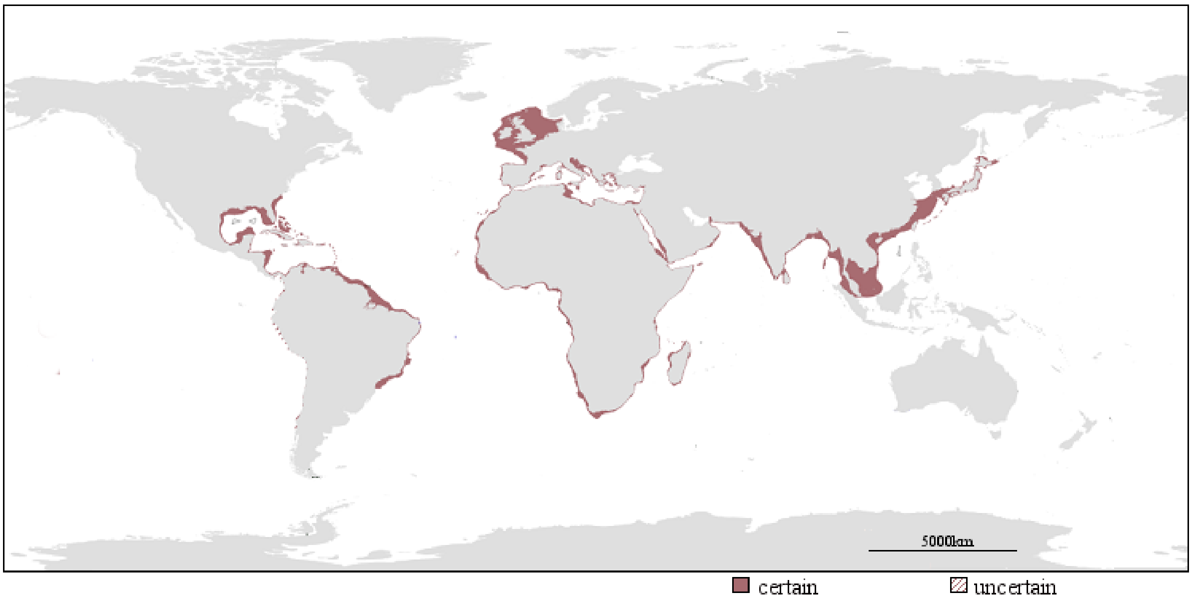

Figure 1.1: The worldwide geographical distribution of *Octopus vulgaris* as documented by the Food and Agricultural Organization of the United Nations (FAO 2014). Solid regions indicate areas in which the species is known to occur whilst the dashed lines are indicative of areas of distribution uncertainty.

The cosmopolitan distribution (Fig 1.1) of the species may be considered to be indicative of a wide temperature tolerance. Whilst literature on the temperature preference of the species is limited, Valavanis (2002) indicated that *O. vulgaris* occurs within waters between 10-30ºC. However, extreme increases in *O. vulgaris* abundance as a result of a shift in distribution, as was observed within the English Channel, have been attributed to a preference for warmer waters (Rees 1950). Furthermore, the duration of the embryonic and planktonic phase of *O. vulgaris* is considered to be highly dependent on sea surface temperature (SST), with the rate of embryonic development decreasing drastically with a substantial decline in SST (80-87 days at 17°C, 15-42 days at 27°C) (Mangold & Boletzky 1973, Sobrino et al. 2002). Nevertheless, it has been proposed that the species is able to implement an adaptive mechanism in order to regulate the embryonic growth and developmental timing in response to food availability (Boletzky 1994), thereby further emphasizing its adaptability.

Octopus vulgaris is classified as a generalist predator, as it feeds on a wide range of prey species, including molluscs, crustaceans and teleosts (Smale & Buchan 1981, Oosthuizen & Smale 2003, Smith 2003). In South Africa, *O. vulgaris* from False Bay feed primarily on the Cape rock crab (64.6% IRI), *Plagusia chabrus,* while the brown mussel, *Perna perna*, was the dominant prey item on the east coast (Smale & Buchan 1981, Smith 2003). Overall, crustaceans and molluscs were considered the two most important dietary components (Smale & Buchan 1981) in octopus from South Africa, while crustaceans and teleosts were dominant in the Mediterranean (Quetglas et al. 1998). Diet has also been found to be strongly associated with octopus size and developmental stage, habitat and depth (Smale & Buchan 1981, Ambrose 1984, Cortez et al. 1995, Oosthuizen & Smale 2003, Smith 2003), with several studies indicating that prey size and diversity increased with an increase in the development and size of *O. vulgaris* (Smith 2003). As such, smaller octopus are characterised by a more specialised diet, feeding primarily on amphipods, isopods and ichthyoplankton (Oosthuizen 2003, Smith 2003).

Commercial importance of octopus

As commercial catches of finfish decline, there has been an increase in the utilization of cephalopods over the past few decades (Rodhouse 2001, Agnew *et al.* 2005). For example, there was a 300% rise in the cephalopod catch in the English Channel between 1983 and 2003 and a concomitant 25% decline in finfish catch (Payne et al. 2006). Despite the relatively low contribution of octopus (~13%) to global cephalopod catches, the worldwide demand appears to be steadily increasing (Tsangridis et al. 2002, Pierce et al. 2010, Sauer et al. 2011). Octopus are caught using a broad range of fishing techniques and gears including seine and trammel nets, trawls, long lines and hooks, pots and traps, fishing gaffs and spears (Tsangridis et al. 2002). Large scale fisheries primarily targeting *O. vulgaris* are found to be predominantly distributed along the northwest coast of Africa, in the Mediterranean Sea and around the coast of Japan (Quetglas et al. 1998, Oosthuizen 2003). However, a considerable amount of small-scale fishing for this species also occurs around the globe (Oosthuizen 2003). The high export potential to Asian and European markets, as has been documented by the Mauritanian octopus fishery (Kazmierczak et al. 1997), coupled with the high percentage of edible proportion (80-85% of total weight) in comparison to teleosts (40-75%) (Vaz-Pires et al. 2004) allows for *Octopus* species to be considered a potentially valuable, untapped fisheries resource in southern Africa.

Until recently, the exploitation of *O. vulgaris* consisted mainly of subsistence and recreational fisheries in South Africa (Oosthuizen & Smale 2003, Sauer et al. 2011). However, the Department of Agriculture, Forestry and Fisheries has implemented an experimental octopus pot fishery with the intention of developing a commercial octopus fishery within the near future (Oosthuizen 2003). In the northern Benguela sub-system, the species appears to primarily be harvested by subsistence and recreational fishers.

Project Aim

In the context of a larger project, which aims to understand the influence of the formation of the Benguela on the evolution of warm-temperate marine species, the broad temperature tolerance, extensive larval dispersal capability, short lifespan, rapid population turnover, high growth rates and adaptive life history characteristics of *O. vulgaris* make this species an important candidate for comparisons with other marine organisms.

The high dispersal ability, during the early life history phases, and “broad” temperature tolerance of the species may promote panmixia in the region. However, if these characteristics are not able to overcome the Benguela cold water barrier, as has been observed for a number of other species, the rapid life history of the octopus may provide it with an improved ability to adapt to historical and contemporary environmental change when compared with longer-lived species.

The aims of this study were:

1) to determine whether the oceanographic features of the Benguela Current have influenced the population connectivity of *O. vulgaris* across the region,
2) to identify the historical mechanisms which may have driven the observed patterns,
3) to contribute to the knowledge of the systematic relationships across the genus *Octopus*,
4) To understand whether the observed genetic divergence is accompanied by morphological differentiation.

In order to achieve this, comparative genetic and morphological analyses were conducted to evaluate population connectivity and sub-structuring, and to determine the evolutionary history of *O. vulgaris* in the Benguela region.

Thesis Outline

The present thesis is divided into five chapters:

Chapter 1- General Introduction: The General Introduction defines the scope of the project, provides background information on the study species and outlines the overall aim of the study.

Chapter 2- General Methodology: This chapter outlines the general methods and materials, provides an overview of the major oceanographic features of the Benguela Current and a brief history of the study area.

Chapter 3- Population Genetic Substructure & Evolutionary History: This chapter assesses the population structure, evolutionary history and phylogenetic relationship of *O. vulgaris* in the Benguela Current region, based on mtDNA markers.

Chapter 4 – Morphological Comparison: Chapter four investigates the morphological variability of *O. vulgaris* in the Benguela Current region, using morphometric and meristic characters.

Chapter 5- General Discussion: This chapter discusses the findings of the two data orientated chapters in relation to the theoretical body of knowledge, with consideration of their implications for fisheries management and conservation, at both a regional and global scale. Limitations of the study and the areas of potential future research are discussed.

CHAPTER 2

General Methods and Materials

Sample collection, fixation and preservation

In order to gain a comprehensive understanding of the effects of the oceanographic features of the Benguela Current on population connectivity, genetic diversity and evolutionary history of *Octopus vulgaris*, a total of 168 specimens were collected across the Benguela region from Namibe (15º 11'S, 12º 08'E) in southern Angola, to Hamburg (33º 17'S, 27º 28'E), in the Eastern Cape, South Africa (Figure 2.1, Table 2.1). Samples were obtained during spring low tide, primarily in the nearshore intertidal zone, between May 2012 and March 2013. In regions which proved difficult to sample, specimens were obtained from local subsistence fishermen and through the collaboration with local research institutes. Specimens were collected using an octopus gaff which consisted of a 1m aluminium or wooden rod, with an 8/0 hook and a pink or red plastic skirt fitted on the end.

Due to the cognitive abilities and intelligence associated with these marine invertebrates, ethical consideration with regards to euthanasia is vital (Mather & Anderson 2007, Moltschaniwskyj et al. 2007). Over-anaesthesia is a common method of euthanasia for *O. vulgaris* and is of preference in morphological studies due to the importance of anatomical characteristics in this field. Octopus were therefore euthanized in a 5% EtOH-seawater solution, prior to fixation (Oosthuizen 2003).

Effective fixation and tissue preservation guidelines are fundamental in studies relating to the identification, biological and anatomical attributes of a species (Roper & Sweeney 1983). The fixation and preservation of fresh octopus is a common problem in morphological analyses due to the soft bodied nature and lack of a skeleton of specimens of the Order Octopoda (Roper & Sweeney 1983). Consistency in preservation and fixation is therefore often regarded as a major impediment for systematic comparison studies on cephalopods (Roper & Sweeney 1983). Since different preservation and fixation methods have been found to influence morphological measurements (Andriguetto & Haimovici 1988), consistent, appropriate methods are a prerequisite (Roper & Sweeney 1983, O'Shea 1997).

Besides selecting preservation and fixation methods which do not influence the morphological measurements, the primary aim of fixation is to prevent post-mortem decay and autolysis of specimen tissue, and since post-mortem decay of octopus occurs relatively quickly (Roper & Sweeney 1983), timely fixation is critical in order to maintain the integrity of the anatomical structure of the specimen (Roper & Sweeney 1983). Three primary cephalopod fixatives have been identified: formalin, ethanol and freezing (Roper & Sweeney 1983). Although formalin is the most favoured fixative in cephalopod studies (Roper & Sweeney 1983), this method has several major drawbacks such as: i) the adverse health effects associated with formaldehyde, as formalin is regarded as a toxic, carcinogenic and allergenic chemical; ii) the tendency to result in the loss of colour, decalcification and degradation of specimens as a consequence of the formation of formic acid, which arises due to the oxidation of formaldehyde when exposed to atmospheric oxygen and/or amino acids and proteins; and iii) as with alternative chemical fixatives, fixation using formalin has several logistical issues, as this process requires not only large containers to ensure that specimens can be appropriately laid out, with the arms fully extended, but also a significant amount of time, as tentacles must be fixed in phases to prevent coiling. In the process, the tentacles are partially submerged in the fixative, removed and straightened and then further submerged until the entire length of the arm is considered sufficiently fixed (Roper & Sweeney 1983).

The use of ethanol as a cephalopod fixative is not well supported as: i) it is often regarded as a desiccating chemical, resulting in the dehydration of tissues thereby leading to high levels of fixation-induced anatomical deformation; ii) tissue penetration appears to be delayed, specifically in larger specimens, and as such internal degradation is therefore common, despite the fixation of external anatomical characteristics (Roper & Sweeney 1983, Voight 2001).

Finally, freezing is often considered a favourable fixation method due to the convenience and efficiency of this technique (Roper & Sweeney 1983). However, freezing should not be regarded as a preservation technique and additional methods should be sought to preserve the specimens for long-term storage.

In the present study, due to the problems associated with chemical fixing, due to the dependence on external sources for specimen collection and the difficulties associated with the transportation of chemicals in Angola, freezing was selected for the fixation of the specimens. For this, all individuals were frozen in labelled air-tight polyethylene bags directly after collection. Specimens remained frozen at -20°C for approximately three months prior to laboratory analyses. Further long-term preservation techniques were however not required as specimens were discarded post measurement and dissection.

Samples for genetic analyses were obtained prior to fixation from tentacle clippings of approximately 1.5cm, from the first left arm, and stored in 95% EtOH. Additional *O. vulgaris* tissue samples were obtained from existing genetic collections in order to gain a comprehensive understanding of the phylogeography and phylogenetic relationships within the study species (Table 2.1). Furthermore, in order to further investigate phylogenetic relations within the genus, *O. tetricus* samples from north-eastern Tasmania, Australia, and available sequences of different *Octopus* species were obtained.

Table 2.1: Sampling locations, locality codes and sample frequencies (*n*) for *O. vulgaris* collected for the comparative genetic and morphometric study.

Country	Sampling locality	Locality code	Whole samples (n)	Genetic Samples (n)	Genetic samples (n): existing collections	GPS coordinates
Angola	Namibe	NBE	18	13	-	15° 11'S, 12° 08'E
	Baía dos Tigres	BdT	61	20	-	16° 36'S, 11° 44'E
South Africa	Western Cape	WC	34	20	-	-
	Port Alfred	EC	29	7	-	33° 36'S, 26° 53'E
	Kleinemonde	EC	11	10	-	33° 33'S, 27° 1'E
	Hamburg	EC	13	6	-	33° 17'S, 27° 28'E
	Durban	DBN	-	-	8	-
Senegal	-	SEN	-	-	6	-
-	Mediterranean Sea	MED	-	-	3	-
Portugal	Oporto	POR	-	-	1	-

Description of the study site

Major oceanographic features of the Benguela Current upwelling system

The Benguela Current is one of the four major eastern boundary coastal upwelling regimes of the world (Shannon & O'Toole 2003, Shillington et al. 2006, Hutchings et al. 2009). This upwelling current system is located off the south-western coast of the African continent between 5-37ºS, 0-26ºE and extends across the coast of three countries: southern Angola, Namibia and the west coast of South Africa (Lass et al. 2000, Shillington et al. 2006, Hutchings et al. 2009). It comprises a cold, broad northward flowing oceanic current of the South Atlantic subtropical gyre (Fennel 1999) and is thought to originate near the Cape of Good Hope. The Benguela Current system is influenced primarily by waters from the South Atlantic Current and to a lesser extent by waters from the Agulhas Current and Antarctic Circumpolar Current (Berger et al. 1998, Shannon & O'Toole 2003). The Benguela Current is unique from the other eastern boundary currents as it is bounded, both in the north and the south, by warm equatorial waters from the Atlantic and Indian Oceans (Shannon & O'Toole 2003, Shillington et al. 2006, Hutchings et al. 2009). It comprises an offshore and inshore component (Figure 2.1). The inshore component, often referred to as the Benguela Coastal Current (BCC) is characterised by a wind-driven coastal upwelling regime, the periodicity and intensity of which is highly influenced by local climatic conditions (Little et al. 1997, Griffiths et al. 2010). The BCC exhibits substantial mean monthly sea surface temperature variability, ranging between 10°C to 18°C, in comparison to its offshore counterpart (15.4°C to 20.1°C)(Griffiths et al. 2010). The offshore component, known as the Benguela Oceanic Current (BOC) is a wide, slow, equatorward flow which diverges from the coast forming the eastern portion of the South Atlantic Subtropical Gyre (Little et al. 1997, Griffiths et al. 2010). The upwelling of cold, low-oxygen, nutrient rich South Atlantic Central waters (SACW) to the surface of the Benguela system is driven primarily by the local southerly and south-easterly trade winds which allow for the offshore transportation of warmer, nutrient poor surface waters (Summerhayes et al. 1995, Hutchings et al. 2009).

The characteristics of the Benguela Current upwelling system, as with the Humboldt, Canary and California currents, promote high levels of biological productivity (maxima ~3g C m^{-2} day^{-1}) (Abrams & Griffiths 1981, Carr & Kearns 2003). Areas with high levels of primary productivity, like the Benguela region, are generally characterised by low biodiversity when compared to the warmer, western boundary counterparts (Sakko 1998, Cury et al. 2000). Reduced levels of species richness, within systems such as these, can be attributed to the high levels of physical stress, as a result of environmental perturbations, within upwelling ecosystems (Baird et al. 1991, Sakko 1998, Moloney et al. 2005).

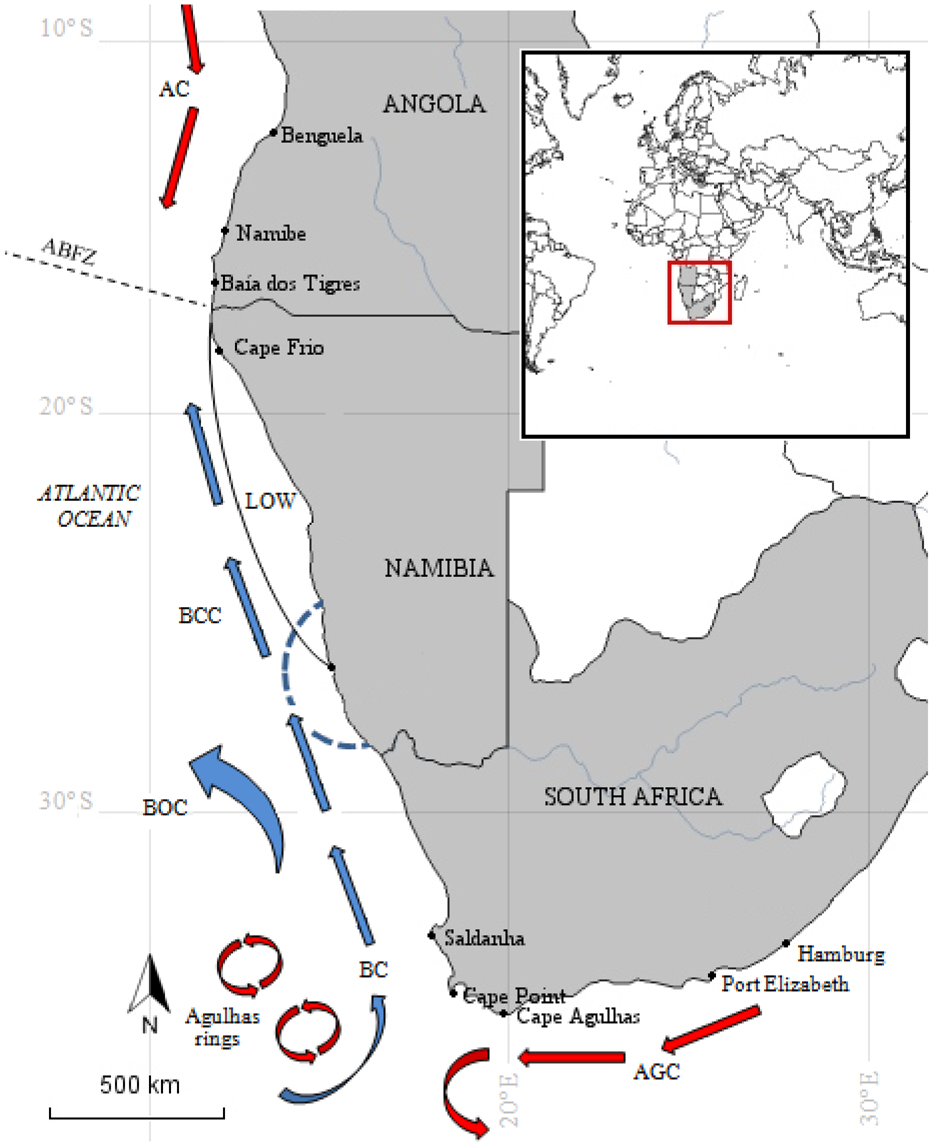

Figure 2.1: Map illustrating the major oceanographic features of the Benguela upwelling system. AC = Angola Current, AGC= Agulhas Current, ABFZ = Angola-Benguela frontal zone BC = Benguela Current, BCC = Benguela Coastal Current, BOC = Benguela Oceanic Current, LOW = Low Oxygen Water (adapted from Shannon et al. (2006)).

In the north, the Benguela upwelling regime converges with the warm, saline, southward flowing waters of the Angola Current forming the Angola-Benguela Frontal Zone (ABFZ) (Ekau et al. 2001, John et al. 2004). This dynamic frontal zone, which corresponds to invariably steep sea surface temperature (SST) gradients, is considered to be a permanent feature within this region, with the midpoint of the front lying between 14ºS and 16ºS (John et al. 2001, Veitch et al. 2006, Lass et al. 2000). The central point of this thermal frontal system exhibits seasonal latitudinal shifts, with a northward movement being observed during winter and early spring, whilst southerly transitions are brought about by the intrusions of warm, saline Angolan waters (Lass et al. 2000, Shannon & O'Toole 2003). Despite the relative stability in the positioning of the centre of this oceanographic feature, as a result of the orientation of the coastline coupled with the regions bathymetry and wind stress, the ABFZ is known to demonstrate high levels of both seasonal and interannual variability (Bianchi 1992, Veitch 2002). Such variability is seen to result in physical and chemical fluctuations in the offshore extension of the frontal region and shifts in the width and intensity of the zone (Hutchings et al. 2009, Veitch et al. 2006). Although primarily a surface and subsurface feature (~50m), the front has been found to extend up to depths of 200m (Lass et al. 2000, Veitch 2002). The ABFZ has been documented to extend as far as 250km offshore in a south-westerly direction off the coast during warmer summer months, with this extension increasing drastically during cooler winter periods (Ekau et al. 2001, Veitch 2002). The movement offshore results in the widening of the frontal zone and a consequent reduction in thermal gradient intensity (Veitch 2002). Intense thermal gradients are therefore predominantly associated with the narrower regions of the front and are largely considered to be characteristic of coastal waters of the region (Veitch 2002). Drastic Angola Current water intrusions, referred to as Benguela Niños, have been documented approximately once every 10 years (Lass et al. 2000). The advection of warm, nutrient poor equatorial waters onto the Namibian shelf has been shown to reduce biological productivity (Duncombe Rae 2005, Hutchings et al. 2009), but may also provide conduits for connectivity of warm temperate species on either side of the cold water Benguela barrier.

Three primary semi-permanent upwelling centres, Cape Frio, Lüderitz and Cape Point have been identified in the Benguela (Baird et al. 1991, Duncombe Rae 2005), and seem to exhibit various levels of spatio-temporal variability. The position of these cells appears to be influenced by the width of the inner continental shelf, with the majority of these upwelling centres at narrow zones, such as those found south of Lüderitz and off the Cape Peninsula (Lutjeharms et al. 1991, Hutchings et al. 2009). The Lüderitz cell is considered to be the most pronounced due to the persistent perennial nature and intensity of its upwelling regime (Lutjeharms et al. 1991, Dingle et al. 1996, Duncombe Rae 2005, Lett et al. 2007). It is driven by persistently strong winds, elevated turbulence and high levels of offshore advection. The extreme conditions of the Lüderitz upwelling cell, and consequently the high levels of mixing within the water column, are considered to have negative impacts for retention, concentration and enrichment, subsequently leading to decreased levels of primary and secondary production within the region (Lett et al. 2007, Hutchings et al. 2009). The permanent Lüderitz upwelling cell separates the Benguela Current into two substantial dissimilar oceanographic and biological sub-systems (West et al. 2004, Duncombe Rae 2005, Lett et al. 2007): the northern (NB) and southern Benguela (SB) sub-systems (Baird et al. 1991, Ekau & Verheye 2005, Lett et al. 2007, Hutchings et al. 2009).

The position of the NB and SB boundaries are highly variable and shift in position and intensity throughout the year (Veitch 2002). Such changes are thought to be related to the upwelling intensity and the strength of the warmer currents situated on either side of the system (Veitch et al. 2006). The NB, which lies north of the main upwelling cells off the Namibian coastline, extends from the upper boundary of the Lüderitz upwelling zone to central Angola (Figure 2.1) (Hutchings et al. 2009). The NB is influenced by three main oceanographic features: i) the ABFZ, which exhibits a latitudinal displacement southward during the austral summer and drives the NB boundary southward to approximately 18ºS, ii) the presence of low oxygen waters (LOW) off the continental shelf of northern Namibia as a consequence of multiple interlinking biological and hydrographical processes, which have been documented to extend to depths of up to 100-150m and with conditions persisting for approximately 90km offshore, iii) and lastly the occurrence of upwelling cells on the southern extremities, in particular a perennial upwelling event which weakens during late summer (Sakko 1998, West et al. 2004, Hutchings et al. 2009).

The SB extends from central Namibia to Port Elizabeth, which is situated on the south coast of South Africa (Figure 2.1); this includes the southern region of the Agulhas Current, which extends from Port Elizabeth south towards the Cape Agulhas-Cape Point transitional zone. The Agulhas Bank (Figure 2.1) is therefore considered to form the southern boundary of the Benguela system (Hutchings et al. 2009; Ruijter et al. 1999). The widening of the continental shelf and the Agulhas Bank begins approximately in Algoa Bay (Figure 2.1), which extends from the Bird Island Group in the north and Cape Recife in the south. The dynamic nature of the convergence of the characteristics of the Agulhas on the northeast and the nature of the Agulhas Bank on the southwest of Algoa Bay allows for this region to be considered a major transitional zone (Teske et al. 2006, Goschen & Schumann 2011).

The SB is highly influenced by the Agulhas current as warm, saline waters detach periodically within the region of the Agulhas retroflection, forming what is known as warm core Agulhas rings (Ruijter et al. 1999, Hutchings et al. 2009). These shear edge features not only play a vital role in global thermohaline circulation patterns but are also critical to inter-ocean exchange between the Indian and South Atlantic subtropical gyres (Hutchings et al. 2009, Beal et al. 2011). Extending to depths of up to 4km, with diameters of approximately 500km, these anti-cyclonic features have substantial local and global environmental impacts (Ruijter et al. 1999). The SB system follows a pulsing upwelling regime, which is fundamental in regulating biological productivity within the surface layers (West et al. 2004). Upwelling intensity within the SB increases during the austral spring and summer (West et al. 2004). LOW events in the SB, which are caused by the decay of biological matter, are not as definitive as those found along the northern coasts of Namibia and depletion within this region is rare (Shannon & O'Toole 2003, Hutchings et al. 2009).

In summary, the major oceanographic features of the Benguela system divide the region into three major regions: i) the NB subsystem; ii) the Lüderitz upwelling area, and finally iii) the SB subsystem. For the purpose of this study, greater resolution was required to understand the population sub-structuring and connectivity across the Benguela Current system. Therefore, these three zones can be further subdivided, according to differential oceanographic processes and the presence of potential biogeographical barriers, to form six main sampling regions:

i) the upper NB, which extends north of Tômbua until Namibe. This region exhibits a perennial upwelling regime, which intensifies during the winter months (Hutchings et al. 2009). The region is characterised by coarse substrates interspersed with rocky outcrops and coral reefs (Bianchi 1992). Difficulties with sampling due to accessibility limitations within the region lead to specimens being obtained from local fish markets only in Namibe (NBE).

ii) the central region of the NB, ranging from Tômbua to the Cunene River mouth, is associated with the presence of the ABFZ. This region therefore exhibits substantially lower bottom temperatures, in comparison to further north (Bianchi 1992). Finer silts and clays are found to dominate the lower reaches of this region, near Baía dos Tigres; however this substrate type is seen to be replaced by coarser sand extending up to Tômbua (Bianchi 1992). Baía dos Tigres (BdT) a shallow, coastal embayment (Simmons et al. 2006), was the primary sampling locality within this region (Figure

2.1). Specimens were collected during a single sampling trip in July of 2012.

iii) the lower NB, ranging from the Cunene River mouth to the northern boundaries of the Lüderitz upwelling cell are characterised by LOW and perennial upwelling (Kristmannsson 1999, Ekau & Verheye 2005). Due to financial, logistical and time constraints, it was not possible to obtain samples from this region.

iv) the cool, temperate West Coast, or western reaches of the SB, ranging from the southern boundary of Lüderitz to Cape Point is characterised by strong, persistent upwelling which peaks during late summer/autumn; however this region is seen to be highly influenced by oceanographic features of the Agulhas Current, such as the formation of anti-cyclonic rings (Hutchings et al. 2009). LOW events have been documented to occur within this region (Hutchings et al. 2009). Due to time and financial restrictions sampling within this region was limited and yielded no specimens. Local fishermen and fishmongers were approached, however due to the delay in the implementation of the experimental octopus fishery, specimens from this particular region were unavailable during the course of this study.

v) the warm, temperate Western Cape (WC), extending from Cape Point to Port Elizabeth, situated in the Eastern Cape, is seen to encompass the broad Agulhas bank, of which the inner shelf is characterised by a strengthened, summer upwelling regime (Hutchings et al. 2009). Colder waters are found to dominate the eastern extremities of this region due to the upwelling of cool, nutrient rich waters associated with the offshore divergence of the Agulhas Current (Lutjeharms et al. 2000, Hutchings et al. 2009). Extensive sampling was carried out on the inshore region; however, as the frequency of octopus collected was relatively low, and due to time restrictions, specimens within this region were primarily obtained through the collaboration with the South African Department of Agriculture, Forestry and Fisheries (DAFF).

vi) north of Port Elizabeth, the Eastern Cape region (EC), is seen to be dominated by the warm waters of the Agulhas Current (Teske et al. 2006). Specimens were collected from three localities: Port Alfred, Kleinemonde and Hamburg, and additional genetic samples were obtained from fishermen within the region.

Changing State of the Benguela

The Benguela Current is regarded as one of the oldest upwelling systems of the world, with geochemical and sedimentological studies suggesting that the establishment of persistent upwelling along the south-western coast of Africa, occurred during the early-late Miocene, approximately 12 Mya (Siesser 1980, Krammer et al. 2006). This epoch was characterised by prominent changes in the global climate system such as an increase in the amplitude of Antarctic ice volume, a shifting in boundary conditions due to the onset of the uplifting of the Isthmus of Panama, as well as a decline in sea surface and bottom water temperatures (Diester-Haass et al. 2004, Krammer et al. 2006, Kastanja et al. 2006). Such drastic changes had major implications on global atmospheric and oceanic circulatory patterns (Flower & Kennett 1994, Krammer et al. 2006). In particular, the substantial accumulation of East Antarctic ice sheets during the middle Miocene, from ~ 14.8 to 12.9 Mya, subsequently led to the onset of accelerated polar and deep water cooling, resulting in the strengthening of oceanic surface circulatory patterns and influencing the commencement of upwelling events within the south-eastern Atlantic (Flower & Kennett 1994, Zachos et al. 2001). Sea levels, during the establishment of the current, reached 250m higher than current levels (Siesser 1980). A considerable change in the positioning and intensity of the system was documented since the establishment of the Benguela Current (Berger & Richter 1998). During the late Miocene and Pliocene, the Benguela upwelling signal extended north of the Walvis Ridge; however, this was only seen to occur during glacial phases (Diester-Haass 1988). Sedimentology, micropaleontology and organic geochemistry studies indicated a significant strengthening of the upwelling intensity within the region ~10 Ma and this is thought to have coincided with an increase in local productivity (Siesser 1980, Krammer et al. 2006).

Significant spatial and temporal changes in productivity have been noted since the establishment of the south-western upwelling system in the Benguela region (Krammer et al. 2006). Drastic declines in productivity were noted between 12 – 9 Mya (Robert et al. 2005, Krammer et al. 2006). Decreases in productivity within the region, as well as in the equatorial waters of the Indian and Pacific Oceans, were thought to be a consequence of the late Miocene ‘Carbonate Crash’ (Diester-Haass 1988, Robert et al. 2005, Kastanja et al. 2006). The term ‘Carbonate Crash’ is used to describe a rapid decline in the rate of carbonate mass accumulation, however a number of hypotheses have been formulated in attempt to account for this phenomenon (Diester-Haass et al. 2004, Robert et al. 2005). Such hypotheses include the potential of a biogenic bloom, increased levels of dissolution linked to differential deepwater circulatory patterns or the substantial shoaling of the lysocline and calcite compensation depths in these regions (Diester-Haass et al. 2004, Krammer et al. 2006). However, the rapid declines in calcium carbonate ($CaCO_3$) in the Benguela region between 9.6-9.0 Mya, have been associated with substantial increases in smectite (Robert et al. 2005, Krammer et al. 2006). Smectite is considered to be a good indicator of the terrigenous input from the Orange River system (Robert et al. 2005). High levels of smectite in the south-eastern Atlantic may have potentially led to the decline in oxygen concentration in the waters of the Benguela, subsequently resulting in decreased levels of productivity (Diester-Haass et al. 2004, Robert et al. 2005). Primary productivity and consequently marine biological productivity as a whole, began to increase substantially around 7 Mya (Diester-Haass et al. 2004). This major paleoceanographic event, classified as a ‘Biogenic Bloom’, is thought to be attributed to the enhanced levels of paleoproductivity around the globe (Diester-Haass et al. 2004).

The late Miocene-Early Pliocene, approximately 6 Mya, was characterised by a continuation of the global cooling trend, with an increase in the volume of the Arctic and west-Antarctica ice sheets (Zachos et al. 2001). Major marine regressions during this period were attributed to the glaciation of the Antarctic (Siesser 1980, Diester-Haass 1988). The decline in sea level was later further amplified during the Pliocene-Pleistocene transition period ~3-2 Mya, resulting in sea levels 300m below modern elevations (Siesser 1980). The transition from the warm Pliocene to the cold Pleistocene, commenced with the closure of the Central American Seaway (CAS) ~3 Mya, resulting in extensive changes in the global climatic system (Marlow et al. 2000, Zachos et al. 2001, Filippelli & Flores 2009). The closure of the CAS led to the strengthening of North Atlantic Deep Water Production (NADW) and to the intensification of the Atlantic meridional overturning circulatory pattern (Marlow et al. 2000). The onset of the intensified global cooling trend was seen to occur approximately 3.2 Mya, accompanied by substantial glaciation in the Northern Hemisphere (NHG) (Marlow et al. 2000, Zachos et al. 2001). This cooling, coupled with the effects of the closure of the CAS, are thought to have driven the strengthening of upwelling events along the south-western coast of Africa and resulted in a decline in temperature and an increase in productivity in the region, between 2.1 and 1.9 Mya (Berger et al. 1998, Marlow et al. 2000). In addition, the strengthening of the ocean currents consequently led to the northward shift of the Antarctic frontal system and the

Southern Atlantic Subtropical Convergence Zone (Marlow et al. 2000, Jahn et al. 2003) and the enhancement of the upwelling intensity in the region (Jahn et al. 2003).

The contemporary Benguela is therefore thought to have formed ~2 Mya, due to the strengthening of the oceanic characteristics of the region (Krammer et al. 2006, Etourneau et al. 2009). Looking back from the present conditions, the Plio-Pleiostocene can be characterised as a geological period in which major variability in upwelling intensity, productivity and nutrient cycling occurred in the Benguela region (Filippelli & Flores 2009). This geological period can be subdivided into five successive stages. The mid-Pliocene, 4.6-3.2 Mya, is the first of the major Plio-Pleistocene phases (Marlow et al. 2000). During this period the Benguela region was warmer with sea surface temperatures (SST) averaging 26ºC (Marlow et al. 2000). However there were three major cooling episodes (4.1, 3.7 and 3.2 Mya) during this geological interval (Marlow et al. 2000, Filippelli & Flores 2009). Subsequent to the warm mid-Pliocene phase between 3.2 and 2.1 Mya, the region underwent a moderate cooling phase with the onset of a glacial period (Marlow et al. 2000). The rapid decline in SST (2-3ºC, over 200 thousand years (Kya)) resulted in the onset of the third stage, the early Pleistocene (2 -1.4 Mya) (Marlow et al. 2000, Krammer et al. 2006). The fourth stage, the mid Pleistocene, ranging from 1.3-0.6 Mya, included a crash is SSTs, associated with the effects of an intensified glacial-stage cooling, with intensified cooling periods occurring through both the glacial and interglacial phases between 0.8 and 0.6 Mya (Marlow et al. 2000, Jahn et al. 2003). Between 0.9 and 0.4 Mya the Benguela system was considered relatively unstable due to the establishment of the mid-Pleistocene Revolution (Jahn et al. 2003), after which the signal of the Quaternary 100 Ky Glacial-Interglacial (G-IG) cycles became fully established at approximately 0.5-0.1 Mya (Marlow et al. 2000, Jahn et al. 2003).

Over the past 150 Ky, upwelling intensity and productivity were at a maximum between 25 and 60 thousand years ago (Kya), during the Marine Isotope Stage (MIS) 3 (Summerhayes et al. 1995, Kirst et al. 1999, West et al. 2004). This period of increased upwelling has been attributed to the strengthening of upwelling-favouring winds as well as a decrease in the intensity, and therefore intrusion, of the warm Angola Current (Kirst et al. 1999). During this stage, sea level was reported to have been 50m below present (Summerhayes et al. 1995). However, sea levels further regressed, reaching its lowest during the Last Glacial Maximum (26.5 – 19 Kya), during the initial stages of MIS 2 (Summerhayes et al. 1995, Clark et al. 2009,). The low sea levels resulted in the exposure of the continental shelf in a number of areas; however this was most extensive south of Cape Agulhas (Dingle & Rogers 1972)). Sea surface temperatures during this geological period were 4-5ºC lower than those of the early Holocene, which was seen to be characterised by a rise in sea levels due to a substantial increase in ice-sheet melting (Farmer et al. 2005). Significant cooling, of 2-3ºC was also documented to have occurred during the brief geological period classified as the Younger Dryas, as well as during the Little Ice Age and the mid-Holocene, due to enhanced levels of upwelling within the Benguela region (Farmer et al. 2005). Sea levels have been documented to have undergone drastic fluctuations during the Holocene, with sea level at the Pleistocene-Holocene transition being characterised by post glacial eustatic rise. Further sea level fluctuation occurred between ~6 and 1.8 Ka, after which sea levels reached contemporary heights. During this period of environmental variability, sea levels reached a maximum level of approximately 2-3m above contemporary mean sea levels ~6 Kya and a minimum ~3.5-2.8 Kya; such fluctuations are likely to have resulted in considerable levels of local coastal remodelling (Miller et al. 1995).

Extreme fluctuations in oceanic circulatory patterns, SST and sea levels within the Benguela Current Region, during the Plio-Pleistocene resulted in major environmental variation (Table 2.2) and subsequently played a major role in shaping population structure, demographic history and phylogenetic relationships of species distributed throughout the system.

Table 2.2: Summary of the major climatic events within the Benguela Current region.

Epoch	Date (Mya)	Feature	Consequence
Mid-Miocene	~14.8 -12.9	Accelerated polar and deep water cooling	Strengthening of oceanic circulatory patterns, commencement of upwelling of SE Atlantic
Miocene	~ 12 – 9	Miocene Carbonate Crash	Decrease in productivity
Late - Miocene	~ 7	Biogenic Bloom	Increase in 1° and marine biological productivity
Late Miocene - Early Pliocene	~ 6	Increase of Arctic & W-Antarctica ice sheet volume	Global cooling, major marine regressions
Pliocene - Pleistocene transition	~ 3	Closure of the America Seaway	Strengthening of NADW production & intensification of Atlantic meridional overturning
Pleistocene	~ 2.1 – 1.9	Formation of contemporary Benguela	Decrease in SST, increase in productivity, shift in Antarctic frontal system & Southern Atlantic Subtropical Convergence Zone
Mid - Pleistocene	~ 1.3 – 0.6	Intensified glacial-stage cooling	SST crash
Mid - Pleistocene	~ 0.9 – 0.4	Establishment of mid-Pleistocene Revolution	Benguela enters unstable phase
Late - Pleistocene	~ 0.5 – 0.1	Establishment of Quaternary 100Ky G-IG cycle	Climatic and oceanic oscillations

CHAPTER 3

Population structure, evolutionary history and phylogenetic relationship of *Octopus vulgaris* across the Benguela Current system

Introduction

The inability to successfully identify and delineate species, stocks and populations can have major implications for current and future fisheries management, sustainability and global biodiversity conservation (Coyle 1997, Carvalho & Hauser 1998, Ward 2000). However, the invention of the polymerase chain reaction (PCR) based molecular approach has played a major role in the development of the field of molecular systematics (Park & Moran 1994, Ward & Grewe 1994, Ward 2000). This biochemical technology utilises a three step thermal cycling protocol allowing for DNA sequences to be obtained rapidly and effectively from small, preserved tissue samples (Schochetman et al. 1988). The increased reliance on the use of molecular applications in fisheries research, which can be demonstrated by the substantial gain in genetic related citations (e.g. Gharrett et al. 2004), is indicative of the efficiency and advantages of molecular techniques within the fisheries field. The development of such molecular approaches allows for effective species delimitation, as well as a comprehensive understanding of population structure and evolutionary history, all of which are critical in the understanding of the ecology of a species (Ward 2000, Wiens 2007, Weigand et al. 2011, Moreira et al. 2011).

Until recently, the majority of current species delimitations have been based on the presence of morphological discontinuities between organisms (Sokal & Crovello 1970, Wiens & Penkrot 2002). The development of modern molecular systematic techniques, coupled with an increase in interest within this field, has led to the strengthening of these traditional taxonomic hypotheses of species delimitation (Hebert & Gregory 2005, Vogler & Monaghan 2007). However, this new complementary approach has highlighted the existence of taxonomic uncertainty within traditionally recognised species, specifically with the onset of molecular based taxonomic methods such as DNA barcoding (Harrison 1991, Féral 2002, Hebert & Gregory 2005, Hoolihan et al. 2006). Instances of ambiguity are often attributed to factors such as a lack of diagnostic characters, the presence of phenotypic plasticity, morphological stasis and the existence of cryptic lineages (Hebert & Gregory 2005, Bickford et al. 2006, Beebee & Rowe 2008, Weigand et al. 2011). Such cases have been demonstrated in a number of studies in which one historically recognised species contained multiple species with deep genetic divergence, but no evident morphological differentiation (Lee & Frost 2002, Santos et al. 2006, Lavoué et al. 2011). The use of molecular techniques to reveal complex patterns, at both the population and species levels, are therefore critical in the development of conservation and management strategies, especially in commercially exploited marine species.

The importance of the application of molecular markers in fisheries research has been acknowledged for an extended period of time (O'Connell & Wright 1997). Genetic markers have primarily been applied to three aspects of fisheries related research: i) stock identification; ii) taxonomy and systematics, and iii) aquaculture (Ferguson & Danzmann 1998). Fisheries management and conservation related genetic studies are primarily focused on identifying and separating stocks as well as investigating population structure in more conventional finfish fisheries (O'Connell & Wright 1997). In order to effectively understand the molecular structuring of a species, one therefore needs to be familiar with the concept of a "stock", a term which is currently characterised by a number of definitions. Central to these definitions are two primary principles: firstly, species are subdivided into localized populations, and secondly, genetic differentiation may occur between local populations which are adaptive (Coyle 1997, Çiftci & Okumus 2002). Furthermore, genetic stocks can be defined as reproductively isolated units which exhibit substantial levels of genetic differentiation resulting in significant differences in allele frequencies among populations or stocks (Coyle 1997). Such differences arise as a consequence of a lack of inter-stock migration and consequently a hindrance of gene flow. However, it is important to note that signals of molecular differentiation may be undermined by the migration of a small proportion of individuals between sub-populations or the recent separation of populations/stocks which would consequently not allow for the accumulation of significant genetic divergence due to the limited period of separation (Ward & Grewe 1994). Marine fish stocks have been documented to exhibit substantially higher levels of migrations per generation (of up to two orders of magnitude more migrants per generation) than their freshwater counterparts (Ward et al. 1994) resulting in relatively low levels of genetic divergence within the marine realm (mean F_{ST} for allozymes is 0.062 for marine fish in comparison to F_{ST} = 0.222 for freshwater fish) (Hauser & Ward 1998). It has, thus, been stipulated that in order for stocks to be effectively determined, an exchange of up to 10% of migrants between populations could potentially provide reasonable justification for the identification of discreet stocks (Hauser & Ward 1998).

The accurate delimitation of genetic stocks is critical for management and conservation at taxon levels below that of the species (Coyle 1997). A wide range of molecular markers such as allozymes, randomly amplified polymorphic DNA (RAPDs), amplification fragment length polymorphism (AFLPs), DNA sequencing (e.g. mitochondrial DNA) and variable number of tandem repeat loci (VNTRs, e.g. microsatellites) are currently used to detect genetic variation among individuals, populations and/or species (O'Connell & Wright 1997, Ferguson & Danzmann 1998, Feral 2002). Of these, mitochondrial DNA and microsatellite DNA markers appear to be the most commonly used (Feral 2002, Zhang & Hewitt 2003, Galtier et al. 2009).

Mitochondrial DNA (mtDNA) is a circular structure which exhibits a non-mendelian pattern of inheritance (Park & Moran 1994, Wan et al. 2004, Galtier et al. 2009). The mitochondrial genome typically contains 36 or 37 genes of which two code for ribosomal RNAs, 22 for transfer RNAs and 12 or 13 subunits of multimeric proteins of the inner mitochondrial membrane (Avise et al. 1987, Wan et al. 2004). The popularity of this molecular marker can be attributed to a number of factors: i) the presence of multiple copies of the mitochondrial genome within a single cell allows for mtDNA to be amplified relatively easily; ii) maternal transmission and the subsequent lower effective population size allows for coalescent processes to occur at a more rapid rate than nuclear DNA (nDNA); iii) as a consequence of the enhanced mutation rates, mtDNA is more likely to exhibit genetic differentiation over a shorter period of time than nuclear genes; iv) the lack of recombination implies that the entire loci is represented by a single genealogy, thereby simplifying within-species differentiation analyses and representation, and lastly v) mtDNA is considered to conform to a neutral model of evolution (Avise et al. 1987, Park & Moran 1994, Galtier et al. 2009, Henriques 2012).

Although the use of molecular markers within marine fisheries has drastically increased over the past decades (Park & Moran 1994, Ward & Grewe 1994), few studies have focussed on commercially important marine invertebrates (Thorpe et al. 2000, Doubleday et al. 2009). The lag in molecular invertebrate studies can be attributed to a number of issues such as the inadequacy of biological information, particularly at the larval stages, the presence of cryptic species within this group and the misjudgement of the importance of invertebrates as a fisheries resource due to the relatively low contribution to global fish landings (Pierce & Guerra 1994, Thorpe et al. 2000). Cephalopods are however significant in many commercial fisheries and three orders, the Octopoda, Sepioidea and Teuthoidea (Boyle 1990, Pierce & Guerra 1994, Thorpe et al. 2000) have significant commercial importance. Many of the species belonging to these orders are highly susceptible to the impacts of overfishing due to short life cycles (Pierce & Guerra 1994).

Within the Octopoda, the Octopodidae is the largest family, and is currently considered the only group of benthic octopi (Guzik et al. 2005). This family comprises of the genus *Octopus*, which consists of approximately 200 octopod species, and the majority of the commercially important species such as *O. dofleini, O. variabilis* and the cosmopolitan *O. vulgaris* (Thorpe et al. 2000, Acosta-Jofré et al. 2012). The taxonomic allocation of species to the genus has been based primarily on discrete morphological characters, such as the presence of an ink sac (Allcock & Piertney 2002). This group is therefore often described as a “catch all genus” (Nesis 1998) due to the high levels of ambiguity, resulting from the reliance on a single diagnostic character for taxonomic classification (Roper & Voss 1983, Warnke et al. 2004, Guzik et al. 2005). The lack of clarity within the family, and subsequently within this genus, highlights the need for major systematic revision within the benthic octopods (Roper & Voss 1983, Boyle & Rodhouse 2005, Guzik et al. 2005, Guerra et al. 2010). *Octopus vulgaris* is, in many instances, regarded as a cryptic species complex (Söller et al. 2000, Cabranes et al. 2008, Leite et al. 2008), with an unclear taxonomic status, which is still considered to be under review (Söller et al. 2000, Warnke et al. 2004).

Due to the widespread distribution of the species (as discussed in Chapter 1), a number of studies using molecular markers have been used to investigate population structure, demographic history and phylogenetic relationships in an attempt to resolve instances of ambiguity within *O. vulgaris* (Teske et al. 2007, Doubleday et al. 2009). Genetically homogenous populations can often be attributed to life history traits, associated with dispersal capabilities, and the absence of marine geographic barriers (Hellberg et al. 2002, Grillo 2011). The sedentary adult stage of *O. vulgaris* and a range of distinct biogeographical discontinuities in the Benguela region (see Chapter 2) may, like in other parts of the world, contribute to the disruption of gene flow in this species (Hellberg et al. 2002, Moreira et al. 2011). However, this may be countered by the distinct larval dispersal phase of *O. vulgaris* which may contribute to connecting individuals on either side of oceanic discontinuities.

Unfortunately, our lack of knowledge on the extent and characteristics of this vital dispersal phase has hampered our prediction of population connectivity. The lack of a comprehensive understanding of this vital dispersal phase in *O. vulgaris* may result in poor management and conservation decisions, leaving the species vulnerable to the effects of overharvesting (González et al. 2005).

However, despite the assumed widespread larval dispersal, significant population structure has been observed in several populations of *O. vulgaris* in the Mediterranean Sea, as well as along the coasts of southern Brazil and North West Africa (Casu et al. 2002, Maltagliati et al. 2002, Murphy et al. 2002, Fadhlaoui-Zid et al. 2012). In these cases, population structure appeared not to coincide with an isolation-by-distance model but rather with the presence of oceanographic features such as strong seasonal upwelling zones, overlapping water masses and the presence of distinct transitional zones (Casu et al. 2002, Maltagliati et al. 2002, Murphy et al. 2002, Fadhlaoui-Zid et al. 2012). The lack of significant population structure within a species with sedentary adults is often attributed to the dispersal capabilities during early larval stages (Higgins et al. 2013). Interestingly, a study conducted by Oosthuizen et al. (2004) identified a single *O. vulgaris* stock along the coast of South Africa, represented by a single haplotype using the mitochondrial cytochrome c oxidase subunit III (*COIII*) gene, despite the presence of a number of biogeographical barriers in the region that are documented to have influenced the structuring of other South African marine fauna (Teske et al. 2011). This pattern was later confirmed by Teske et al. (2007) using the mitochondrial cytochrome c oxidase subunit I (*COI*) and *16s* ribosomal RNA (*16s* rRNA) genes. Differential patterns of population structure between and within species, albeit similarities in life history, highlights the importance of a comprehensive molecular approach in order to effectively understand the population structure and the mechanisms driving these trends (Rocha-Olivares et al. 2000), particularly across highly heterogeneous environments, such as the southern Africa region.

Despite the recent studies regarding population sub-structuring of *O. vulgaris* in southern Africa, none have focused on the influence of the potentially greatest biogeographical barrier in the region, the Benguela Cold Current, on the population connectivity of the species. As mentioned in Chapter 2, the system is an effective barrier to dispersal in a number of marine taxa; including numerous tropical and warm-temperate inshore teleost species (Rocha et al. 2005, Floeter et al. 2008, Henriques 2012) and the sea urchin *Diadema* (Lessios et al. 2001). A comprehensive study conducted by Henriques (2012) indicated deep genetic differentiation, between northern and southern Benguela sampling sites, in four teleost species including *Diplodus capensis*, *Argyrosomus inodorus*, *Atractoscion aequidens* and *Lichia amia*. This structuring was attributed to two primary oceanographic processes, the occurrence of the Lüderitz upwelling cell off central Namibia and the presence of colder sea surface temperatures off southern Namibia (Henriques 2012). Nevertheless, a similar study involving the cephalopod *Loligo reynaudii*, did not show evidence of distinct, significant genetic breaks, raising the question of whether the oceanographic features of the Benguela Current only constitute a barrier to warm-temperate inshore fish species, whilst cephalopod species are able to disperse freely across the system (Shaw et al. 2010, Stonier 2012).

The lack of a comprehensive understanding of population connectivity can have major ecological and evolutionary repercussions for the management of commercially exploited species (Von der Heyden 2009, Grillo 2011). Determining population sub-structuring is therefore critical not only to developing sustainable management policies, but also to understand the ability of a species to adapt to fluctuating environmental conditions, the potential to speciate, the probability of local and/or global extinction as well as the capability to recover from the effects of adverse environmental and anthropogenic influences (Grillo 2011). Differential patterns of population structure between and within species, albeit similarities in life history, highlights the importance of a comprehensive molecular approach, in order to effectively understand the population structure and the mechanisms driving these trends, particularly across highly heterogeneous environments, such as the southern Africa region (Rocha-Olivares et al. 2000).

Using a molecular approach, this chapter aims to investigate population connectivity, identify population sub-structuring, determine the evolutionary history and reconstruct the phylogenetic relationships for *O. vulgaris* within the Benguela region, all of which are fundamental for sustainable management and effective conservation strategies of this potentially valuable fishery resource.

Method and Materials

Sample collection and DNA extraction

Sample collection methods are outlined in Chapter 2. Additional samples of *O. vulgaris*, from Senegal, the Mediterranean Sea, Portugal, Spain and Durban, and of *O. tetricus*, from North-eastern Tasmania, were included in order to conduct a more comprehensive phylogenetic analysis.

Total genomic DNA extraction was carried out using a Wizard® Genomic DNA Purification Kit (Promega, USA). Extraction procedures followed those outlined by the manufacturer. A NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) was used to measure DNA concentration and purity of the extracted product, which were stored at 4˚C to allow for later analyses.

Preliminary investigations were carried out in order to determine optimal extract dilution concentrations. Extracted products from six individuals were diluted to two different concentrations, 1:10 and 1:100. Optimal extract concentration was determined by subjecting 5µl of undiluted and diluted extract to electrophoresis in a 1% agarose gel stained with ethidium bromide. Visual analysis, using band clarity and brightness as an indicator of DNA quality, revealed that a dilution of 1:10 was optimal for further analysis. Subsequently, an aliquot of each extracted product was diluted to a 1:10 concentration, and stored at 4˚C until further analysis.

Genotyping

In order to address the questions proposed in this study, Polymerase Chain Reaction (PCR) amplifications were carried out for five individuals from the northern and southern Benguela regions, for two mitochondrial DNA genes and two nuclear regions, allowing for preliminary investigations into genetic variability in *O. vulgaris* across the Benguela Current region.

A general amplification protocol consisting of a 20µl final solution containing 10µl of KapaTaq ReadyMix DNA Polymerase (Kapa Biosystems, South Africa), 0.5µl of each of the primers (10µM), 6µl water and 3µl of DNA (diluted at 1:10) was conducted for all genes unless amplification protocol optimisation was required as a result of low amplification yields. In order to determine the success of the PCR amplification, approximately 5µl of PCR product was electrophoresed in a 1% ethidium bromide-stained agarose gel. Successful amplification was visually determined by the presence of a single clear band of the expected fragment size. The PCR products resulting in multiple bands were re-run in a 2% ethidium bromide-stained agarose gel for an hour at 80V, after which, fragments of the correct size were excised from the gel and purified using the GeneJet Gel Extraction Kit (Thermo Scientific, USA).

Successfully amplified PCR products were sent to Macrogen Inc. (Seoul, Korea) for purification and sequencing, with the same primers used in the amplification protocol.

Cytochrome b

PCR amplification of the mitochondrial DNA cytochrome *b* (*cytb*) gene was carried out using the following cephalopod-specific primer pair CEF H (TTA TGG KTG RGT RYT DCG TTA T) and CEF L (TAC HCC YCC WAR TTT WYT AGG AAT) (Santaclara et al. 2007), for 20 individuals, when possible, from each of the four sampling localities.

Amplification protocol followed that outlined by Santaclara *et al.* (2007), with an initial denaturation step of 95°C for 3min, followed by 35 cycles at 95°C for 30s, 50ºC for 1min, 72°C for 1.5min, and a final extension step at 72ºC for 7min. Due to poor amplification of some samples, PCR optimisation included increasing DNA concentration (5µl) and the number of cycles (40 cycles).

Cytochrome Oxidase subunit I

The PCR amplification of the mitochondrial DNA cytochrome oxidase I (*COI*) gene was carried out using the invertebrate primer pair LC01490 (GGT CAA CAA ATC ATA AAG ATA TTG G) and HC02198 (TAA ACT TCA GGG TGA CCA AAA AAT CA) (Folmer et al. 1994). The PCR amplification reactions were performed at 95°C for 1min, 35 cycles at 95°C for 1min, 40°C for 1min, 72°C for 1.5min followed by a final extension step at 72°C for 7min (Folmer et al. 1994). No additional optimisation was necessary.

1st Intron of the nuclear S7 ribosomal protein

Due to time constraints and the ready availability of the universal primers S7RPEX1F (TGG CCT CTT CCT TGG CCG TC), S7RPEX2R (AAC TCG TCT GGC TTT TCG CC) and S7RPEX3R (GCC TTC AGG TCA GAG TTC AT) (Chow & Hazama 1998) amplification of the 1st intron of the S7 ribosomal protein gene (*RPS7*) was carried out. Initial amplifications followed the original PCR protocols (Chow & Hazama 1998), consisting of an initial denaturation of 95°C for 1min, followed by 30 cycles of 95°C for 30s, 60°C for 1min, 72°C for 2.5min and a final extension step at 72°C for 10min. Optimization of the amplification protocol consisted of a decrease of annealing temperature (40°C) coupled with an increase in DNA concentration (5 µl) and amplification cycles (40x).

Elongation factor-1 alpha

Amplification of the nuclear elongation factor-1 alpha (*EF-1α*) gene was carried out using the primer pair EF0 (oct) (TCT GGN TGG CAT GGT GAT AAC ATG) and EF1(oct) (AGA YAA YGT TGG TTT YAA CGT WAA GA) (Guzik et al. 2005). These primers were modified by Guzik et al. (2005) from the universal primers EF0 and EF1 of Palumbi (1996) so as to allow for the successful amplification of the *EF-1α* gene within the benthic shallow water octopuses (Octopodinae). Initial amplification protocol consisted of an initial denaturation of 94°C for 3min, 35 cycles of 93°C for 30s, 54°C for 30s, 72°C for 2min and a final extension step of 72 °C for 7min, as stipulated by Guzik *et al.* (2005). As a consequence of low levels of amplification yield, PCR protocol optimization was carried out, resulting in an increase in amplification cycles (40x), a reduction in annealing temperature (44°C) and an increase in secondary denaturation phase time (45s).

Statistical analysis

The retrieved sequences were visually inspected in Chromas Lite v.2.1. Sequences of poor quality were removed from the analysis and, when necessary, sent to be re-sequenced. Multiple sequence alignments were carried out in BIOEDIT v.7.1.11 (Hall 1999), and basic sequence statistics including number of base pairs, polymorphic sites and parsimony informative sites were assessed in MEGA v.5.1 (Kumar et al. 1994).

Population structure and phylogeographic patterns

After assessing the most suitable marker for the inference of population sub-structuring of *O. vulgaris* in the Benguela Current region (*cytb*), molecular diversity was determined by the number of haplotypes (*H*), haplotype diversity (*h*) and nucleotide diversity (π), in Arlequin v.3.1.1 (Excoffier et al. 2005). Haplotype diversity can be defined as the probability that two haplotypes, drawn randomly from a population, are not the same (Zhang & Hewitt 2003), while nucleotide diversity is a measure of the likelihood that two individual homologous nucleotides, drawn at random from a population, will be different (Zhang & Hewitt 2003). Diversity levels were calculated at three hierarchical levels: within sampling locality, within each region and for the entire sampled area.

As the effects of selection on a gene can play a major role in the misinterpretation of results regarding population structure and demographic history (Rand 1996), the neutrality tests Tajima's *D*, Fu's F_S and Ewens-Waterson's *F* were performed to detect deviations from the expectation of the neutral theory, as implemented in Arlequin v.3.1.1 (Excoffier et al. 2005). Statistical significance was determined after 10 000 permutations. Neutrality tests were conducted for each sampling locality, each region and for the entire sampled distribution. The best-fit model of nucleotide substitution was assessed in jModelTest v.0.1 (Posada 2008) using the Akaike information criterion (AIC), with likelihood scores computed using 5 substitution schemes and an optimized maximum likelihood base tree.

Estimation of population sub-structuring and of deviation to the null hypothesis of panmixia were conducted based on Wright's Pairwise F_{ST} estimator and on a hierarchical analysis of molecular variance (AMOVA), in Arlequin v.3.1.1 (Excoffier et al. 2005). Wright's Pairwise F_{ST} is an index of genetic differentiation with values ranging between 0.0 and 1.0 (Wright 1943). A value of 0.0 is indicative of panmixia, samples or populations are therefore represented by identical allelic compositions, whilst a value of 1.0 represents complete differentiation as a result of the lack of shared alleles (Wright 1943, Bird et al. 2011). Calculation of pairwise F_{ST} was therefore conducted in order to determine, quantify and compare genetic divergence between sampling localities, as well as between sampling regions. Statistical significance was assessed after 10 000 permutations. An AMOVA test was used to estimate indices of genetic structure using a hierarchical approach based on allelic differences of haplotypes and their frequencies (Excoffier et al. 1992). A standard (haplotypic) and a locus-by-locus AMOVA were implemented in order to test the hypothesis of genetic variation across the Benguela system (NBE and BdT vs. WC and EC). Unlike the standard AMOVA, the locus-by-locus AMOVA accounts for data at any given locus thereby allowing for a more accurate estimation of population differentiation should there be missing information (Excoffier & Lischer 2010). Statistical significance of each approach was determined after 10 000 permutations.

In order to further investigate the relationship between geographical location and genetic relatedness, reconstruction of phylogeographic patterns were conducted based on the genetic relationships between samples, using a median-joining haplotype network in Network v.4.6.1.1 (Bandelt et al. 1999). Median-joining networks allow for the visualisation of the intraspecific geographic distribution and genealogical relationships of haplotypes (Bandelt et al. 1999). Networks were post-processed, in Network v. 4.6.1.1, using the coalescent theory approach in an attempt to resolve connection ambiguity between haplotypes. Coalescence theory predicts that haplotypes should preferably be linked to the most abundant haplotypes, and those found within the close geographical proximity rather than to rare, distant haplotypes (Crandall & Templeton 1993, Posada & Crandall 2001).

Evolutionary History

Assessment of the evolutionary history of *O. vulgaris* in the Benguela Current region was conducted for the putative populations identified using multiple approaches. First, estimation of summary statistics such as haplotype diversity (h), nucleotide diversity (π), Tajima's D, Fu's F_S and Ewens-Waterson's F neutrality tests was performed for each population, in Arlequin v.3.1.1.1 (Excoffier et al. 2005). Past evolutionary and demographic events result in identifiable patterns within molecular diversity indices (Rogers & Harpending 1992, Grant & Bowen 1998, Schneider & Excoffier 1999). Therefore, using molecular diversity indices (h, π), Bowen & Grant (1998) proposed a demographic classification scheme to infer evolutionary history for marine fishes using four broad categories: i) $h < 0.5$, $\pi < 0.5\%$ which is considered to be indicative of a recent population bottleneck or a founder event, ii) $h > 0.5$, $\pi < 0.5\%$ is characteristic of a population which has undergone rapid growth after a population bottleneck, thereby resulting in the accumulation of mutations, iii) $h < 0.5$, $\pi > 0.5\%$ is representative of a population with few, highly divergent haplotypes as a result of either a bottleneck event within a large, stable population or the occurrence of secondary contact between two, or move, previously isolated populations, iv) $h > 0.5$, $\pi > 0.5\%$ is considered to be evident in populations with a long evolutionary history of large, stable population size or where secondary contact has occurred between previously differentiated allopatric lineages (Grant & Bowen 1998). Furthermore, deviations from the assumption of neutrality allows for a number of neutrality tests to be informative with regards to the occurrence of demographic processes within the recent past. Examples of this would be population expansions or contractions (Rand 1996, Fu 1997). It is therefore unclear whether the observed deviation occurs as a result of selection or population processes (e.g. expansions), as both are seen to result in the excess of rare alleles within populations (Hahn et al. 2002). However, the inclusion of multiple neutrality tests allows for the development of more accurate conclusions, regarding different evolutionary pressures, as each test varies in its ability to detect departures from the neutral theory.

The hypothesis of a past population expansion was tested using the mismatch distribution parameters, as implemented in Arlequin v.3.1.1 (Excoffier et al. 2005). Statistical significance, resulting in the rejection of the null hypothesis of past demographic expansion, was determined after 10 000 parametric permutations using the sum of square deviations (SSD) and the Harpending raggedness index (HRI). A unimodal distribution of pairwise differences is considered characteristic of a past population expansion event, whilst a multimodal mismatch distribution is indicative of a population at mutation-drift equilibrium (Harpending 1994, Schneider & Excoffier 1999).

An approximation of the time elapsed since population expansion was calculated based on the estimated parameters of the mismatch distribution, in Arlequin v.3.1.1 (Excoffier et al. 2005), following $T = 2\mu t$; where T is the time since expansion in mutational units, $2\mu = umt$ where *u* is the mutation rate, *mt* is the sequence length in bp and *t* is generation time. In order to estimate time since expansion in years, a mutation rate of 2% per million years (My), based on values determined by Pérez-Losada et al. (2007) for *Sepia officinalis*, and an estimated generation time of one year were used based on an estimated lifespan of 9-12 months in female *O. vulgaris* and a 12-15 month lifespan in their male counterparts (Smale & Buchan 1981). Finally, inference of demographic changes through time, estimation of time since expansion and time since divergence were further explored using two complementary coalescent-based, Bayesian approaches in BEAST v.1.7.5 (Drummond & Rambaut 2007).

A coalescent Bayesian Skyline Plot (BSP) analysis was used to infer past population demographic changes for the two *O. vulgaris* populations, as implemented in BEAST v.1.7.5

(Drummond & Rambaut 2007). The BSPs allow for the genealogy, demographic history and substitution-model parameters to be co-estimated within a single analysis, thereby potentially increasing demographic history estimation clarity (Ho & Shapiro 2011). Reconstruction of the demographic history using BSP allows for the development of inferences regarding the effects of evolutionary and population-genetic processes and therefore allows users to test, for example, the relationships between historical climatic events and population demography (Ho & Shapiro 2011). All analyses were performed under the HKY substitution model, as indicated by jModelTest (Posada 2008), and enforcing a strict molecular clock with a fixed rate of 2% per My. Furthermore, the data was partitioned into the 1st, 2nd and 3rd codon respectively using BEAUTi v.1.7.5 (Drummond & Rambaut 2007). Three independent analyses were run for 10 000 000 generations with parameters being logged every 1 000 generations. The performance of each of the generated outcomes was analysed using the likelihood of posterior probabilities, and convergence was assessed based on estimates of effective sample sizes (ESS > 200) visualized in Tracer v.1.5 (Drummond et al. 2005).

Estimation of time since most recent common ancestor (tmrca) for the two *O. vulgaris* populations was performed using the coalescent-based approach implemented in BEAST v.1.7.5 (Drummond & Rambaut 2007). Three independent analyses were performed, using a coalescent with constant size tree prior, the HKY substitution model and a strict molecular clock. Analyses were run for 10 000 000 generations with parameters being logged every 1 000 generations with a 10% burn-in phase. Calibration of the molecular clocks predominantly makes use of information from fossil records; however two alternative approaches were employed in the present study as a consequence of a lack of fossil records for *O. vulgaris* within the region. These included a fixed sequence mutation rate of 2% per My (Pérez-Losada et al. 2007) and the calibration of internal nodes based on the key biogeographical events associated with the Benguela current in the last 2 My, while estimating mutation rates. Biogeographical calibrations included three alternative analyses: i) strengthening of the oceanographic features within the region which occurred approximately 2 Mya; ii) an intensified cooling period within the system 0.5 Mya; and iii) the onset of quaternary glacial-interglacial cycles, 0.1 Mya (as discussed in Chapter 2). A normal distribution, with the calibration point as the mean, coupled with a broad standard deviation was enforced for each of the biogeographical calibration analyses, in an attempt to account for a lack of estimation certainty (Kass & Raftery 1995, Henriques 2012). As before, convergence was assessed based on ESS > 200, in Tracer v.1.5 (Drummond et al. 2005).

Independent runs were pooled using LogCombiner v1.7.3, to ensure convergence of the model. Comparison of the models was conducted using Bayes Factors, allowing for the identification of the most suitable model.

Phylogenetic Analyses

As the results from the population structure and phylogeographic analyses identified several highly divergent individuals, it was necessary to conduct additional phylogenetic analyses to investigate the relationships between unidentified individuals found in the study, sampled *O. vulgaris* specimens and a number of *Octopus* spp from around the world. Assessment of the phylogenetic relationships within *O. vulgaris* as well as within the genus *Octopus* was conducted by obtaining numerous sequences from GenBank in order to generate three data sets comprising of two gene regions and differential fragment lengths: i) a 559 bp *cytb* alignment comprising of six Angolan and four South African samples from the present study; outlier-individuals NBE14 (Angola), EC6, EC11 and EC15 (South Africa); *O. vulgaris* sequences from Durban (n = 8), the Mediterranean Sea (n = 3), Portugal (n = 1) and Senegal (n = 6); five *O. tetricus* samples from Australia; and an additional 16 sequences from GenBank from six different *Octopus* species, five *O. vulgaris* sequences from specimens in the Mediterranean and one *Loligo subulata* to be used as an outgroup; ii) a 370 bp *cytb* alignment comprising of six Angolan and four South African samples from the present study; outlier-individuals NBE14, EC6, EC11 and EC15, *O. vulgaris* sequences from Durban (n = 8), the Mediterranean Sea (n = 3), 1 Portugal (n = 1) and Senegal (n = 6), five *O. tetricus* samples from Australia and an additional 26 sequences from GenBank comprising of 12 *Octopus* spp, five *O. vulgaris* sequences and one *Loligo subulata*; and finally iii) a 540 bp *COI* alignment comprising of six Angolan and nine South African samples from the present study; the outlier-individuals NBE14, EC6, EC11 and EC15 and an additional 38 sequences of GenBank comprising of 23 *Octopus* spp, four *O. vulgaris* sequences and one *Loligo subulata* (see Tables 3.8 & 3.9 in the Appendix). As phylogenetic relationships may reflect the gene tree, rather than the true species tree, multiple data sets including multiple gene regions of varying fragment lengths were incorporated in order to allow for a more holistic understanding of the genetic relationship between a number of *Octopus* species and within *O. vulgaris*, and more importantly the placement of the outlier-individuals.

Sequence alignments were carried out in BIOEDIT v.7.1.11 (Hall 1999). Estimation of sequence alignment reliability was performed in MEGA v.5 (Kumar et al. 2008). The percent identity of amino acids play a major role in restricting the number of correctly aligned amino acids within a sequence. Thompson et al. (1999) illustrated that an amino acid identity below 20% resulted in less than 50% of the amino acids being correctly aligned. However, accuracy of phylogenetic reconstructions are found to show very little differentiation when more than 50% of amino acids are correctly aligned. As p-distance is equal to 1-amino acid identity, the 20% limit of amino acid identity, as previously discussed, would result in a p-distance of 0.8 (IMV 2008). Sequence alignments were therefore classified as reliable if a p-distance of less than 0.8 was obtained, due to the high proportion of correctly aligned amino acids (IMV 2008). Determination of the best suited model of evolution was conducted using the Akaike Information Criterion in jModelTest v.0.1 (Posada 2008). Furthermore, in order to assess the suitability of the three datasets for phylogenetic analyses sequence reliability, the substitution saturation index were determined in DAMBE v.5.3.38 using the index of substitution saturation (I_{SS}) (Xia & Lemey 2009). An I_{SS} value close to one is considered to be indicative of sequences which have undergone severe levels of substitution saturation thereby undermining the reliability of the sequences for phylogenetic purposes. The critical I_{SS} value ($I_{SS.C}$) is the point at which the sequences will be unable to reflect the true phylogenetic relationships. Sequences were therefore considered suitable for phylogenetic analysis if $I_{SS} < I_{SS.C}$ (Xia et al. 2003).

Reconstruction of the phylogenetic relationships for each of the datasets was conducted using two methods: Maximum Likelihood (ML) and Bayesian Inference (BI). The use of multiple phylogenetic reconstruction methods allows for a more comprehensive understanding of *Octopus* phylogeny, as congruence between alternative methods increases the reliability of the obtained results by eliminating inference based differences. All ML analyses were performed in PhyML v.3.0 (Guindon et al. 2009) using a neighbour-joining starting tree (BIONJ) and run using an approximate likelihood-ratio test (aLRT) (Guindon & Gascuel 2003, Anisimova & Gascuel 2006). BI analyses of all three data sets were conducted in MrBayes v.3.2.1(Huelsenbeck & Ronquist 2001) using two simultaneous independent runs with four Monte Carlo Markov Chains. All analyses were run for 1 000 000 generations, with a 2 500 iterations burn-in period and diagnostics reported every 1 000 iterations. Assessment of the convergence of runs was performed using the average standard deviations of split frequencies ($p < 0.01$) (Ronquist et al. 2011). FigTree v.1.3.1 was used to graphically represent results generated by MrBayes and PhyML (Rambaut 2009).

Results

Preliminary testing

Of the four markers tested, only the mitochondrial genes (*COI* and *cytb*) resulted in clean, informative sequences and were therefore used exclusively for the analyses. However, *COI* was found to exhibit considerably lower levels of genetic variability (h = 0.468, π = 0.001), in comparison to *cytb* (h = 0.637, π = 0.005), and was therefore excluded from population structure and evolutionary history studies, and later combined with *cytb* for comprehensive phylogenetic analyses. Due to the maternal inheritance of mtDNA, two nuclear genes were included within the current study. However, initial attempts to amplify the nuclear genetic markers (S7 and *EF-1α*) failed to provide informative sequences and were therefore excluded from further analysis.

Population structure and phylogeography

In total, 76 individuals were sequenced for 580bp of the mtDNA *cytb* gene. Of these 580bp, 124bp were variable and 86bp parsimony informative. The majority of variable sites were found in four specific individuals, one from NBE (NBE14) and three from EC (EC6, EC11 and EC15). Such high levels of genetic variability are not commonly found in *O. vulgaris*, and so these individuals were removed from further population and phylogeographic analyses, and included in the phylogenetic analyses, to assess their taxonomic status. The resulting dataset (n = 72) exhibited 13 variable sites (six parsimony informative), resulting in 10 haplotypes.

Overall, haplotype (h) and nucleotide (π) diversity values were relatively small, h = 0.636 and π = 0.005 (Table 3.1). The NBE samples were found to exhibit the highest levels of genetic diversity (h = 0.530, π = 0.004) of the four regions, whilst the lowest levels were found within the WC samples (h = 0.100, π = 0.000) (Table 3.1). When comparing between regions, haplotype and nucleotide diversity was found to be higher in Angolan *O. vulgaris* (h = 0.470, π = 0.003), than in the South African samples (h = 0.146, $\pi < 0.001$) (Table 3.1).

There were no significant deviations from neutrality in the overall dataset (Table 3.1), although a clear deviation from the assumption of neutrality was observed in EC (Tajima's $D = -1.513$, $p < 0.05$; and Fu's $F_S = -1.863$, $p < 0.05$), which caused a significant deviation from neutrality amongst the South African group (Table 3.1). Nevertheless, the absence of a significant result in Ewens-*F* suggested that deviations are unlikely to represent selective pressures.

Table 3.1: Genetic diversity indices and neutrality tests for *O. vulgaris* based on 580bp of mtDNA *cytb* region: sample size (n), number of haplotypes (H), number of private haplotypes (Ph) haplotype diversity (*h*), nucleotide diversity (π), Tajima's test of neutrality (*D)*, Fu's test of neutrality (F_S) and Ewens-Watterson neutrality test (*F*).

	NBE	**BdT**	**Overall ANG**	**WC**	**EC**	**Overall SA**	**Overall**
n	12	20	32	20	20	40	72
H	3	5	6	2	3	4	10
Ph	2	3	6	1	2	4	7
h	0.530	0.442	0.470	0.100	0.195	0.146	0.637
Π	0.004	0.003	0.003	< 0.001	< 0.001	< 0.001	0.005
D	1.003	-0.963	-0.606	-1.164	**-1.513**	**-1.716**	0.349
Fs	2.455	0.106	0.545	-0.879	**-1.863**	**-3.884**	0.369
F	0.514	0.580	0.160	0.905	0.815	0.858	0.160

Analyses of population differentiation revealed very high F_{ST} values between Angola and South Africa ($F_{ST =}$ 0.705, $p < 0.05$), and varied between $F_{ST =}$ 0.682 for BdT – EC, and $F_{ST =}$ 0.729 for NBE – WC and BdT – WC ($p < 0.05$ – Table 3.2). The insignificant F_{ST} value between WC and EC ($F_{ST =}$ -0.01633, $p > 0.05$) suggest homogeneity within the two sampling localities (Table 3.2). This trend was also found to occur within the two northern sites, between NBE and BdT ($F_{ST =}$ -2084, $p > 0.05$). Based on these results the null hypothesis of panmixia could be rejected for the *O. vulgaris* from Angola and South Africa, but not within each region (Table 3.2).

Table 3.2: Pairwise *Fst* values for *O. vulgaris* from each sampling locality across the Benguela region (statistical significance, $p < 0.05$, is highlighted in bold).

	NBE	**BdT**	**WC**	**EC**
NBE	-	-	-	-
BdT	-0.208	-	-	-
WC	**0.729**	**0.729**	-	-
EC	**0.669**	**0.682**	-0.016	-

Results from both the haplotypic and locus-by-locus analysis of molecular variance (AMOVA) indicated that genetic variation among groups (70.76% and 82.89%, respectively) was much larger than the within-population variation (29.67% and 17.01%, respectively) (Table 3.3), when the northern-southern differentiation hypothesis was tested.

Table 3.3: Haplotypic and locus-by-locus analysis of molecular variance (AMOVA) values based on *cytb* data for *O. vulgaris* (statistical significance, $p<0.05$, highlighted in bold).

	Haplotypic	**Locus by locus**
Among groups	70.760	**82.885**
Among pop within groups	-0.450	0.109
Within pop	**29.680**	**17.005**

Reconstruction of haplotype relationships identified two main haplogroups: the Angolan haplogroup and the South African haplogroup (Figure 3.11), corresponding to the same population sub-structuring pattern observed with pairwise F_{ST} and AMOVA analyses. Each haplogroup was characterised by one common, dominant haplotype, from which private haplotypes emerged, and no geographical association was observed within either the Angolan or South African haplogroups. The difference between the two haplogroups ranged between two and six mutational steps, while in the South African haplogroup it averaged one mutational step, and within the Angola haplogroup, haplotypes differed by between one to six mutational steps.

3.4,

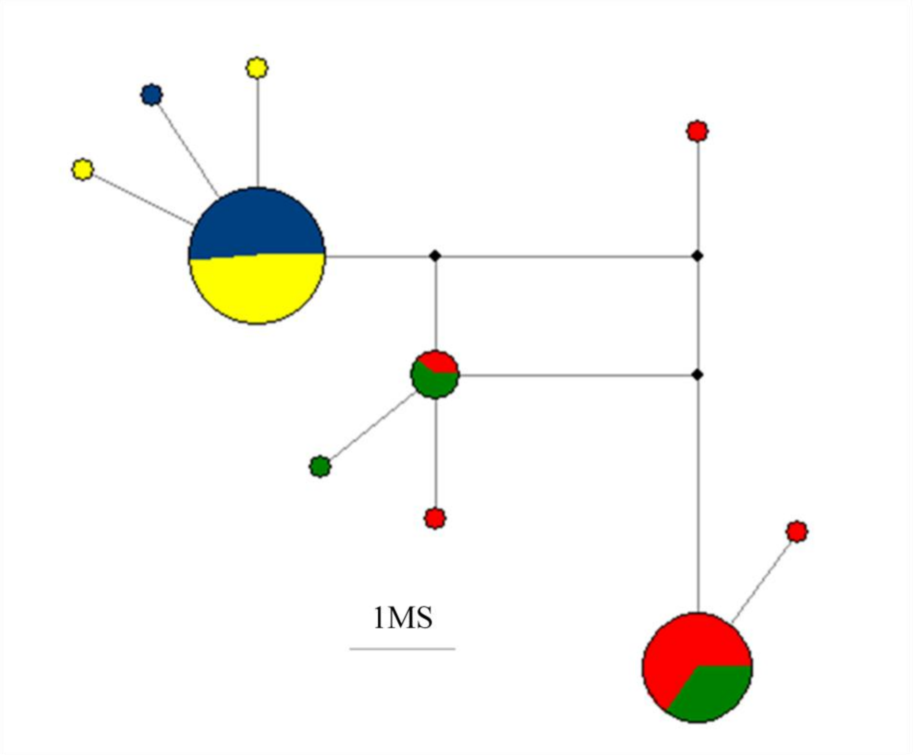

Figure 3.11: A median-joining haplotype network, based on 580bp of *cytb* mtDNA, illustrating the genealogical relationships between *O. vulgaris* in the Bengula region; EC = yellow, WC = blue, NMB = green, BdT = red. Node width is proportional to total haplotype frequency, branch lengths are proportional to mutational steps and black dots correspond to extinct/unsampled haplotypes.

Evolutionary History

Due to the presence of significant genetic differentiation across the Benguela Current region, assessment of the demographic history was performed for each of the identified populations separately.

The haplotype (*h*) and nucleotide (π) diversity levels were higher for the northern population (ANG) (Table 3.4). Only the southern population (SA) exhibited deviations from the null assumption of neutrality (Tajima's *D* = -1.716 and Fu's F_{ST} = -3.884, p<0.05) (Table 3.4). These findings were further supported by the unimodal mismatch distribution analyses, where the null hypothesis of a past population expansion was accepted for the SA population (Table 3.4,

Figure 3.12). Estimated time since expansion, based on mismatch distribution, indicated that expansion of the SA population occurred approximately 129.31 Kya (**Error! Reference source not found.**Table 3.4). In contrast, the mismatch distribution analyses rejected the null hypothesis of population expansion for the ANG population (SSD = 0.320, $p < 0.05$). This was further supported by the absence of a unimodal distribution of pairwise differences (

Figure 3.12) and thus the time since expansion could not be determined for this population.

Table 3.4: Demographic history results for northern and southern Benguela populations of *O. vulgaris* using 580bp of mtDNA *cytb* gene: haplotype diversity (*h*), nucleotide diversity (π), Tajima's (*D)*, Fu's (F_S) and Ewens-Watterson's (*F*) tests of neutrality. Mismatch parameters include time since expansion in mutational units (τ), population size prior to expansion (θ_0), population size after expansion (θ_1) and time since expansion in thousands of years T (Ky) with upper and lower 95% confidence intervals in parenthesis. Statistically significant values highlighted in bold.

	ANG	**SA**
h	0.470	0.146
π	0.003	< 0.001
D	-0.606	**-1.716**
Fs	0.545	**-3.884**
F	0.160	0.857
Mean	1.792	0.150
Variance	4.351	0.135
SSD	**0.320**	< 0.001
HRI	0.346	0.525
τ	0.000 (0.000-0.500)	3.000 (0.422-3.500)
θ_0	0.000	0.000
θ_1	99999.000	0.179
T(Ky)	-	129.310 (18.190-150.860)

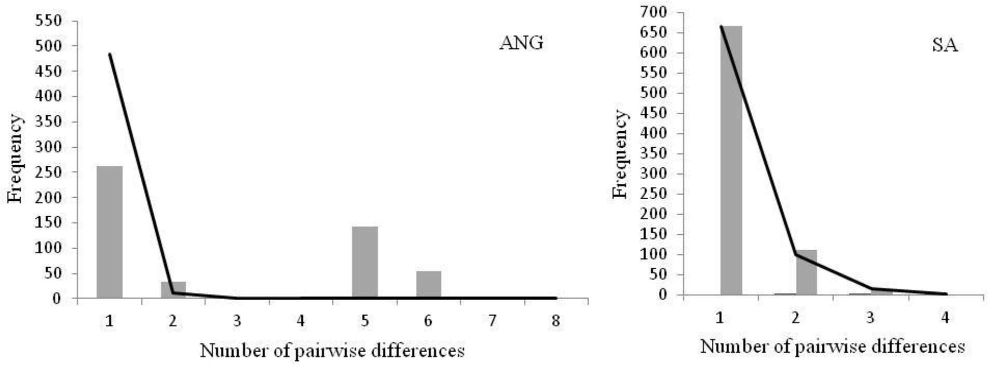

Figure 3.12: Mismatch distributions of pairwise differences for Angolan (ANG) and South African (SA) *O. vulgaris* populations, based on 580bp of mtDNA *cytb* gene. Grey bars are indicative of the observed frequency of pairwise differences while solid lines represent the expected distribution under the demographic expansion model.

Assessment of demographic change through time, based on Bayesian Skyline Plots, was only possible to be calculated for the ANG population, and revealed a clear population expansion around 75 Ka until present day (Figure 3.13). Due to insufficient genetic diversity within the South African population, BSP was unable to determine changes in population size through time.

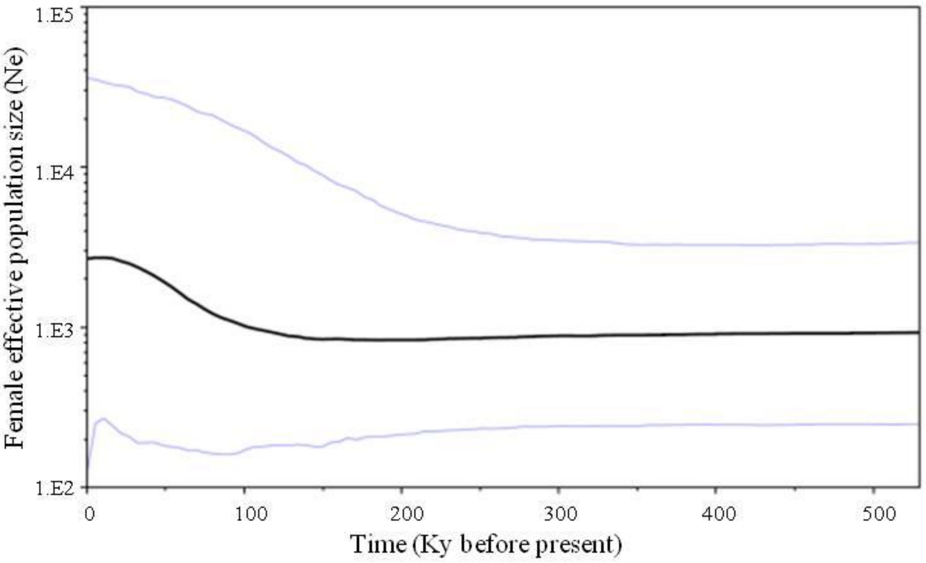

Figure 3.13: Bayesian skyline plot illustrating the change in effective population size through time for Angolan *O. vulgaris*. The solid line is indicative of the mean estimate of population size through time, and the 95% highest posterior density interval is represented by the grey lines.

Despite the different calibration methods, the posterior probability likelihoods were similar (ranging between -845.148 and -845.632) and suggested that all models provided a similar fit. Nevertheless, the Bayes Factor analyses results indicated that the fixed mutation rate of 2% per My provided the best fit (Table 3.5). Within the biogeographical calibration models, the 2 My hypothesis surfaced as the most likely model, based on the Bayes Factor analyses and the estimated mutation rate (1.26% per My) (Table 3.5).

Table 3.5: Bayes Factor and standard error (SE) for alternative molecular clock calibration models, based on 540bp of mtDNA *cytb* gene, for *O. vulgaris* in the Benguela Current region.

	SE	Fixed Diverg	Model 1	Model 2	Model 3
Fixed Divergence	± 0.129	-	0.387	0.242	0.249
Model 1- 2My	± 0.149	-0.387	-	-0.145	-0.138
Model 2 - 0.5My	± 0.123	-0.242	0.145	-	0.008
Model 3 - 0.1My	± 0.119	-0.249	0.138	-0.008	-

Based on the fixed mutation rate, the tmrca between the northern and southern populations was estimated at 231 Ka (95% CI: 98 – 384 Ka). However, the confidence intervals, which were represented by upper and lower 95% highest posterior distribution, were broad for all calibration techniques as expected due to the variable nature of the data (Table 3.6).

Table 3.6: Estimates of time since most recent common ancestor (tmrca) in millions of years (My) under four alternative models: ln (likelihood) represents the posterior probabilities of each of the calibration approaches.

Calibration Method	ln (likelihood)	tmrca (My)
Fixed 2%	-845.081	0.231 (0.098 -0.384)
2 My	-845.505	1 (0.500-2.239)
0.5 My	-845.632	0.232 (0.100-0.585)

Phylogenetic analysis

The *cytb* phylogenetic trees identified a highly robust *O. vulgaris*-type clade and a second clade containing species from the Indo-West Pacific Oceans (Figures 3.4. to 3.7). The *O. vulgaris* clade included samples from Angola, Senegal, South Africa, Portugal, Spain and the Mediterranean, independently of the approach and dataset used (Figures 3.4 to 3.7). In all cases *O. tetricus* and the outlier-individual NBE14 remained part of this clade, with *O. vulgaris* from South Africa appearing as the closest relative (Figures 3.4 to 3.7). This relationship was seen to be strongly supported regardless of the number of species and size of the fragment that was included in the analysis (Figures 3.4 to 3.7). Interestingly, *O. vulgaris* specimens from Angola and Senegal appeared to be more closely related to specimens from the Mediterranean than from specimens from South Africa, with the exception of two South African individuals which clustered with *O. tetricus* (Figures 3.4 to 3.7). Despite an overall similarity in tree topology the decrease in the number of trifurcations in the ML trees suggested that the ML method resolved the relationship within the *O. vulgaris* clade better than the BI approach, regardless of the number of species used (Figures 3.4 to 3.7).

Outlier-individuals EC6, EC11 and EC15 grouped into the Indo-West Pacific Clade, independently of the analysis and dataset used (Figures 3.4 to 3.7). Both, EC6 and EC11 clustered with *O. minor*, while EC15 was included with *O. macropus* using both approaches (Figures 3.4 to 3.7).

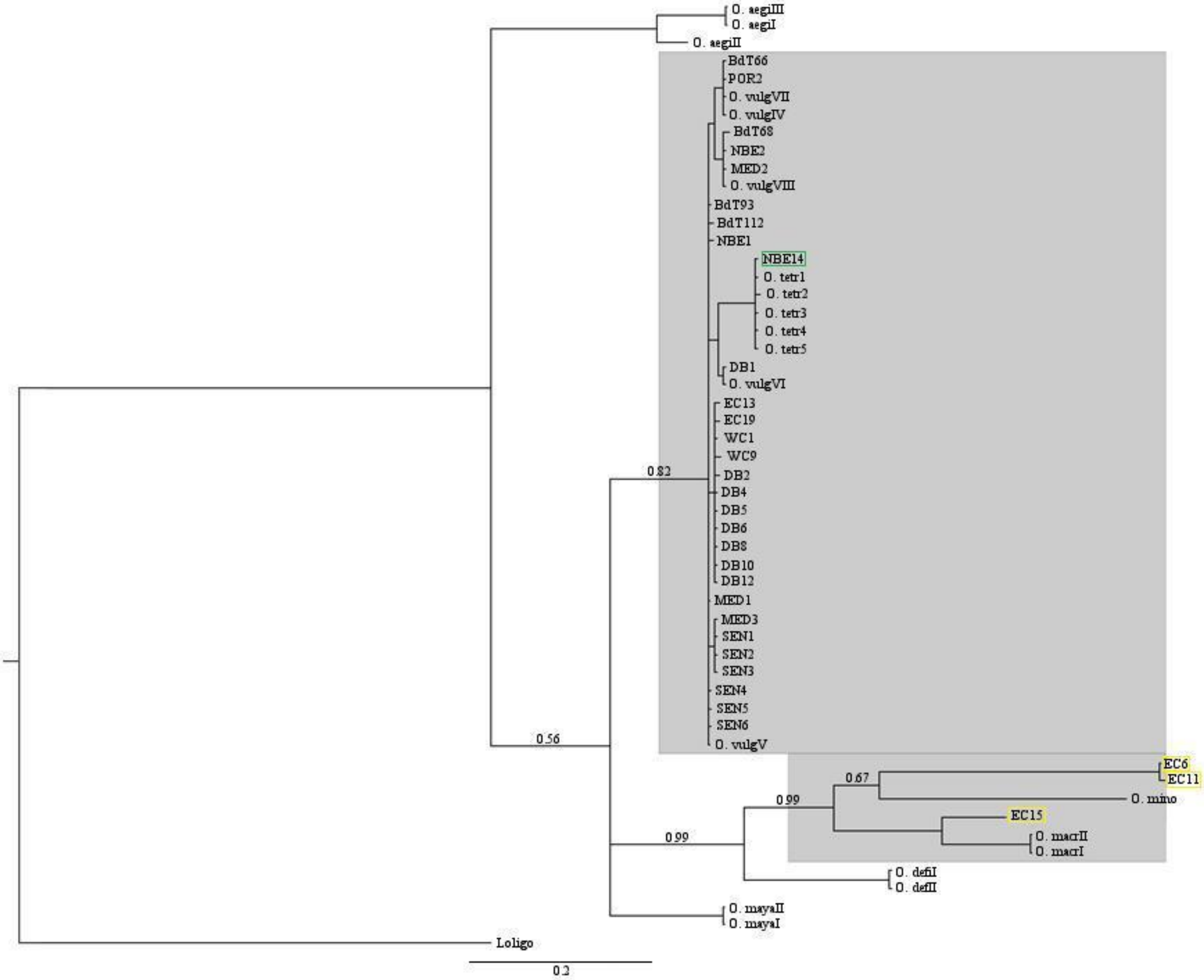

Figure 3.14: Bayesian phylogenetic reconstruction of the relationship between seven *Octopus* species (see Table 3.8 for species codes), based on 559bp mtDNA *cytb* dataset. Node support represented by posterior probabilities. Areas highlighted in grey represent the two major clades identified by phylogenetic analyses; the *O. vulgaris* clade and the Indo-West Pacific clade. Coloured boxes highlight outlier-individuals. Outgroup: *Loligo subulata*.

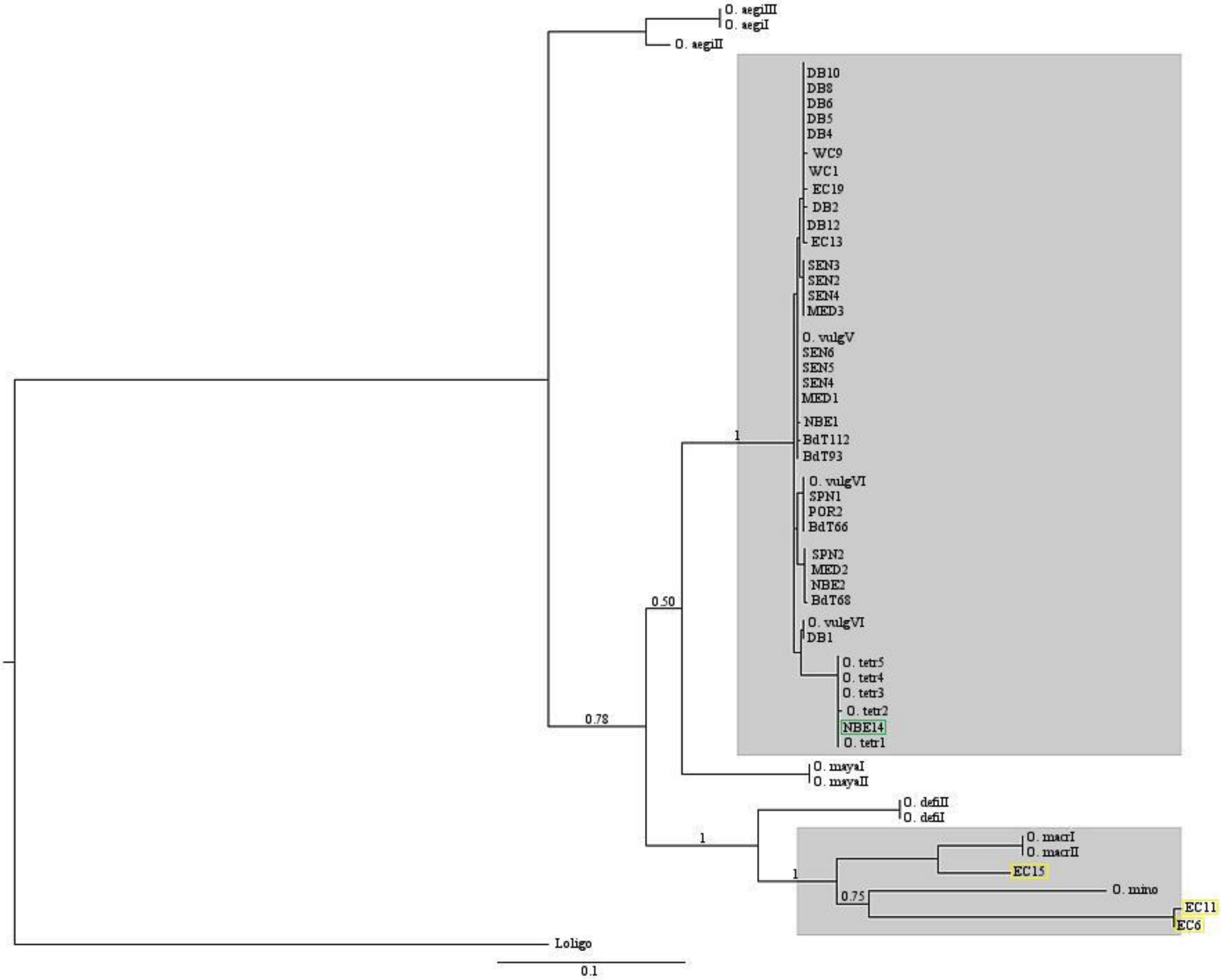

Figure 3.15: Maximum likelihood reconstruction of the relationship between seven *Octopus* species (see Table 3.8 for species codes), based on 559bp mtDNA *cytb* dataset. Node support represented by aLRT values. Areas highlighted in grey represent the two major clades identified by phylogenetic analyses; the *O. vulgaris* clade and the Indo-West Pacific clade . Coloured boxes highlight outlier-individuals. Outgroup: *Loligo subulata*.

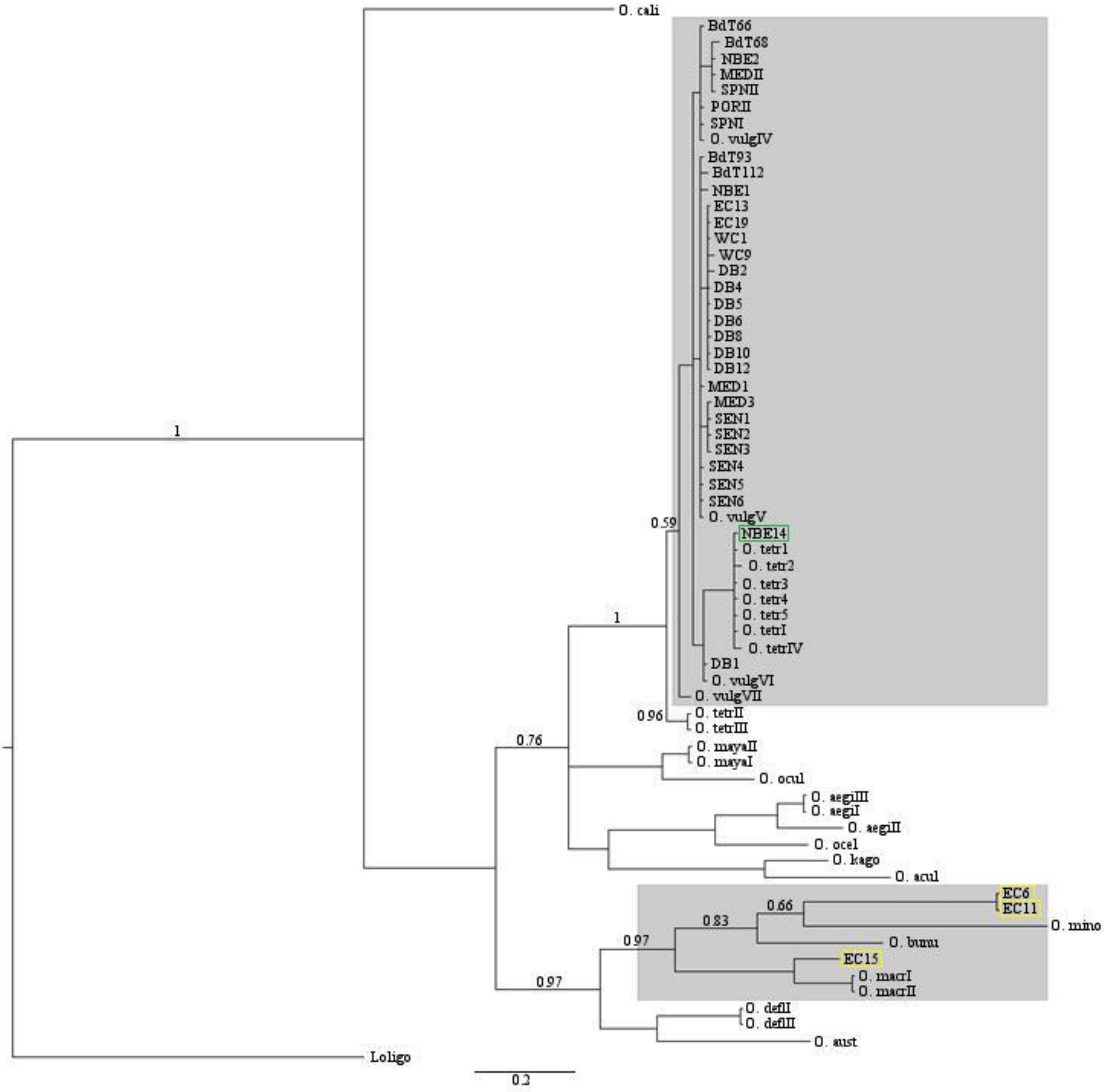

Figure 3.16: Bayesian phylogenetic reconstruction of the relationship between 13 *Octopus* species (see Table 3.8 for species codes), based on 370bp mtDNA *cytb* dataset. Node support represented by posterior probabilities values. Areas highlighted in grey represent the two major clades identified by phylogenetic analyses; the *O. vulgaris* clade and the Indo-West Pacific clade . Coloured boxes highlight outlier-individuals. Outgroup: *Loligo subulata*.

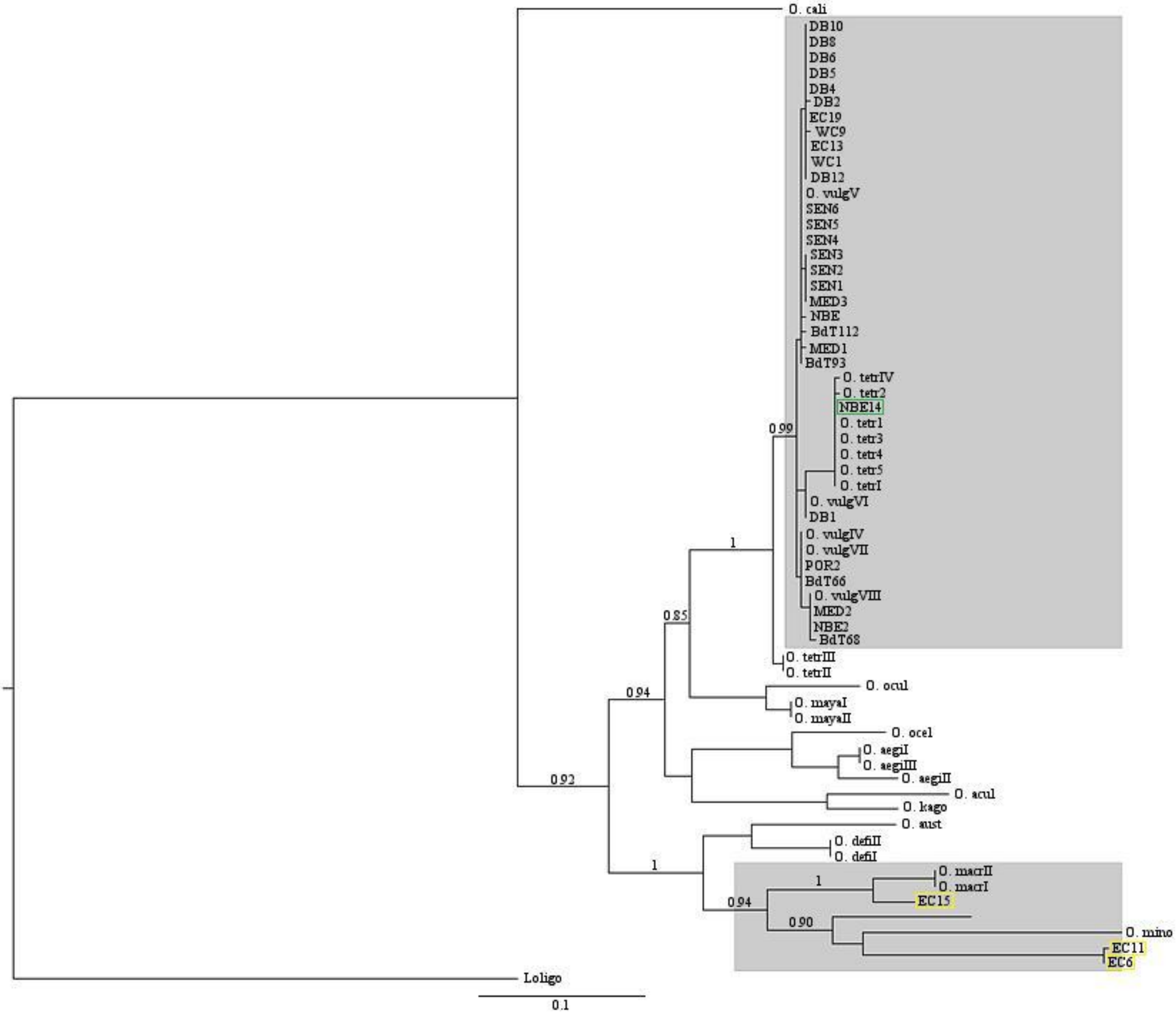

Figure 3.17: Maximum likelihood reconstruction of the relationship between 13 *Octopus* species (see Table 3.8 for species codes), based on 370bp mtDNA *cytb* dataset. Node support represented by aLRT values. Areas highlighted in grey represent the two major clades identified by phylogenetic analyses; the *O. vulgaris* clade and the Indo-West Pacific clade . Coloured boxes highlight outlier-individuals. Outgroup: *Loligo subulata.*

Reconstruction of phylogenetic relationships based on *COI* sequences revealed a similar strongly supported *O. vulgaris*–type clade, however *O. areolatus* and *O. oshimai* specimens from the northwest Pacific were found to fall within this cluster (Figures 3.8 and 3.9). As observed for the *cytb* reconstruction, the outlier-individual NBE14 was included with the *O. vulgaris* clade and not with the *O. tetricus* specimens (Figures 3.8 and 3.9). On the contrary, specimens identified as *O. oshimai and O. areolatus* were included in the *O. vulgaris* clade, and found to be closely related to *O. vulgaris* specimens from the NW Pacific and the Eastern Mediterranean (Figures 3.8 and 3.9). The *O. vulgaris* from South Africa and Angola appeared more closely related to each other than to *O. vulgaris* from the Eastern Mediterranean and NW Pacific (Figures 3.8 and 3.9). Outlier-individuals EC6, EC11 and EC15 clustered in an isolated clade, closely related to Indo-West Pacific species *O. sasakii*. *O.minor, O. variabilis* and *O. fusiformis*, when using both approaches (Figures 3.8 and 3.9). As observed for the *cytb* dataset, the BI approach resulted in a less resolved tree, than the ML method.

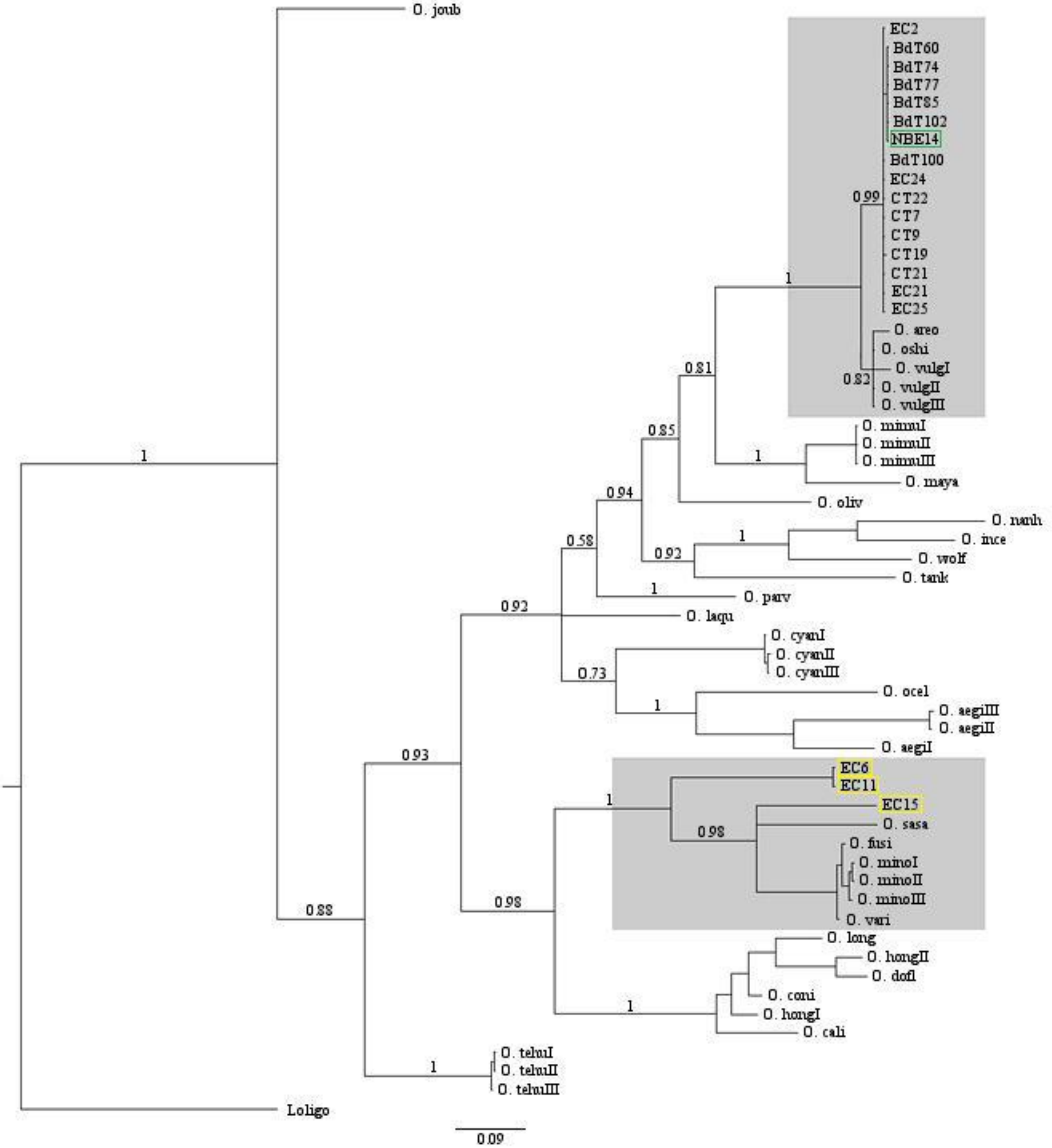

Figure 3.18: Bayesian phylogenetic reconstruction of the relationship between 24 *Octopus* species (see Table 3.9 for species codes), based on 540bp mtDNA *COI* dataset. Node support represented by posterior probabilities values. Areas highlighted in grey represent the two major clades identified by phylogenetic analyses; the *O. vulgaris* clade and the Indo-West Pacific clade . Coloured boxes highlight outlier-individuals. Outgroup: *Loligo subulata*.

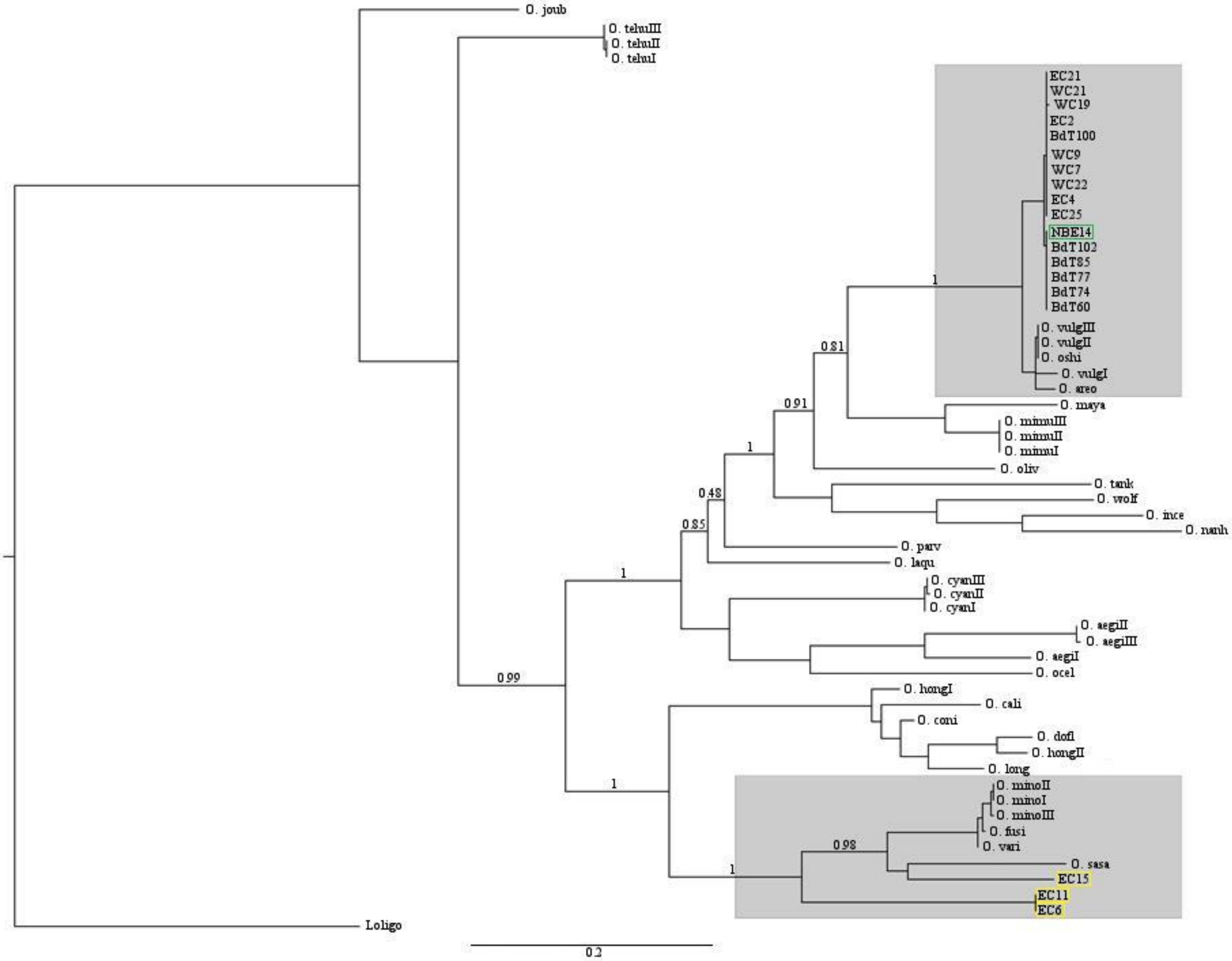

Figure 3.19: Maximum likelihood reconstruction of the relationship between 24 *Octopus* species (see Table 3.9 for species codes), based on 540bp mtDNA *COI* dataset Node support represented by aLRT values. Areas highlighted in grey represent the two major clades identified by phylogenetic analyses; the *O. vulgaris* clade and the Indo-West Pacific clade . Coloured boxes highlight outlier-individuals. Outgroup: *Loligo subulata*.

Discussion

Low levels of both inter and intraspecific differentiation are common assumptions within the marine realm due to the lack of distinct barriers to dispersion (Waples 1998, Grillo 2011). However the advent and utilisation of molecular techniques has played a major role in undermining this assumption and revealing complex patterns of population structure across both large and small geographic distances (Hellberg 2009, Evans et al. 2010, Grillo 2011). Knowledge of connectivity within the marine environment is critical to development and implementation of effective management strategies, thereby emphasizing the need for population structure and evolutionary history research. Despite similar distributions, life history attributes and biology, high levels of variability have been observed within and between closely related species, and thus species-specific investigations are required (Evans et al. 2010, Teske et al. 2010).

Inferences regarding the evolutionary history of a species are often highly dependent on the chosen gene region (Hellberg 2009, Petit & Excoffier 2009). The inclusion of multiple molecular markers therefore provides a more holistic approach to the interpretation of a species population structure, evolutionary history and phylogenetic relationships. Within the current study, four markers were tested; however due to the inability to obtain clean, informative sequences for the S7 ribosomal protein gene and the elongation factor-1 alpha these two nuclear markers were excluded from further analysis. Low levels of variability, possibly due to the conservative nature of the gene (Begg & Waldman 1999) or, alternatively, as a result of selective forces (Amos & Harwood 1998), were observed in *COI* sequences during preliminary studies. *COI* was therefore excluded from population structure and evolutionary history analyses, as *cytb* was found to be more informative and thus used in a subsequent population survey. The comparison of the results found for *cytb* and *COI* in the phylogenetic reconstructions indicated that there was a comparative lack of variability in the *COI* gene. This finding is significant as it places into question the suitability of the use of the *COI* marker for DNA barcoding purposes in *Octopus* spp.

Population structure and phylogeography of O. vulgaris in the Benguela Current region

Genetic variability is fundamental to a populations' viability and adaptability (Holderegger et al. 2006). Populations characterised by extremely low levels of genetic diversity are often considered to be highly susceptible to extinction, as a consequence of a decreased ability to adapt to changing environmental processes (Hellberg 2009, Grillo 2011). Whilst the importance of maintaining molecular variability should not be undermined, it is important to note that genetic homogeneity cannot be unequivocally linked to high extinction risks. This can be seen to be demonstrated by a number of species, such as the cheetah (*Acinonyx jubatus*), the wandering and the Amsterdam albatrosses (*Diomedea xulans* and *Diomedea amsterdamensis*), which continue to thrive despite extremely low levels of genetic variability (Merola 1994, Milot et al. 2007).

The low levels of overall genetic diversity (h = 0.637, π = 0.005) observed for *O. vulgaris* in the Benguela Current, could be attributed to the nature of the genetic marker used, however low levels of diversity in comparison to other invertebrates, have been noted in a number of cephalopod species (Boyle & Boletzky 1996, Allcock et al. 1997, Sands et al. 2003, Doubleday et al. 2009). The lack of genetic variability, frequently observed within this class has been suggested to be an impediment to the progress of cephalopod molecular population studies (Shaw et al. 1999, Oosthuizen et al. 2004). The assessment of genetic diversity of South African *O. vulgaris* distributed between Cape Point and Hamburg in this study revealed low levels of haplotype and nucleotide diversity (h = 0.146, $\pi < 0.001$), and was represented by a single dominant haplotype, and three private haplotypes. Despite moderate levels of haplotype diversity (h = 0.470) in the northern Benguela sampling localities, nucleotide diversity (π = 0.003) also remained relatively low. This is assumed to be indicative of the small differences between the haplotypes; this assumption was further supported by the network analysis, which predominantly consisted of single haplotype differences within each haplogroup. Furthermore, the higher levels of genetic diversity exhibited by the Angolan population may indicate that the observed low genetic diversity in the South African population could be attributed to factors other than the nature of the genetic marker. It is unlikely that the observed results are an artefact of sample size, as regions with lower sample sizes do not necessarily correspond to a decrease in genetic diversity (Teske et al. 2007, von der Heyden 2007a, Groeneveld et al. 2006). Therefore, lower levels of genetic diversity observed within the South African population could be a consequence of a loss of heterozygosity due to demographic factors, such as a recent population bottleneck or founder event (Ramel 1998, Teske et al. 2007).

The assessment of population structure and phylogeographic patterns across the Benguela Current region indicated the presence of significant genetic differentiation in *O. vulgaris.* Results from the minimum spanning network, regional pairwise F_{ST} and the locus-by-locus AMOVA supported the hypothesis of a northern-southern Benguela population sub-structure. Pairwise F_{ST} comparisons for individual sampling localities indicated a significant differentiation between localities from different regions, however within each region F_{ST} values were considerably lower and were found to support the hypothesis of panmixia within each region (South Africa: $F_{ST} = 0.0163$, $p > 0.05$; Angola: $F_{ST} = 0.208$, $p > 0.05$). The significant pairwise $F_{ST} = 0.705$ ($p < 0.05$) detected between Angolan and South African populations suggest high genetic divergence between these two populations. This value is far greater than the overall mean F_{ST} of marine fish (approximation of mean $F_{ST} = 0.062$) (Ward 2000). However it was similar to values reported for populations of *Loligo forbesi* on the shelf of the North East Atlantic and the Azores ($F_{ST} = 0.789$) (Shaw et al. 1999). Furthermore a study by Murphy et al. (2002) detected a significant F_{ST} of 0.010 ($p = 0.005$) for *O. vulgaris* populations along the northwest African coast. Direct comparison between mtDNA F_{ST} and that of microsatellites should be interpreted with caution, as the two markers are substantially different due to, among other features, the amount of within-sample variation (Shaw et al. 1999). Nevertheless, the classification scheme outlined by Wright (1978) indicates that a value greater than $F_{ST} = 0.25$ should be considered representative of 'very great genetic differentiation'.

These findings were further supported by the results of the locus-by-locus AMOVA, which indicated that the northern and southern structure was explained by 82.89% of the variation between groups and by the phylogeographic analyses. The lack of statistical significance for the haplotypic AMOVA, at the among-group level, and the difference in results between the two approaches may be a consequence of missing data. Locus-by-locus AMOVA results therefore may provide a more accurate representation of population genetic structure. Furthermore, the haplotype network illustrated a lack of shared haplotypes between northern and southern populations, with both populations being dominated by a single haplotype, and few private haplotypes.

The lack of genetic structure along the coast of South Africa confirms previous findings by Oosthuizen et al. (2004) and Teske et al. (2007), who attributed the lack of molecular variability to the potential conservative nature of the *COIII*, *COI* and *16s* genes of the mtDNA in *O. vulgaris*. A number of potential phylogeographic barriers, associated with oceanographic features, have been identified along the South African coast and are considered to play a major role in limiting gene flow across the region in a number of species (Evans et al. 2004, Teske et al. 2006, 2011). However, the lack of variation and structure within *O. vulgaris* along the South African coast is indicative of high levels of interbreeding and gene flow. Genetic homogeneity along the South African coast has been observed for several species, being generally associated with the dispersal capabilities and life history of a species in the larval and adult phases (Tolley et al. 2005).

Based on the high regional pairwise F_{ST} ($F_{ST} =$ 0.705, $p < 0.005$), the increased amount of among-group variation within the AMOVA and the identification of distinct haplogroups in the minimum spanning network, the hypothesis of a panmictic population of *O. vulgaris* throughout the Benguela region can be rejected. A separation between northern and southern Benguela fauna, as a result of oceanographic or temperature changes during the Pleistocene, has been reported for several warm-temperate coastal species including teleosts such as *Lichia amia*, *Diplodus capensis* and *Atractoscion aequidens* (Henriques 2012), as well as invertebrates such as the sea urchin *Diadema* (Lessios et al. 2003). Research suggests that the intensification of the cold upwelling cell, located off the southwest coast of Africa, during the Pliocene, played a major role in contemporary patterns of differentiation observed across the Benguela region (Lessios et al. 2003). The presence of upwelling zones, along the north-western coast of Africa, are considered to play a major role in limiting the dispersal of octopus paralarvae, thereby resulting in the presence of significant genetic structure between Mauritanian and Western Saharan *O. vulgaris* fisheries (Murphy et al. 2002). It is therefore likely that dispersal of *O. vulgaris* paralarvae across the Benguela region is currently restricted by the presence of the Lüderitz upwelling cell located off the coast of central Namibia. Upwelling cells are known to contribute to the breakdown of gene flow among populations, as they disrupt the longshore transport of pelagic phases, such as larvae, driving them offshore, where the environment is unsuitable for recruitment and survival (Lett et al. 2007). However, the lack of samples between Baía dos Tigres and Cape Point do not allow for inferences regarding the exact position of the geographic barrier responsible for the genetic structuring of *O. vulgaris* in the Benguela region.

Evolutionary History

Results from mismatch distribution analyses, Tajima's D and Fu's F_S tests suggested that the South African *O. vulgaris* population has undergone a past population expansion, as has been previously demonstrated by Teske et al. (2007). These findings were further supported by the slightly star-shaped haplotype network and the unimodal nature of the mismatch distribution plot. The observed low haplotype and nucleotide diversity levels ($h < 0.5$, $\pi < 0.5\%$) was also congruent of a recent population expansion or founder event (Grant & Bowen 1998). Time since expansion was estimated to have occurred during the late Pleistocene at approximately 129.31 Kya. Climatic oscillations during the Pleistocene have resulted in major climatic variations within the Benguela Current (Dingle et al. 1996, Marlow et al. 2000). A reduction in sea surface temperatures and sea level, following the warmer interglacial periods, and large fluctuations in circulation patterns occurred in the Benguela Current region during the late Pleistocene (Marlow et al. 2000). Such dramatic changes can have major implications on the distributional patterns and stability of marine populations (Hansen & Hemmer-Hansen 2007, Larmuseau et al. 2009). However the effects of these cyclic climatic fluctuations are thought to be species-specific and highly dependent on the nature of such events (Roy et al. 1996, von der Heyden et al. 2007b).

Estimated time since expansion for South African *O. vulgaris* was documented to coincide with the last interglacial period (LIG), the Eemian, which was estimated to have occurred roughly 125 Kya (Kopp et al. 2009, Dutton & Lambeck 2012). This period was characterized by higher sea surface temperatures (+ 0.9°C on the southeast coast, +3.8°C on the south coast of South Africa) and a eustatic sea level rise of approximately 4-6m higher than currently observed (Teske et al. 2003, Dutton & Lambeck 2012). The rise in sea level and sea surface temperatures could be identified as potential drivers of population expansion as a consequence of either an extension in the species' biogeographical boundaries or a shift in the latitudinal abundance of the species. Despite the subsequent cooler, glacial period and climatic fluctuations which occurred to date, it would appear that the established population was not greatly influenced by any later events, allowing for further expansion to occur. Alternatively, due to the high levels of variation associated with the approximation of time since expansion (18.19 – 150.86 Kya), demographic expansion may have occurred more recently during the last glacial maxima (LGM), which occurred approximately 18 to 22 Kya. Demographic changes associated with the LGM have been exhibited in a number of other

South African marine organisms including the shallow-water Cape hake, *Merluccius capensis*, the Cape fur seal, *Arctocephalus pusillus pusillus*, and the spiny lobster, *Palinurus delagoe* (Matthee et al. 2005, Gopal et al. 2006, von der Heyden et al. 2007b). However, the high levels of variation associated with the estimation of the time since expansion, as demonstrated by the broad confidence intervals, suggests a very conservative approximation of the demographic history of South African *O. vulgaris*. The accuracy of the approximation of a species' demographic history has a major impact on the efficiency of management and conservation efforts, and as such, inferences regarding evolutionary history should always be interpreted with caution (Johnson et al. 2007). The accuracy of mismatch distribution analyses are often undermined, as the obtained histograms are said to be greatly influenced by early bifurcations within the gene tree (Avise 2000). Assessment of evolutionary history and time since divergence was therefore further investigated using a Bayesian approach. However, it is important to note that skyline plots do not take into consideration the error associated with phylogenetic reconstruction, as demographic history is inferred from an estimated genealogy, rather than the sampled gene sequences (Drummond et al. 2005) and are therefore of limited use for populations with low genetic diversity levels. The inclusion of multiple methods to infer the demographic history of *O. vulgaris* aimed to overcome the drawbacks associated with each of the approaches in an attempt to gain a more accurate understanding.

Bayesian skyline plots indicated that the Angolan population has experienced gradual demographic growth since, approximately, 75 Kya. Population growth may be attributed to higher sea surface levels within the region, an outcome of the previously mentioned interglacial period. The continental shelf off southern Angola is documented to be relatively narrow (Wysokiński 1986), thereby potentially limiting the availability of a suitable habitat for *O. vulgaris*. A rise in sea level was therefore likely to have been coupled with the widening of the shelf, which in turn had the potential to increase the availability of a suitable habitat and shelter to the species. Alternatively, an increase in the width of the continental shelf due to sea level rise may have allowed for an extension of the distributional range of *O. vulgaris*, which may have been previously restricted by depth preferences. *O. vulgaris* is considered a shallow water species, and is documented to occupy depths of 0-100m, with abundance decreasing drastically thereafter, and completely limited at depths of 200m or more (Katsanevakis & Verriopoulos 2004a). Despite little being known about the effects of depth on the distributional patterns of *O. vulgaris*, depth has been found to be an important factor limiting the distribution and population structure of the Antarctic octopus *Pareledone turqueti* in the southern Ocean (Allcock et al. 1997).

Estimation of time since most recent ancestor, revealed that separation between Angolan and South African *O. vulgaris* populations occurred approximately 231 Kya, when using a fixed mutation rate of 2% per My. Biogeographical calibration of internal nodes resulted in little differentiation in posterior probability likelihoods, and despite marginal support for the second biogeographical calibration model (0.5 My), the moderately high clock rate of 5.7% per My associated with this calibration technique was considered biologically unrealistic when compared to rates determined by Pérez-Losada et al. (2007) for *S. officinalis.* Alternatively the first model, which made use of a 2 My internal node calibration, yielded a more likely divergence rate of 1.3% per My indicating that separation was likely to have occurred between 231 Ka to 1Ma, during the middle Pleistocene (Marlow et al. 2000).

The mid-Pleistocene, which extended from approximately 0.6 to 1.3 Mya, was characterized by a drastic decrease in sea surface temperatures, and was accompanied by an increase in the amplitude of global ice volume, specifically during glacial stages (Marlow et al. 2000, Petrick et al. 2013). Mid-Pleistocene sea levels were estimated to have dropped drastically during glacial periods and remain relatively stable during interglacial stages (Sosdian & Rosenthal 2009). Cooling during this epoch was documented to have intensified around approximately 0.9 Mya, and was maintained during both the glacial and interglacial maxima (Marlow et al. 2000, McClymont et al. 2013). At around 0.6 Mya, fluctuation in sea surface temperatures was observed as a result of the establishment of 100 Kya glacial-interglacial cycles (Marlow et al. 2000). Such a drastic change in temperature, as the one associated with the onset of the mid-Pleistocene, may have had major implications for species within the Benguela Current region. Furthermore, the commencement of the second most recent glacial age, the Wolstonian glacial period or the Santa Maria period, ~200 Kya subsequently led to a drop in SST (Pisias & Moore 1981) and sea level, potentially reinforcing/maintaining the observed divergence pattern between the northern and southern Benguela sub-systems. The observed time since divergence between northern and southern sub-systems for *O. vulgaris*, using a fixed divergence rate, is comparable to that of *L. amia* and *D. capensis* populations (Henriques 2012). Climatic fluctuations, throughout the Benguela region, during the mid-late Pleistocene therefore can be considered to have played a major role in influencing population connectivity, of a number of marine species, between the northern and southern Benguela.

The response to environmental fluctuations is often considered to be specific to the species' physiological thresholds; species may therefore either be able to adapt to the change or disperse to more favourable conditions (Grillo 2011). However, dispersal capabilities may limit movement to a preferred habitat, either as a consequence of life history attributes or environmental discontinuities, thereby resulting in the local extinction of populations which are unable to adapt to the change in conditions (Hellberg 2009, Grillo 2011). To date, the distributional limitations of *O. vulgaris* remains relatively unresolved (Warnke et al. 2004), and the effects of temperature on both the abundance and distribution of the species is therefore relatively unclear. Nevertheless, the species has been described as a warm-water multi-ocean species (Haimovici & Perez 1991, Sobrino et al. 2002). Despite the global distribution of the species, which may be considered indicative of a wide temperature tolerance, a number of authors have documented an expansion in the range of *O. vulgaris* to coincide with the increase in temperatures of previously uninhabited waters (Robin & Denis 1999, Oviatti 2004). Contemporary patterns therefore may be a consequence of a historical shift in distributional patterns in response to a drastic decline in sea surface temperatures within the Benguela current, during the mid-Pleistocene. Whilst it is unclear whether the species may have been able to tolerate such changes, it is likely that the preference for warmer waters (Haimovici & Perez 1991), would drive *O. vulgaris* to disperse to either the warmer northern or southern boundaries of the cold Benguela Current. This northern-southern Benguela distributional pattern may have been further supported by variations in upwelling intensity, which were likely to have arisen during fluctuations associated with glacial-interglacial cycles. The intensity of the upwelling regime in the Benguela Current has been documented to have undergone significant changes due to fluctuations in the intensity and zonality of trade winds as a consequence of climatic oscillations (van Leeuwen 1989, Hutchings et al. 2009). Dispersal between the northern and southern Benguela has been limited in a number of species due to the occurrence of the Lüderitz upwelling cell (Henriques 2012). Further intensification of this cell was therefore likely to enforce previously established distributions which may have arisen as a consequence of colder sea surface temperatures and stronger upwelling events.

Phylogenetic status of O. vulgaris in the Benguela Current region

Evidence from both the *cytb* and the *COI* based phylogenetic reconstructions suggest the presence of two main clades: an *O.* vulgaris type clade and an Indo-West Pacific clade. Interestingly, in all analyses the outlier-individual NBE14 from Angola grouped within the *O. vulgaris* clade, while the unidentified Eastern Cape individuals (EC6, EC11 and EC15) fell within the Indo-West Pacific clade. These findings suggest that these three specimens may be an introduction of exotic species in South African waters from Asia. A study conducted by Teske et al. (2007), examining the phylogeographic patterns of *O. vulgaris* within South Africa, also identified the presence of a divergent lineage near Durban port. As found within this study, Teske et al. (2007) suggested that the low numbers of this divergent lineage, in comparison to that of the *O. vulgaris* lineage, could be attributed to a species introduction due to the presence of major shipping lanes along the coast of the South Africa. Furthermore, the patchy distribution exhibited by these outlier individuals was seen to coincide with major harbours within the sampling range. The potential implication of the introduction of non-indigenous octopus species is further discussed in Chapter 5.

In all phylogenetic reconstructions *O. vulgaris* was found to be a non-monophyletic group, as demonstrated by Carlini et al. (2001), suggesting the need for a thorough systematic revision of the genus. For example, the Australian *Octopus tetricus* (Gould, 1852) specimens were found to consistently fall within the *O. vulgaris* clade in all *cytb* reconstruction methods, as observed by Guerra et al. (2010) and Guzik et al. (2005). However, when the *COI* dataset was used, *O. tetricus* no longer grouped with the *O. vulgaris* clade, while *O. areolatus* (de Haan in d'Orbigny, 1841) and *O. oshimai* (Sasaki, 1929) did. Furthermore, *O. vulgaris* specimens from Japanese and Chinese waters were found to be more closely related to *O. areolatus* and *O. oshimai* specimens from the Northwest Pacific, than to *O. vulgaris* specimens off Angola and South Africa.

The phylogenetic placement of *O. tetricus* within the *O. vulgaris* species group in the *cytb* data set is unlikely to be a result of a misidentification of the specimens, as a variety of tissue and GenBank samples were used in this study. The similar results in other phylogenetic studies on this genus (Guzik et al. 2005, Guerra et al. 2010), which are in agreement with these, suggests that the results are robust. The species is considered to exhibit differential morphology when compared with *O. vulgaris* descriptions (Guerra et al. 2010). Guerra et al. (2010) proposed that the high levels of observed branch support for *O. tetricus* clustering with *O. vulgaris* may indicate that the *O. vulgaris* species group is monophyletic. However, since comparative studies investigating morphological and molecular differentiation between these two species is lacking, further research may therefore be required to resolve this issue. Adaptation to alternative environments may result in high levels of morphological variation despite molecular similarities (Wiens & Penkrot 2002), and species descriptions based purely on morphological characters therefore may not be supported by molecular data (Hillis 1987). The placement of *O. tetricus* within the *O. vulgaris* clade therefore may potentially be a result of a previous classification error. Inferences regarding the cause of the placement of *O. tetricus* should be made with caution due to the lack of extensive literature surrounding this species.

Octopus areolatus (also known as *O. fangsiao* d'Orbigny, 1839-41) is considered to be another species surrounded by taxonomic confusion (Norman & Lu 2000, Roeleveld et al. 2003). The *COI* phylogenetic pattern which suggested that this species was grouped with the *O. vulgaris* clade was similar to those of Guerra et al (2010). This may be explained by a number of factors including the misidentification of *O. vulgaris* specimens as *O. areolatus*. The lack of comprehensive *Octopus* species identification guides, coupled with high morphological plasticity and lack of distinct taxonomic characters within this genus, is likely to lead to misidentification of specimens (Roper 1983). Misidentification has the potential to constitute a major impediment to fisheries management and conservation efforts within the genus. Alternatively the *O. vulgaris* specimens from Japan, China and the eastern Mediterranean which fell within the *O. areolatus/O. oshimai* group, may have been misidentified. It is also possible that the resultant phylogenetic incongruence may be a consequence of incomplete lineage sorting leading to the misplacement of the species (Guerra et al. 2010). This explanation may also be also be applicable to *Octopus oshimai*, whose lack of phylogenetic resolution is thought to be a consequence of its unresolved taxonomic status (Norman & Lu 2000).

All phylogenetic reconstruction approaches in this study contained polytomies and therefore a lack phylogenetic resolution. Polytomies, which are defined as multifuricating rather than bifuricating relationships within phylogenetic reconstructions, can take on two different forms, either soft or hard polytomies (Walsh & Friesen 2001, Braun & Kimball 2001). Soft polytomies are often considered to be a result of insufficient phylogenetic information, which in turn, limits the ability to infer interspecific relationships (Braun & Kimball 2001). On the other hand, hard polytomies are thought to be the outcome of true multiple concurrent speciation events resulting in a phylogenetic tree in which branching order cannot be effectively determined (Braun & Kimball 2001). Whitfield & Lockhart (2007) identified a number of factors that cause a lack of phylogenetic resolution when rapid radiation is not evident: i) use of an unsuitable marker; ii) discordance between data sets; iii) poorly constructed relationships due to poor methodology or model selection; and lastly iv) the use of inadequate data as a result of a number of factors such as the inclusion of highly incomplete taxa sampling.

Deviations to the assumption of neutrality as exhibited by the significant Tajima's D and Fu's F_S may therefore undermine marker suitability in the case of *cytb*. In this instance, substitutional saturation may mask the phylogenetic signal of functionally critical sites. This decreases the ability to describe the true evolutionary history of a species due to atypical differences between sequences (Rokas et al. 2003). However, as polytomies were observed in both *cytb* and *COI* data sets in this study, despite different approaches, marker suitability could most likely be excluded as a confounding factor, along with poor phylogenetic reconstruction methodology. The similarity in tree topology despite marker choice or reconstruction method, coupled with moderate to high levels of branch support, at shallower and deeper nodes, and low I_{SS} values indicate that sequence substitutional saturation may potentially be eliminated as the cause of poor reconstruction resolution. (Betancur-R 2010) suggested that it is difficult to exclude the possibility of inadequate data as a potential cause of unresolved phylogenetic relationships. However it is important to note that within the current study, despite the inclusion of a high number of species, polytomous branching patterns were still observed within the resultant topologies. Data may therefore be inadequate, in which case further studies including different markers may be required in order to resolve *Octopus* phylogenetic relationships. It is however important to note that rapid radiation events have been suggested within the Cephalopoda (Bonnaud et al. 1997). Carlini & Graves (1999) found extensive polytomous branching within oegopsid squids and attributed this multifuricating pattern to three life history attributes: i) the presence of cryptic speciation; ii) accelerated rates of evolution; and finally iii) cosmopolitan distributional patterns. All three of these life history attributes have been associated within *O. vulgaris*, and it is therefore possible that these three factors may be driving the occurrence of hard or near-hard polytomies within the current phylogenetic reconstructions (Belcari & Cuccu 2002). Unresolved relationships, illustrated by polytomous branching patterns, may therefore be indicative of a number of historical simultaneous speciation events, within the *Octopus* genus.

Concluding remarks

Genetic stock identification and assessment, on a temporal and spatial scale, of a species is critical for the development of sustainable fisheries around the world (Çiftci & Okumus 2002, von der Heyden et al. 2007b). Genetic analyses have played a key role in determining population connectivity levels, demographic histories and phylogenetic relationships of important fish stocks, however cephalopod data is limited (Ward 2000, Doubleday et al. 2009). The reliance upon *Octopus* as a fishery resource is considered to be on the rise due to the decline in more conventional finfish stocks, highlighting the need for comprehensive stock identification studies within the Cephalopoda.

The current study makes use of mtDNA to highlight the presence of two *O. vulgaris* populations within the Benguela region. However, due to the lack of sampling in Namibia, determination of the location of the biogeographic barrier responsible for the observed northern and southern structuring could not be accurately determined. Previous research within the Benguela has highlighted the Lüderitz upwelling zone as a barrier to dispersal in a number of species. Should this be the case for *O. vulgaris*, management protocols could be developed independently in Angola and South Africa.

Despite the observed molecular differences within the system, it is important to incorporate alternative stock identification approaches so as to provide a holistic approach towards management efforts (Begg & Waldman 1999). The ability to distinguish between species and stocks, based on morphological characters, is critical for the development of management strategies.

CHAPTER 4

Morphological comparison of *Octopus vulgaris* in a heterogeneous environment: the Benguela Current System

Introduction

Systematics can effectively be described as the study of biological diversity and of the evolutionary relationships, between and within both contemporary and historical organisms (Winston 1999). Understanding the systematics of any marine organism is therefore fundamental to the sustainable management of fisheries and the conservation of oceanic biodiversity (Roeleveld 1998, Baron & Re 2002). However, systematics is only of value if rigorous and if the accuracy is built on a foundation of comprehensive morphological and molecular research (Rosen & DeBach 1973).

Comparative morphological studies are critical in determining taxonomic units, distinguishing between species, as well as identifying populations and management stocks (Baron & Re 2002). The extent to which morphology has contributed towards the identification and discrimination of species has fluctuated over the past few decades (Strauss & Bond 1990). The morphological species concept, developed by Linnaeus, identifies species based on shared anatomical characteristics. The concept is based on the idea that in the absence of gene flow, populations are likely to be distinguishable due to the development of a number of localised, adaptive morphological traits (Freeman 2005). Although fundamental flaws in this concept have been recognized, morphology continues to form a vital component of taxonomy, and is considered the foundation of modern classification systems (or alpha taxonomy) (Scotland et al. 2003).

Extensive and accurate morphological analyses rely on the measurement of a comprehensive set of characters. Rohlf and Marcus (1993) recognised the importance of character choice and identify three important elements: repeatability, applicability and the ability to encompass important characteristics across the entire organism. The development of cephalopod description guidelines by Roper and Voss (1983) allowed for the identification and standardization of a comprehensive set of morphometric and meristic characters. These variables continue to guide morphological studies within this class; however the ability of these characters to discriminate between such morphologically plastic organisms has been questioned in recent years (Allcock et al. 2008, Doubleday et al. 2009).

Since its initial morphological description by Cuvier in 1797, there has been little taxonomic clarity in *Octopus vulgaris* (Warnke & Söller 2000, Doubleday et al. 2009). Theoretically, an analysis of morphometric variation should provide insight into the population structure within a species and potentially identify the presence of cryptic diversity. However, the soft bodied nature and lack of solid structures in *O. vulgaris* are thought to have undermined the efficacy of cephalopod taxonomy (Roper 1983). Furthermore, there are considered to be several factors that introduce bias in taxonomic studies of this nature, such as the use of poor preservation and fixation techniques and insufficient sample sizes (Roper 1983). An extensive literature review, combined with preliminary investigations allowed for the identification of confounding factors, within such a study. Recognition of the potential sources of bias allowed for error to be reduced, and where possible, completely eliminated in order to provide a more accurate representation of the levels of intra-specific morphological variation in *O. vulgaris* distributed throughout the Benguela Current region.

Intra-specific morphological variation arises as a consequence of either adaptive or non-adaptive processes, and can occur at a number of taxonomic levels such as between species, within species, as well as among individuals within populations (Mobley et al. 2011). Intra-specific morphological differentiation often results as a consequence of the distributional range of a species spanning across heterogeneous environments (Eklöv & Svanbäck 2006). It is important to note that the complex interactions between environmental conditions, genetic traits and phenotypic plasticity are all considered to be primary mechanisms driving the evolution of the anatomical structure of any organism (Mobley et al. 2011).

The high levels of spatial and temporal environmental variability across the Benguela Current region and the deep genetic divergence between northern and southern Benguela sub-systems (as discussed in Chapter 3) have the potential to play a major role in the development of inter-population morphological variation. Furthermore, patterns of morphological variation can, in many instances, be used to infer evolutionary and phylogenetic relationships between species, populations and individuals.

The aim of this present chapter is therefore to determine whether the deep genetic divergence, observed in Chapter 3, would correspond to morphological variation between the identified northern and southern Benguela *O. vulgaris* populations. Furthermore, the project aims to determine whether alpha taxonomy is of use within *Octopus* systematics.

Method and Materials

Details of the collection methods and sampling localities are described in Chapter 2.

Several preliminary studies were conducted to test the effect of the repeatability of morphological measurements, freezing, and sexual dimorphism on the morphological measurements.

The repeatability of morphological measurement was tested by repeating a set of 16 morphometric characters (detailed below) on a total of 20 specimens on the same day. Specimens were maintained at a temperature of 4°C between measurements in order to minimize the effects of degradation. Octopus specimens were measured in a random order, in order to reduce any potential bias associated with specimen recognition.

The effect of freezing was evaluated based on the results from 20 individuals. A total of 16 morphometric measurements (detailed below) were taken from fresh specimens and these were repeated once the specimens had remained frozen at -20°C for three weeks.

Furthermore, a set of 14 morphometric measurements (detailed below) were recorded for 20 male and 20 female *O. vulgaris* of similar sizes, in order to investigate the effects of sexual dimorphism within the study species.

A dependent t-test was used to investigate the effects of freezing and morphological variation on each of the chosen 16 morphometric variables. Effects of sexual dimorphism were investigated using an independent t-test. Characters which were classified as sexually dimorphic ($p < 0.05$) were standardized using an allometric adjustment equation and tested using a dependent t-test to determine the efficacy of data transformation in removing the differences between sexes.

Morphometrics

All samples were measured approximately three months after freezing in order to reduce any fixation discrepancies. Samples were partially defrosted, rinsed and blotted dry for laboratory analysis. The total body weight of each octopus was recorded to the nearest 0.01g. Morphometric analysis included a total of 60 measurements (Table 4.1). Character choice and measurement description were guided by Guerra *et al.* (2010), Leite *et al.* (2008), Roper and Voss (1983) for soft part analysis (Table 4.1). Hard part morphometry included both the upper and lower beak, consisting of eight measurements (Figure 4.1) (Lefkaditou & Bekas 2004, Doubleday et al. 2009, Perales-Raya et al. 2010). Once all soft part morphometric variables had been recorded, octopi were dissected ventrally to allow for gill lamellae counts to be conducted. All beaks were removed and stored in 70% ethanol, to allow for hard part morphometry at a later stage.

Morphometric measurements were conducted using a digital caliper (Insize Electronic Caliper Series 1112, 0.01 mm) by a single worker. All measurements which exceeded 300mm were measured to the nearest 0.1cm using a tape measure. Normal sucker diameter was determined by measuring the sucker found in both rows at a quarter, half and three quarters of the total arm length for the second left arm in order to standardise the data. Suckers with a width less than 2cm were photographed under reflected light using a dissection microscope at 10x magnification. SigmaScan Pro v.5 was then used to determine the width of suckers to the nearest 0.01mm.

The coiling of arms is a common problem associated with morphological studies on octopus (Roper & Sweeney 1983). Arm length (AL) was therefore obtained by running a piece of string down the midline of the tentacle, from the beak to the tip of the arm, the length of the string was then determined to the nearest 0.1cm using a tape measure. Total length (TL) was impacted by the coiling associated with the tentacles. A more accurate representation of the TL was obtained by adding the dorsal mantle length (DML), head length (HL) and the length of the longest arm.

Distinguishing or unusual characters were identified, noted and photographed when deemed necessary.

Table 4.1: Definition of soft part morphometric measurements recorded for *O. vulgaris.*

Character	Description	Abbreviation
Total length	Length from the apex of the mantle to the tip of the longest arm	TL
Total body weight	Weight of the whole octopus	TBW
Dorsal mantle length	Length from the dorsal midpoint between the eyes to the tip of the mantle	DML
Ventral mantle length	Length from the pallial aperture to the ventral tip of the mantle	VML
Mantle width	Width of the mantle at the greatest point	MW
Head length	Dorsal length from the midpoint between the eyes to the point of attachment between the first left and right arm	HL
Head width	Width at the widest point across the head	HW
Funnel length	Length of the funnel from the anterior opening to the posterior edge	FuL
Free funnel length	Length from the anterior opening of the funnel to the point of mantle attachment	FfuL
Pallial aperture	Width between the points of attachment of the mantle to the head	PA
Eye diameter	Diameter of the eye across the bulbous	EDI
Eye orifice diameter	Diameter of the eye opening	EO
Arm width	Width of the stoutest arm, at the widest point	AW
Arm length	Length from the beak to the tip of the arm	AL
Web depth	Length from the beak to the midpoint	WD
Web extension	Length from the beak to the furthest most point that the web extends along either side of the arm	WE
Enlarged sucker diameter	Diameter of the largest sucker on a designated arm	SDE
Normal sucker diameter	Diameter of the sucker located in pre-determined distances along the arm	¼ L& R, ½ L & R, ¾ L& R

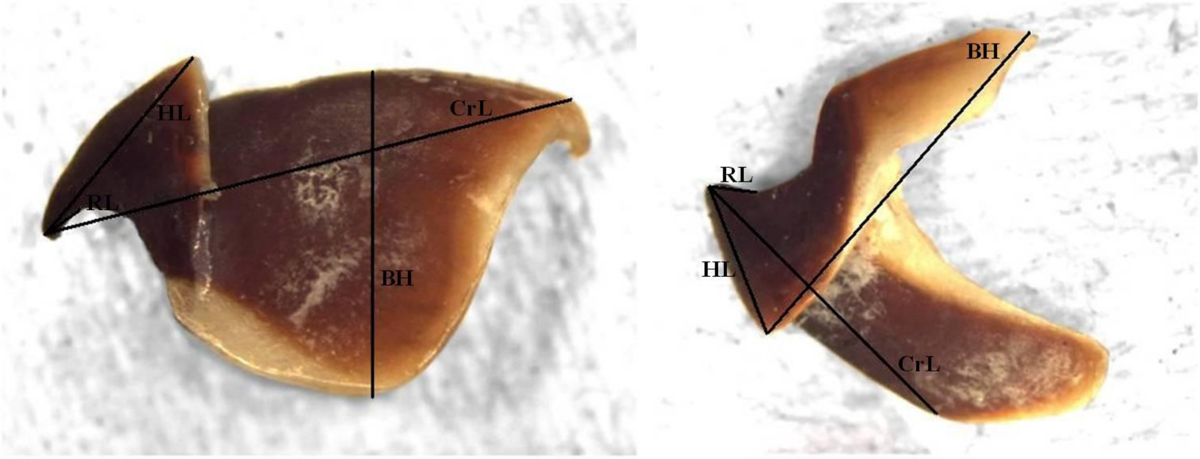

Figure 4.1: Upper (left) and lower (right) beak morphometry: beak height (BH), hood length (HL), crest length (CrL) and rostrum length (RL).

Meristics

Meristics analyses comprised three counts (Table 4.2). Where necessary, a dissection microscope was used.

Table 4.2: Description of meristic counts of *O. vulgaris.*

Character	Description	Abbreviation
Arm sucker count	Number of suckers on the designated arm	ASC
Gill lamellae count	Number of gill lamellae on outer demibranch	GiLC
Sucker row count	Number of sucker rows	SRC

Data Analysis

Statistical analyses were performed using Statistica v.11; however relationships between morphological variables and comparisons with additional morphometric studies were carried out using Microsoft Excel. Prior to morphological statistical analysis, genetic screening was conducted in Network to identify and exclude any specimens which exhibited extreme genetic differentiation from *O. vulgaris*.

Morphometrics and Meristics

Data analysis was guided by the techniques of Simon et al. (2010). As previous research has highlighted the issues surrounding the use of indices to discriminate between species, subspecies and populations, a multivariate approach was therefore undertaken (Baron & Re 2002, Allcock et al. 2008, Liao et al. 2010).

Prior to statistical analysis, box plots and regression analyses were used to identify the presence of outliers and extreme values within the data set. Identified data points were verified by referring to the original data sheets and re-measuring of the character, when possible. Outliers were classified as any variable which exceeded UBV+1.5*(UBV-LBV) or which were less than LBV+1.5*(UBV-LBV), where UBV represents the median + 75th percentile and LBV is defined as the median- 25th percentile. Extreme values were identified as any data point which were greater than UBV+3*(UBV-LBV) or less than LBV+3*(UBV-LBV). Due to the morphological variation and measurement error associated with measuring soft parts, outliers were not excluded from further analysis (Yezerinac et al. 1992, Jordaens et al. 2002, Allcock et al. 2008).

Morphometric and meristic data were analyzed separately as meristic variables were not adjusted according to size. Meristic data consists of discrete variables that are fixed early on in the developmental stages of most marine organisms (Turan et al. 2006, Simon et al. 2010). Effective morphological comparisons of specimens across a wide size range requires that morphometric measurements be independent of individual size (Simon et al. 2010). Allometry is therefore of particular importance when conducting any morphological study, as ontogenic development is often associated with a change in the shape or form of an organism (Chiu et al. 2002, Hassell et al. 2012). Recognition of the importance of allometric growth and the need for size independent data is therefore critical for the credibility and repeatability of morphometric research.

Standardization of morphometric measurements can be achieved through a variety of methods, including logarithmic transformation (Bookstein et al. 1985) and the use of ratios (Lleonart et al. 2000). However, allometric adjustment is the preferred method in the majority of studies (Reist 1985, Lleonart et al. 2000, Simon et al. 2010). Morphometric variables were therefore standardized using the overall mean total length of 606.07mm and the following allometric equation (Simon et al. 2010).

$$Y'_{ij} = logY_{ij} - b_j(logTL_i - log\overline{TL}),$$

where Y'_{ij} is the standardized value of character j for the i^{th} individual, Y_{ij} is the unstandardized morphometric value, b_j is the pooled regression coefficient of $LogY$ on $logTL$, TL_i is the total length of the i^{th} individual, and $\overline{TL}$ is the mean total length.

The efficacy of standardization was determined for each character by comparing the coefficient of determination before and after size transformation.

Measurements from the beaks were analysed separately from soft body parts as previous research indicates a difference in the discriminatory power between the two anatomical components (Vega et al. 2002).

A stepwise discriminant function analysis (DFA) was used to eliminate redundant characters from further analyses ($p > 0.05$). Non-redundant characters were then subjected to a principle component analysis (PCA) based on a variance-covariance matrix. The PCA was used to detect the overall morphological patterns, as well as identify characteristics which contributed towards the observed variability. Further, DFA allowed for the classification success to be determined.

Results

Preliminary Investigation

The WE character was the only variable which was significantly different between fresh and frozen samples ($p<0.05$) (Table 4.3) However, the repeatability of the readings was questionable, as significant differences ($p<0.05$) were observed between the first and second readings for a number of characters, including TL, TBW, WD and WE (Table 4.4). Sexual dimorphism was also observed as there were significant differences in seven of the 14 characters between male and female octopus (Table 4.5). However allometric adjustment, using the overall mean TL, eliminated the difference previously observed between sexes ($p <0.05$) (Table 4.8).

Table 4.7: Morphometric comparisons between fresh and frozen *O. vulgaris* based on 16 morphometric measurements (SD – standard deviation). Values in bold indicate significant differences ($p < 0.05$). See table 4.1 for measurements codes.

	Fresh		Frozen		
Character	Mean	SD	Mean	SD	p-value
TL	17.08	7.74	17.15	6.91	0.84
TBW	52.65	61.64	52.25	63.02	0.66
HL	19.24	7.32	18.80	6.90	0.38
HW	27.12	11.99	26.90	12.72	0.81
DML	33.50	14.15	34.11	12.79	0.28
VML	24.30	9.81	33.18	37.65	0.31
MW	28.63	11.48	28.05	11.46	0.14
FuL	14.98	6.56	15.46	6.13	0.40
FfuL	8.31	3.11	8.70	3.71	0.29
Edi	9.94	4.31	10.47	3.56	0.37
EO	2.01	0.83	2.22	0.71	0.39
PA	16.73	6.87	17.10	6.04	0.61
AW	7.57	2.93	7.68	2.83	0.58
AL	12.02	6.11	12.76	5.37	0.45
WD	27.69	15.63	25.89	12.93	0.17
WE	12.02	32.53	36.18	48.62	**0.01**

Table 4.4: Repeatability of 16 morphological measurements in *O. vulgaris* (SD – standard deviation). Values in bold indicate significant differences ($p < 0.05$). See table 4.1 for measurements codes.

	Reading 1		Reading 2		
Character	Mean	SD	Mean	SD	p-value
TL	18.29	8.08	19.23	9.21	**0.01**
TBW	58.91	68.61	57.03	66.95	**< 0.01**
HL	27.97	13.34	27.28	13.59	0.42
HW	19.30	7.09	19.87	7.39	0.22
DML	35.46	13.93	37.03	16.69	0.16
VML	33.87	36.83	24.87	11.02	0.28
MW	28.56	11.40	29.82	13.70	0.24
FuL	16.15	6.76	17.49	7.99	**0.02**
FfuL	9.23	4.04	9.35	3.58	0.74
Edi	10.78	3.75	11.23	3.87	0.11
EO	2.31	0.80	2.51	0.67	0.23
PA	17.20	6.11	17.90	7.41	0.35
AW	7.88	2.90	7.88	2.57	1.00
AL	13.49	6.22	13.75	7.16	0.66
WD	28.42	15.34	32.14	18.74	**0.02**
WE	31.64	42.55	5.64	1.19	**< 0.01**

Table 4.5: Comparisons of 14 morphometric measurements (SD – standard deviation) between males and females *O. vulgaris* from South Africa. Values in bold indicate significant differences ($p < 0.05$). See table 4.1 for measurements codes.

	Female		Male		
Character	Mean	SD	Mean	SD	p-value
HL	57.54	24.48	39.63	16.08	**< 0.01**
HW	37.81	13.15	28.98	12.61	**0.04**
DML	69.89	33.35	52.70	20.64	0.057
VML	56.39	22.73	39.21	13.47	**< 0.01**
MW	58.61	21.19	44.68	18.15	**0.03**
FuL	30.36	18.30	25.70	11.25	0.34
FfuL	17.53	7.66	13.96	5.59	0.10
Edi	19.47	5.30	14.65	4.32	**< 0.01**
EO	3.86	1.51	3.04	1.21	0.07
PA	29.03	22.85	26.66	13.34	0.69
AW	16.38	6.31	11.86	4.49	**0.01**
AL	27.58	11.16	20.01	8.58	**0.02**
WD	52.03	22.51	43.30	20.39	0.21
WE	95.54	39.31	86.75	41.12	0.49

Table 4.8: Comparison of the variation in 14 morphometric characters of male and female *O. vulgaris* after allometric size adjustments (SD – standard deviation). Values in bold indicate significant differences ($p < 0.05$). See table 4.1 for measurements codes.

	Female		Male		
Character	Mean	SD	Mean	SD	p-value
HL	4.27	0.16	4.20	0.27	0.33
HW	3.82	0.21	3.79	0.19	0.63
VML	4.27	0.11	4.23	0.12	0.35
MW	4.32	0.19	4.33	0.09	0.77
Edi	3.15	0.10	3.08	0.13	0.06
AW	3.02	0.22	3.00	0.12	0.73
AL	3.55	0.11	3.56	0.24	0.88

Morphometrics

Soft parts

Octopus ranged in size between 406.83-1042.92mm with specimens from the EC smaller than those collected in NBE (Table 4.7). As a consequence of the difference in sizes between locations, octopus from NBE were generally associated with the highest means for all soft and hard part morphometric measurements, and the EC specimens with the lowest average measurements (Table 4.7).

Table 4.7: Descriptive statistics of soft part morphometrics for *O. vulgaris* collected from Namibe (NBE) and Baía dos Tigres (BdT) in Angola and from the Western (WC) and Eastern Cape (EC) of South Africa (SD – standard deviation). See table 4.1 for measurements codes.

	NBE					BdT					WC					EC				
Character	Mean	Median	Min	Max	SD	Mean	Median	Min	Max	SD	Mean	Median	Min	Max	SD	Mean	Median	Min	Max	SD
TL	1 042.92	1 079.60	502.24	1 405.82	287.86	497.30	477.03	116.19	961.00	170.07	703.29	715.19	177.90	846.03	118.67	406.83	399.78	106.74	894.24	207.87
HL	116.64	118.03	56.22	173.84	32.34	61.99	58.68	12.90	188.00	26.87	72.18	76.72	21.61	98.25	18.75	47.96	44.26	14.12	118.18	24.24
HW	57.03	54.44	26.73	84.80	14.86	42.12	40.48	10.60	84.00	15.39	41.68	41.48	10.81	57.85	9.84	32.67	31.28	11.34	66.46	14.53
DML	167.10	175.87	65.82	233.00	46.12	83.30	82.55	21.29	136.78	25.62	116.23	117.30	35.29	146.00	20.00	60.34	55.09	17.09	146.99	32.32
VML	113.02	108.03	48.52	197.81	39.27	63.42	60.11	15.36	103.00	20.64	81.62	83.04	17.07	113.24	16.59	46.57	45.10	13.27	106.02	23.29
MW	115.81	123.45	57.57	141.30	26.64	63.71	63.05	18.17	126.00	20.74	85.00	87.94	14.47	115.57	16.36	52.56	51.72	15.38	109.87	25.54
FuL	79.44	84.04	29.69	109.70	22.71	37.03	36.96	5.97	62.59	13.88	58.96	60.45	13.05	90.37	11.79	28.15	23.08	8.41	72.21	18.28
Fful	45.44	49.16	21.39	71.92	12.92	23.96	23.32	4.68	41.43	7.67	34.19	33.96	6.71	47.11	7.25	15.87	14.30	3.86	39.57	9.40
Edi	27.70	28.55	20.06	35.66	4.61	21.90	21.79	6.32	37.96	6.79	21.50	21.27	6.17	36.73	4.89	16.68	16.25	5.54	31.20	6.31
Edo	11.68	11.98	8.18	16.51	2.45	4.78	4.48	1.44	14.24	2.29	9.12	8.99	1.05	22.14	3.26	3.50	3.08	0.97	9.70	1.98
PA	88.93	90.80	66.77	108.30	11.63	42.41	41.87	4.90	78.08	14.66	55.74	54.55	8.37	85.86	12.32	28.39	26.35	0.77	75.18	21.38
AW	30.89	30.57	16.47	43.60	8.23	19.75	18.98	4.02	48.00	8.14	20.24	20.62	6.86	35.70	4.65	14.13	13.57	3.98	28.04	6.72
AL	599.23	608.00	217.00	1 057.00	202.88	292.48	278.00	57.00	649.00	114.71	412.85	409.00	430.00	634.00	105.36	244.43	225.80	16.00	963.50	140.21
¼	17.88	15.71	4.97	44.72	8.81	9.88	9.31	1.47	23.83	4.19	11.69	11.91	2.80	19.62	3.10	7.93	6.71	1.00	16.68	4.45
½	9.24	9.72	2.16	16.50	3.67	6.48	6.18	0.92	13.87	2.91	6.65	6.79	0.96	11.01	1.85	4.29	3.36	0.55	10.11	2.71
¾	3.06	3.05	0.83	6.51	1.38	2.38	1.89	0.42	8.39	1.55	2.19	2.13	0.92	5.12	0.76	2.45	2.00	0.21	10.33	2.15
Sde	21.35	20.45	5.83	44.72	10.33	11.87	10.87	3.01	23.83	4.81	13.35	13.65	1.99	20.91	3.73	9.81	9.35	2.25	23.23	5.27
WD	109.25	104.58	0.76	195.42	39.81	63.51	63.00	4.06	161.00	29.31	77.56	78.51	16.10	151.51	25.90	45.28	39.98	0.90	127.86	30.37
WE	157.68	158.00	44.00	743.00	67.67	117.27	98.00	9.00	403.00	71.50	93.60	77.50	9.00	370.00	64.75	93.60	77.50	9.00	370.00	64.75

The overall Wilk's λ was significant (λ = 0.19, p < 0.01), indicating relatively high levels of variance between octopus collected from different regions, with 19% of the total variability being unexplained. Of the 20 variables subjected to the forward DFA, seven morphometric characters (Edi, Fful, Edo, DML, ¾R, AW, PA) were extracted from the model (p < 0.05).

The first three principle components (PC) explained 64.81% of the total morphological variation of the soft parts (Table 4.8). PC1 accounted for 25.83% and all of the loadings were negative. A total of 23.71% of the morphological variation was explained by PC2 (Table 4.8), with the measurements Fful, Edo and DML (large negative values) exhibiting the highest correlation with this component. PC3 comprised primarily of negative loadings and described 15.26% of the variation (Table 4.8). The measurement Edo (large negative loading), ¾R and PA (large positive loadings) were observed to be the main discriminatory variables within PC3 (Table 4.8). The PC1 and PC2 scatter-plot showed a high level of overlap between the four regions (Figure 4.2).

Table 4.8: Principle component loadings, eigenvalues and cumulative variance for the seven soft-part morphometric characters of *O. vulgaris* collected from Namibe (NBE) and Baía dos Tigres (BdT) in Angola and the Western Cape (WC) and Eastern Cape (EC) of South Africa. See table 4.1 for measurements codes.

Morphometric character	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Edi	-0.73	0.38	-0.35	-0.00	0.14	0.08	0.42
FfuL	-0.48	-0.58	-0.19	-0.28	0.08	-0.56	-0.06
Edo	-0.17	-0.51	-0.59	0.55	0.05	0.18	-0.16
DML	-0.12	-0.69	-0.35	-0.20	0.47	0.34	0.07
AW	-0.72	0.43	-0.04	-0.28	0.02	0.23	-0.39
¾R	-0.45	0.23	0.58	0.55	0.22	-0.23	-0.05
PA	-0.45	0.23	0.58	0.55	0.22	-0.23	-0.05
Eigen value	1.81	1.66	1.07	0.81	0.67	0.60	0.38
% total variance	25.83	23.71	15.26	11.58	9.61	8.57	5.43
Cumulative %	25.83	49.54	64.81	76.39	86.00	94.57	100.00

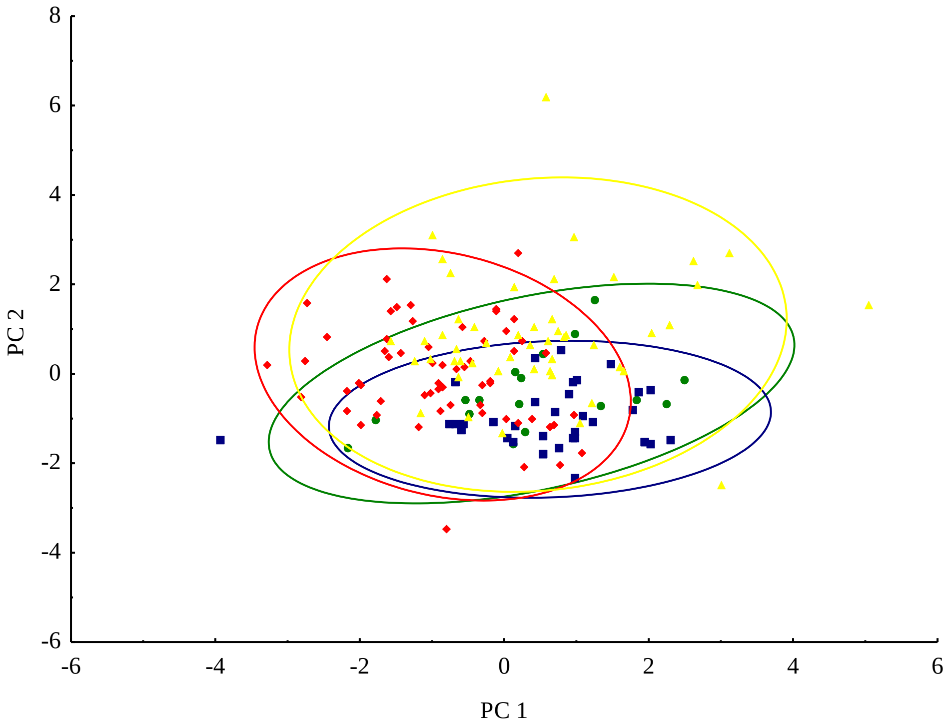

Figure 4.2: Principle component analysis scatter-plot (with 95% confidence ellipsoids) for seven morphometric measurements for *O. vulgaris* specimens from Namibe (NBE = green) and Baía dos Tigres (BdT = red) in Angola and the Western Cape (WC = blue) and Eastern Cape (EC = yellow) of South Africa.

The DFA detected significant levels of discrimination between *O. vulgaris* from the four different sampling sites ($\lambda = 0.34$ approx, $p < 0.01$) (Table 4.9). Of these, the DML (Partial $\lambda = 0.90$, $p < 0.01$) and AW (Partial $\lambda = 0.92$, $p < 0.01$) were important discriminatory characters. PA was the only variable that showed no significant difference ($p = 0.14$) between individuals from the four sampling localities.

Table 4.9: Effectiveness of seven morphological variables in distinguishing between *O. vulgaris* from four different localities across the Benguela current. Number of variables in the model = 8, Wilk's λ = 0.34 approx, F (21,439) = 9.59, p <0.01. See table 4.1 for measurements codes.

Character	Wilks' λ	Partial λ	F-remove	p-value	Tolerance
Edi	0.43	0.78	14.19	<0.01	0.67
FfuL	0.38	0.88	6.63	<0.01	0.90
Edo	0.41	0.83	10.33	<0.01	0.83
DML	0.38	0.90	5.83	<0.01	0.86
¾R	0.39	0.87	7.43	<0.01	0.83
AW	0.37	0.92	4.15	<0.01	0.78
PA	0.35	0.96	1.87	0.14	0.87

The first two canonical variables (CV) cumulatively described 98% of the morphological variation in *O.vulgaris* (Table 4.10) The CV1 accounted for 66% of the total variation, with Edi being identified as an important discriminatory characteristic for this function. There were low levels of discrimination between *O. vulgaris* from different localities, which was illustrated by the lack of separation and high level of overlap between the 95% confidence ellipsoids (Figure 4.). The CV2 described 32% of the variation, while ¾R contributed little to the discrimination between octopus from different regions across the Benguela (Table 4.10).

Table 4.10: Standardized canonical coefficients and classification functions for the soft- part morphometric variables, extracted from the forward stepwise DFA, for *O. vulgaris* collected from Namibe (NBE) and Baía dos Tigres (BdT) in Angola and the Western Cape (WC) and Eastern Cape (EC) of South Africa. See table 4.1 for measurements codes.

Character	Standard coefficients			Classification functions			
	CV1	CV2	CV3	NBE	BdT	WC	EC
Edi	0.76	-0.34	-0.45	147.34	158.47	146.64	152.15
Fful	0.26	0.45	0.27	-17.33	-21.24	-16.66	-19.757
Edo	-0.53	0.32	0.26	193.24	196.77	192.77	190.58
DML	0.42	-0.35	-0.01	-21.46	-22.89	-21.58	-20.84
¾R	-0.48	0.01	0.68	101.78	104.83	98.79	100.10
AW	0.42	0.22	0.44	1.63	1.907	1.28	0.81
PA	0.21	0.47	0.03				
Eigen value	0.96	0.47	0.03				
Cum. Prop	0.66	0.98	1.00				
Constant				-877.32	-939.95	-873.112	-862.05

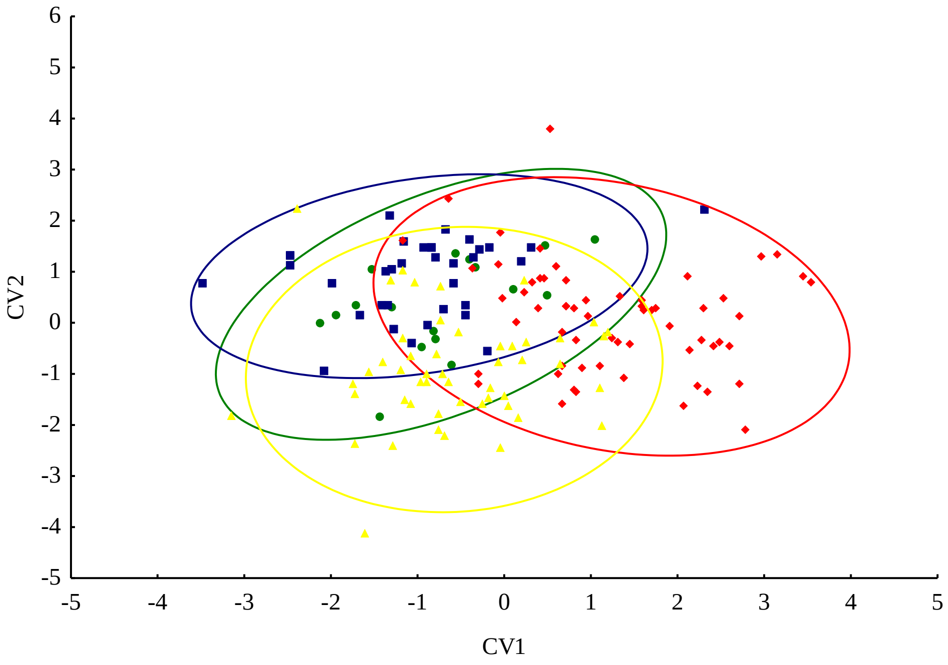

Figure 4.3: Canonical scores with 95% confidence ellipsoids for soft-part morphometric data for *O. vulgaris* collected from Namibe (NBE = green) and Baía dos Tigres (BdT = red) in Angola and the Western Cape (WC = blue) and Eastern Cape (EC = yellow) of South Africa.

The DFA assigned 69.9% of the octopus correctly to their localities (Table 4.). The highest classification success was for individuals from BdT (85.3%), while all individuals from NBE were misclassified.

Table 4.11: Classification results of the DFA using soft part morphometric characters to distinguish between *O. vulgaris* from two localities in southern Angola (NBE & BdT) and two localities in South Africa (WC & EC).

Group	n	Percentage correctly classified, with number of correctly classified individuals in parenthesis			
		NBE	BdT	WC	EC
NBE	17	**0 (0)**	23.5 (4)	47.0 (8)	29.4 (5)
BdT	61	0 (0)	**85.3 (52)**	9.8 (6)	4.92 (3)
WC	35	0 (0)	8.6 (3)	**74.3 (26)**	17.1 (6)
EC	50	0 (0)	18.0 (9)	10.0 (5)	**72.0 (36)**
				Total correct (%)	**69.9**

Hard parts

The overall trends for the soft part analyses were similar to the beak analysis. Generally the larger octopus from NBE had the highest mean character lengths/widths, while the smaller octopus from the EC had the lowest mean values (Table 4.12). The standard deviation was, in all cases, highest for octopus from the WC and the lowest in octopus from the EC (Table 4.12).

Table 4.12: Descriptive statistics of hard part morphometric variables for *O. vulgaris* collected from Namibe (NBE) and Baía dos Tigres (BdT) in Angola and the Western Cape (WC) and Eastern Cape (EC) of South Africa (SD- standard deviation). See table 4.1 for measurements codes.

	NBE					BdT					WC					EC				
Character	Mean	Median	Min	Max	SD	Mean	Median	Min	Max	SD	Mean	Median	Min	Max	SD	Mean	Median	Min	Max	SD
UB-HL	9.10	9.56	4.90	11.24	1.93	6.82	7.06	0.90	10.39	2.17	7.48	7.57	2.67	9.74	1.15	5.32	5.21	1.64	10.56	2.40
UB- H	15.20	16.17	8.77	18.46	2.98	10.68	10.80	2.19	17.00	3.31	11.33	11.36	3.41	13.66	1.79	8.28	8.00	2.69	15.46	3.72
UB- CrL	26.45	28.13	13.24	33.66	6.04	17.71	17.29	3.92	29.22	5.90	19.44	19.52	6.49	24.32	2.85	13.43	13.03	4.91	27.09	5.86
UB- RL	7.70	8.10	4.63	9.25	1.50	5.64	5.57	1.38	9.29	1.60	6.03	6.10	1.98	7.70	0.91	4.27	4.27	1.40	8.04	1.80
LB- HL	6.76	7.33	3.49	9.71	1.81	5.26	5.29	1.30	8.56	1.69	5.56	5.70	1.84	7.55	1.00	3.98	3.90	1.13	8.25	1.86
LB- H	19.85	20.18	11.03	25.85	4.41	13.13	13.25	3.70	20.71	3.80	14.04	13.97	5.15	17.07	2.05	10.46	9.88	3.30	19.55	4.71
LB-CrL	15.40	16.76	8.23	20.10	3.44	10.76	10.38	2.63	17.30	3.43	12.03	12.39	3.56	14.84	1.86	8.58	8.38	2.72	16.43	3.77
LB- RL	7.41	8.30	3.84	9.90	1.87	5.64	5.70	1.13	8.85	1.80	6.06	6.06	2.05	7.98	0.98	4.42	4.33	1.07	8.69	2.06

The overall Wilk’s λ indicated a significant difference between octopus from different localities (λ =0.38, p < 0.01). Four (LB-HL, LB-H, LB-CrL, UB-H) of the seven morphometric characters were extracted by the DFA (p < 0.05).

The PC1 accounted for 87.5% of the variation, while the other three components described little of the distribution (Table 4.13). All PC1 loadings were highly negatively correlated and contributed similarly to the discrimination. The PC1 and PC2 scatter-plot revealed a large amount of overlap in all four hard-part morphometric characters between the four sampling sites (Figure 4.4).

Table 4.13: Principle component loadings, eigenvalues and cumulative variance for the four hard-part morphometric characters of *O. vulgaris* collected from NBE and BdT (Angola) and WC and EC (South Africa). See table 4.1 for measurements codes.

Morphometric character	PC1	PC2	PC3	PC4
LB- HL	-0.93	-0.33	0.04	-0.16
LB- H	-0.93	0.18	0.33	0.03
LB-CrL	-0.95	-0.10	-0.13	0.26
UB- H	-0.93	0.25	-0.24	-0.13
Eigen value	3.50	0.21	0.18	0.11
% total variance	87.5	5.2	4.6	2.7
Cumulative %	87.3	92.7	97.3	100.00

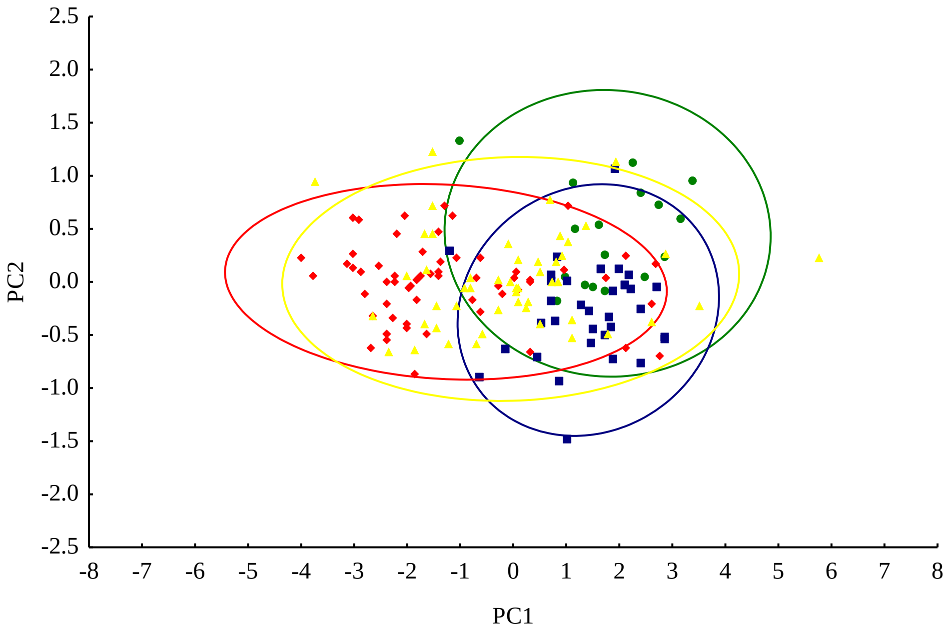

Figure 4.4: Principle component analysis scatter-plot, with 95% confidence ellipsoids, of four hard part morphometric measurements collected from *O. vulgaris* specimens collected from Namibe (NBE = green) and Baía dos Tigres (BdT = red) in Angola and the Western Cape (WC = blue) and Eastern Cape (EC = yellow) of South Africa.

There were significant levels of discrimination between *O. vulgaris* from the four different sampling localities ($\lambda = 0.43$ approx, $p < 0.01$) (Table 4.14). Although LB-CrL (Partial $\lambda = 0.94$, $p = 0.01$) and UB-H (Partial $\lambda = 0.94$, $p = 0.02$) were important discriminatory characters, significant differentiation ($p < 0.01$) between octopus from different localities was only found for LB-HL and LB-H (Table 4.14).

Table 4.14: The effectiveness of 4 morphological variables, associated with the beak, in distinguishing between *O. vulgaris* from four different localities across the Benguela Current. Number of variables in the model = 8, Wilk's $\lambda = 0.43$ approx, F (12,413) = 12.92, $p < 0.01$. See table 4.1 for measurements codes.

Character	Wilks' λ	Partial λ	F-remove	p-value	Tolerance
LB-HL	0.52	0.83	10.32	<0.01	0.29
LB-H	0.51	0.84	10.03	<0.01	0.31
LB-CrL	0.46	0.94	3.60	0.01	0.22
UB-H	0.46	0.94	3.27	0.02	0.33

The first two canonical variables (CV) cumulatively accounted for 96% of the total morphological variation (Table 4.). The CV1 described 73% of the variation between octopus from different sampling localities, with the variable LB-HL identified as the most important discriminatory characteristic for this function. LB-H contributed the least to the discrimination between the four sampling sites for this function. High levels of overlap were observed for all four sites (Figure 4.**5**).

Table 4.15: Standardized canonical coefficients and classification functions for the hard- part morphometric variables of *O. vulgaris* extracted from the forward stepwise DFA.

Character	Standard coefficients			Classification functions			
	R1	R2	R3	NBE	BdT	WC	EC
LB- HL	0.73	1.13	1.24	-247.64	-225.50	-228.77	-234.58
LB- H	0.12	-1.49	0.13	292.56	277.69	263.71	275.47
LB-CrL	-0.36	0.65	-1.98	219.97	220.32	231.73	231.90
UB- H	0.58	-0.38	0.35	29.62	37.28	24.67	30.54
Eigen value	0.73	0.30	0.04				
Cum. Prop	0.68	0.96	1.00				
Constant				-485.82	-500.78	-456.48	-491.42

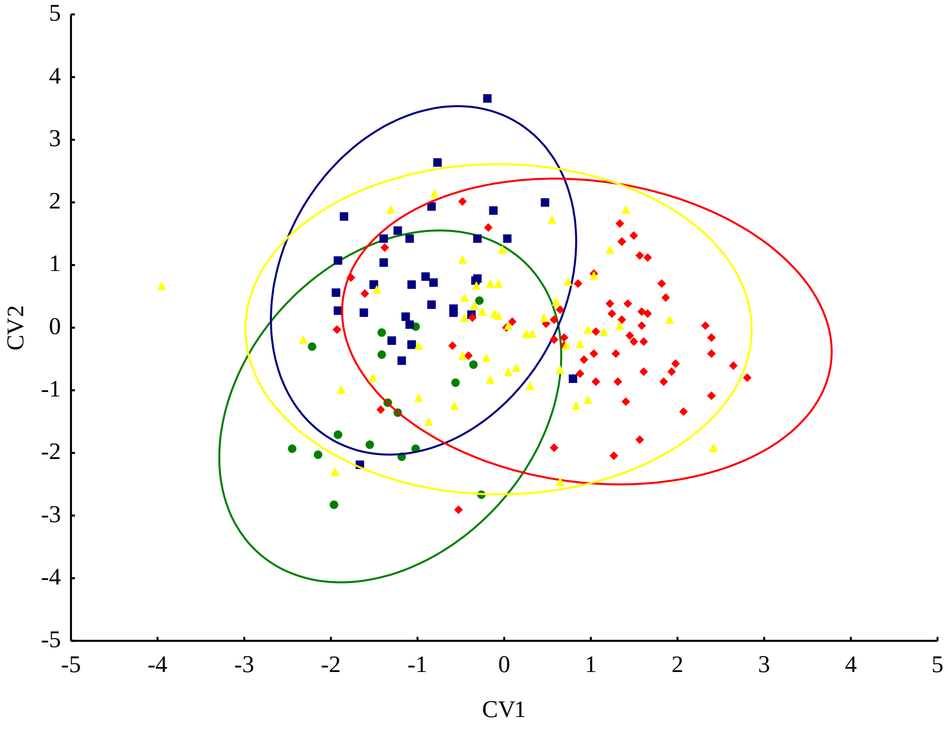

Figure 4.5: Canonical scores with 95% confidence ellipsoids for hard-part morphometric data for *O. vulgaris* collected from Namibe (NBE = green) and Baía dos Tigres (BdT = red) in Angola and the Western Cape (WC = blue) and Eastern Cape (EC = yellow) of South Africa.

Overall, the DFA assigned 66.3% of octopi correctly to their localities. The classification success was highest (82.0%) for specimens from BdT and lowest for individuals from EC (50.0%) (Table 4.16).

Table 4.9: Classification results of the discriminant function analysis using hard-part morphometric characters to distinguish between *O. vulgaris* from two localities in southern Angola , Namibe (NBE) and Baía dos Tigres (BdT) and two localities in South Africa, the Western Cape (WC) and Eastern Cape (EC).

Group	n	Percentage correctly classified, with number of correctly classified individuals in parenthesis			
		NBE	BdT	WC	EC
NBE	17	**52.9 (9)**	5.9 (1)	11.8 (2)	29.4 (5)
BdT	61	3.3 (2)	**82.0 (50)**	9.8 (6)	4.9 (3)
WC	35	2.9 (1)	5.7 (2)	**68.6 (24)**	22.9 (8)
EC	50	4.0 (2)	36.0 (18)	10.0 (5)	**50.0 (25)**
				Total correct (%)	**66.3**

Meristics

The number of sucker rows (SRC) was constant (n= 2) between all octopus collected from all four localities and was therefore excluded from any further analysis (Table 4.17). The arm sucker count (ASC) was seen to range between 203.60 and 225.21, with EC and WC specimens having on average the lowest and highest number of suckers per tentacle, respectively (Table 4.17). However, standard deviation for WC was considerably much lower than that of specimens for other localities. The GiLC was constant for all specimens (n = 9), except for one specimen from the EC (n = 6) (Table 4.17).

There was no differentiation in the meristic characteristics of octopus collected from different sampling localities (Wilk's $\lambda = 0.96$, $p < 0.71$) and thus no further analyses was deemed necessary.

Table 4.17: Descriptive statistics of meristic variables for *O. vulgaris* collected from two localities in southern Angola , Namibe (NBE) and Baía dos Tigres (BdT) and two localities in South Africa, the Western Cape (WC) and Eastern Cape (EC). (SD- standard deviation). See table 4.1 for measurement codes.

	NBE					BdT					WC					EC				
Character	Mean	Median	Min	Max	SD	Mean	Median	Min	Max	SD	Mean	Median	Min	Max	SD	Mean	Median	Min	Max	SD
ASC	216.47	229	111	227	46.63	220.27	228.5	92	295	41.21	225.21	228	130	295	28.71	203.6	215.5	85	294	45.94
SRC	2	2	2	2	0	2	2	2	2	0	2	2	2	2	0	2	2	2	2	0
GiLC	9	9	9	9	0	9	9	9	9	0	9	9	9	9	0	8.98	9	6	12	0.64

Discussion

Preliminary Investigation

As discussed in Chapter 2, fixation is a critical component of any morphological study in order to ensure that the anatomical structure and nature of the specimen is maintained; however, this fundamental process can play a major role in undermining the accuracy of comparative morphometric research. Although freezing was shown to have no significant effect on 15 of the 16 morphometric variables (Table 4.3), the inconsistent results observed in the second preliminary study (Table 4.4) places into question the reliability of taxonomic methods for these cephalopods due to the low levels of repeatability. Inconsistency between measurements, as observed within the current study, may be a consequence of either random or systematic measurement error (ME) (Muñoz-Muñoz & Perpiñán 2010). Random measurement error is generally associated with unpredictable fluctuations between measurements, whilst systematic error primarily results in directional and predictable variation (Muñoz-Muñoz & Perpiñán 2010). Identifying and understanding the causes and limitations associated with these different types of ME is critical in maintaining the credibility of any study.

Various factors have been found to decrease measurement precision and repeatability within soft part morphometrics, thereby potentially undermining the role of morphometry as a taxonomic tool (Roper & Sweeney 1983, Yezerinac et al. 1992, Arnqvist & Martensson 1998, Jordaens et al. 2002, Liao et al. 2010, Muñoz-Muñoz & Perpiñán 2010). Preservation technique, reader bias, accuracy of measuring instruments, flexibility of anatomical structures and a lack of definitive landmarks all play a major role in determining the reliability of morphometric studies (Roper & Sweeney 1983, Yezerinac et al. 1992, Arnqvist & Martensson 1998, Jordaens et al. 2002, Liao et al. 2010, Muñoz-Muñoz & Perpiñán 2010). As the reader, the measuring implements and the preservation technique remained standardized throughout the duration of the study, the significant differences in morphometric measurements could most likely be attributed to the structure of the study specimens. Jordaens *et al.* (2002) investigated the high levels of morphometric variation associated with soft, flexible characters, and concluded that such structures should be used provisionally and with extreme prudence within comparative morphometric studies, due to the high level of potential bias they may introduce into the results. All characteristics which were found to exhibit significant differences ($p < 0.05$) between readings were therefore excluded from further analyses.

Allometric growth (Lleonart et al. 2000) and sexual dimorphism (Hassell et al. 2012) have been identified as two factors that potentially have major effects on the results of comparative morphometric studies. Recognition of confounding factors such as allometry and sex is a critical component of experimental design (Baron & Re 2002). Ecological pressures, discrepancies in reproductive demands, and the outcomes of sexual selection have been characterised as the three primary processes driving shape dimorphism (Hassell et al. 2012). Of the 14 morphometric characters tested for sexual dimorphism, half were found to exhibit significant ($p < 0.05$) differences between male and female octopus (Table 4.5). Such significant differences could however be attributed to sexual size dimorphism. However, the allometric bias correction factor (Simon et al. 2010) appeared to eliminate the effects of shape dimorphism (Table 4.6) and the previously sexual dimorphic characters were no longer significant ($p > 0.05$) once this standardisation was applied. The size transformed data was therefore analysed for combined sexes and the sample size was therefore maintained. Since sample size is considered to be of utmost importance for soft bodied morphometric studies by providing more credible results (Jordaens et al. 2002, Liao et al. 2010), the amalgamation of the male and female data was critical for robust results in this study.

Comparative morphometrics

The morphometric analysis of the soft-parts and hard-structures provided similar overall patterns of discrimination for *O. vulgaris* collected from the four different localities. In both cases, there were high levels of overlap (Figure 4.2, Figure 4.4) and little morphological differentiation between sampling sites. This trend was supported by the inability of the DFA to correctly assign octopus to their home region. The lack of significant regional phenotypic differentiation emphasizes the inability of morphometric and meristic characteristics to distinguish between *O.* vulgaris at a population level. Allcock et al. (2008) highlighted the inability to distinguish between cephalopods at a species level when relying upon morphometric and meristic variables. Morphological uniformity, both within and between species is common for animals within the class cephalopoda and the lack of hard structures coupled with high levels of character plasticity are considered to be the primary factors undermining the use of morphology within cephalopod systematics (Allcock et al. 2008). Roper (1983) suggested that comprehensive population structure and speciation studies within this genus require a more integrative systematic approach.

The absence of geographical variability in morphometric attributes, in any organism, could arise as a consequence of two alternative mechanisms: the lack of significant population sub-structuring within the species across the study region (Palumbi 2003), or the outcome of genetic differentiation masked by morphological stasis (Jackson & Cheetham 1999, Santos et al. 2006, Lavoué et al. 2011).

The inability to discriminate between species, subspecies or even populations based on morphological characteristics due to low levels of genetic diversity has been demonstrated by a number of species (e.g. king weak – *Macrodon ancylodon*; copepod – *Eurytemora affinis*; and the African freshwater butterfly fish – *Pantodon buchholzi*) (Chouinard et al. 1996, Taylor & Dodson 1994, Bowen et al. 2001). Low levels of genetic divergence over an extensive geographical distributional range, such as the Benguela system, is a common occurrence within marine environments (Grillo 2011, Palumbi 2003). Absence of population structure, driven by genetic differentiation, is often associated with a lack of effective reproductive barriers and an increased capability to disperse throughout the environment (Grillo 2011). Marine organisms, particularly those with extended planktonic larval stages such as *O. vulgaris*, often exhibit low levels of genetic structure due to the ability to disperse across increased spatial scales (Santos et al. 2006, Von der Heyden 2009). However, as highlighted in the previous chapter (Chapter 3), significant genetic differentiation was found to occur between northern and southern Benguela *O. vulgaris* populations.

Advances in molecular research have unveiled major issues surrounding the use of morphology to infer taxonomic relationships, thereby further undermining the applicability of the morphological species concept (MSC) (Harrison 1991, Féral 2002, Hebert & Gregory 2005, Hoolihan et al. 2006). Similarities in the morphology between species, subspecies or populations which are characterised by significant levels of genetic differentiation may thus be the outcome of a number of possibilities, such as: a delayed morphological evolutionary response to recently genetically diverged populations (Chouinard et al. 1996, Bickford et al. 2006); niche uniformity throughout the geographical range (Chouinard et al. 1996, Bowen et al. 2001) or finally, the occurrence of stabilizing selection for homogenous phenotypes (Taylor & Dodson 1994).

While it remains relatively unclear which of these mechanisms are driving the observed similarity in phenotype in *O. vulgaris* across the Benguela Current region, the inability to morphometrically distinguish between eastern and western Atlantic *O. vulgaris* populations

(Warnke et al. 2004), coupled with the cosmopolitan distribution of the species (as outlined in Chapter 1) and the diversity of ecological niches throughout its distribution, suggests that the morphological stasis is unlikely to be associated with niche homogeneity. The question therefore arises: could the flexible anatomical structure, which allows for the remarkable adaptability of life history and behavioural attributes of this species, be the result of morphological conservatism of the optimal phenotypic expression? In order to gain a comprehensive understanding of octopus morphological conservatism, further research should aim to investigate the roles of adaptive and non-adaptive evolutionary mechanisms responsible for the maintenance of morphological uniformity between widespread, historically isolated populations.

Ultimately these findings highlight the inability to determine the taxonomic status of *O. vulgaris* based solely on morphometric and meristic characteristics. The high levels of morphological plasticity within populations suggest that morphology is not a suitable tool for species, sub-species and population identification within the genus *Octopus*.

CHAPTER 5

General Discussion

The results of this study provide evidence to support the hypothesis that the strengthening of the Benguela Current upwelling system during the Pleistocene played a major role in the allopatric distribution and diversification of *O. vulgaris*. However, in spite of the significant genetic differentiation observed between northern and southern populations (Chapter 3), *O. vulgaris* throughout the region exhibited considerable morphological stasis (Chapter 4).

The presence of a significant northern-southern Benguela molecular structure has been observed in several coastal warm-temperate species (Henriques 2012) (Table 5.1). Compared with these, the timing of the separation (tmrca) of the northern and southern population of *O. vulgaris* was similar to that of the teleosts *Lichia amia* and *Diplodus capensis.* The similarity in the tmrca suggests that i) the Benguela current represents an important biogeographical barrier to a number of marine taxa; ii) interspecific similarities in the influence of the cold upwelling system on the population structure and demographic history may arise as a result of common biological or life history traits; and lastly iii) the observed interspecific similarities in demographic history may be a result of differential mutation rates, between taxa, however due to the nature of the current dataset definitive inferences cannot be made.

The movement patterns of marine animals, during all life history stages, have a major impact on their genetic structure (Luiz et al. 2012, Liggins et al. 2013). The movement patterns of the three species with a similar tmrca are different at the egg stage (*L. amia* – pelagic, *D. capensis* – pelagic, *O. vulgaris* – benthic) (Carvalho & Reis 2003, Potts et al. 2008, Richardson 2011). Whist all three species are characterised by a pelagic larval phases (Carvalho & Reis 2003, Potts et al. 2008, Richardson 2011), the duration of this life history phase may play a critical role in the connectivity, and subsequently the population structure and demographic history, among and within marine taxa (i.e. interpopulation, interspecific etc.). The pelagic larval duration (PLD) of *L. amia* cannot be accounted for as the time span of this life history phase remains unknown for this species (Connell 2007). The PLD of *D. capensis* has been documented to extend up to 28 days (Pattrick & Strydom 2009). Whilst this comparable to values recorded for *O. vulgaris* distributed within warmer waters, inferences regarding the role of the duration of the pelagic larval phase in the observed interspecific demographic history similarities should be made with caution due to the high variability associated with the PLD of *O. vulgaris* paralarvae (as discussed in Chapter 1). The post-settlement movement patterns are relatively similar for *O. vulgaris* and *D. capensis,* with both species exhibiting relatively high levels of residency, and movements generally being restricted to within a small home range (Cabranes et al. 2008, Watt-Pringle, 2009). *Lichia amia* however, undergo extensive seasonal migrations during both juvenile and adult phases (Smith 2008). It is therefore unlikely that the observed similarities in divergence are a result of movement behaviour. Based on these findings, one would expect *L. amia* to have the most recent tmrca since adults would be able to migrate long distances alongshore and retain connectivity between populations. It is therefore unlikely that the contemporary genetic structure can be well explained using the movement patterns of the species.

Temperature tolerance is often regarded as one of the primary mechanisms shaping distribution within marine species (Maree et al. 2000, Briggs and Bowen 2012). Physiological tolerance, such as thermal limitations, at the egg, larvae and adult phases may have resulted in the similar divergence time of these three species. Whilst *O. vulgaris* is considered to have a relatively broad temperature range (10-30 ºC), recent studies suggest that paralarvae do not appear to be reared in water temperatures below 19ºC, regardless of location (Iglesias et al. 2007). The reliance on warmer waters for paralarval rearing, coupled with the plague within the English Channel (as discussed in Chapter 1), highlights the importance of warmer waters for the species. *Diplodus capensis* larvae appear to have a similar temperature tolerance to *O. vulgaris* with optimal *D. capensis* recruitment occurring within waters between 17 and 20ºC (Richardson 2011). Despite the lack of literature available on the thermal tolerance of *L. amia*, the species is thought to show preference towards warm-temperate and to a lesser extent subtropical estuarine systems (Harrison 2005). It is therefore likely that the similar thermal range of these three species drove divergence of northern and southern populations in a cooling Bengeula system. This concurs with the findings of Henriques (2012) who concluded that the occurrence of persistent upwelling of cold water (as discussed in Chapter 2) along the Namibian and western coast of South Africa may be the driver of this northern-southern Benguela separation.

Despite similarities in the time since divergence between *L. amia*, *O. vulgaris* and *D. capensis*, *A. aequidens* was found to have diverged prior to the mid-late Pleistocene divergence exhibited by the remaining three coastal species (Table 5.1). Henriques (2012) attributed the early divergence of *A. aequidens* to the benthopelagic lifestyle of the species and the presence of LOW along the Namibian coastline, which may have been emphasized by the drop in sea level during the Miocene-Pleistocene.

As *O. vulgaris* is regarded as a benthic species it is likely that events during the Miocene-Pleistocene would have resulted in similar divergence patterns as was exhibited by *A. aequidens*. However, as a species that is capable of moving to and surviving in shallow, turbulent and more oxygenated water, it is possible that *O. vulgaris* may have survived these LOW periods. In addition, there may be other explanations for the differential divergence patterns observed for these two species. Firstly, it is unclear as to when *O. vulgaris* became fully established along the south-western coast of Africa. A difference in tmrca between *A. aequidens* and *O. vulgaris* may therefore be a result of a more recent extension of the historical distributional range of *O. vulgaris* into the Benguela Current region. Secondly, inter-specific ecological, physiological and behavioural differences may result in varying degrees of gene flow between the sub-systems of the Benguela, thus resulting in non-simultaneous divergence events. For example, Knowlton & Weigt (1998) found that ecological differences, such as habitat depth, may play a fundamental role in the development of differential divergence rates between sister species of snapping shrimp, within the genus *Alpheus*, isolated by the Isthmus of Panama. *Atractoscion aequidens* and *O. vulgaris* exhibit similar depth ranges, however *O. vulgaris* is found within the shallower coastal waters. This may have played a fundamental role in maintaining connectivity throughout the region. Furthermore, Marko & Moran (2009) suggested that species within the genus *Acar* characterised by a non-planktonic larval stage, a characteristic which is considered to be associated with higher speciation rates, were found to exhibit the highest post-Isthmian rates of diversification; however, both *O. vulgaris* and *A. aequidens* are characterised by planktonic larvae. Finally, it is important to recognise that the exclusive use of mtDNA genes to infer tmrca may result in a biased representation of inter and intra-specific divergence (Knowlton & Weigt 1998, Marko & Moran 2009). Such bias is thought to be introduced as a consequence of incomplete lineage sorting of ancestral polymorphisms (Edwards & Beerli 2000). Subsequently nuclear unlinked loci may be considered fundamental to the attainment of more reliable time since divergence estimations (Marko & Moran 2009). Inter-specific comparisons of tmrca within the current study should therefore be made with caution due to the considerable reliance upon mtDNA.

Surprisingly, there was no evidence of significant isolation between the northern and southern Benguela populations of another cephalopod species, *L. reynaudii.* Several molecular studies have shown that migrations in squids may be extensive, while migrations in *O. vulgaris* are more locally restricted (Semmens et al 2007). A number of factors therefore may be critical in facilitating connectivity across the cold water barrier situated along the west coast of southern Africa. Unlike *O. vulgaris,* which is largely resident during its adult life (Semmens et al. 2007), the chokka squid, *L. reynaudii*, is a highly mobile species capable of migrating great distances (100's-1000's of km) as an adult (Shaw et al. 2010). Furthermore, in comparison to *O. vulgaris*, *L. reynaudii* appears to have a much lower thermal range during its egg, larval and adult stages. Roberts (2004) highlighted the importance of upwelled waters, situated along the cold ridge within the Agulhas Bank system, to the recruitment success of *L. reynaudii*. Additionally, spawning events have been linked to the upwelling of cold, clear waters with squid migrating into deeper waters should conditions not be suitable (i.e. out of the optimal thermal range, high turbidity) (Augustyn & Roel 1998). As *L. reynaudii* are able to move into waters up to 300m deep (Roberts 2004), and are known to undergo extensive longshore migrations (Downey et al. 2010) it may be possible for them to move around the cold water habitats created during intense coastal upwelling events. In contrast, *O. vulgaris* are seldom found at great depths (as discussed in Chapter 1) and all available movement information suggests that the adults may undertake offshore-inshore migrations, rather than longshore migrations (Cabranes et al. 2008). These types of movement patterns would not promote movement around cold water barriers.

Amongst the teleost species, a northern-southern Benguela morphological divergence was also found in *D. capensis,* thereby supporting the molecular findings, whilst *L. amia* and *A. aequidens* exhibited only a slight differentiation, despite molecular variation between northern and southern populations (Table 5.1), suggesting that there is variability in the level of morphometric plasticity between teleost species. This appears to be similar amongst the cephalopods. The low morphological differentiation of *O. vulgaris* across the Benguela, despite a distinct molecular divergence, is in stark contrast to *L. reynaudii* as significant morphological differentiation was observed between specimens in Angola, the west coast and south coast of South Africa (van der Vyver, submitted) despite a lack of deep genetic divergence. These morphological differences were considered to be an adaptive evolutionary response to local environmental conditions (van der Vyver, submitted) and this variation has been documented in a number of squid species distributed across extensive geographical ranges (Carvalho & Pitcher 1989, Hernández-García & Castro 1998). The lack of morphological difference between the southern and northern Benguela populations of *O. vulgaris* may however be attributed to the measurement error associated with the anatomical structures of *O. vulgaris* (see Chapter 1 and 4). Another potential explanation is the broad morphological variation even within octopus found in the same location. This morphological variability amongst individuals may explain the lack of significance in the findings and suggests that these animals are extremely plastic, adapting to the immediate habitats associated with their home range.

Table 5.1: Comparison of molecular and morphological structure and time since most recent common ancestor (tmrca) of a number of cephalopod and teleost species distributed across the Benguela region. N-S = Northern- southern Benguela structure, IBD = Isolation by distance.

	Genetic structure	tmrca (Mya)	Morphological differentiation	References
O. vulgaris	N-S	~ 0.23 – 1.00	No differentiation	This study
L. amia	N-S	~0.20– 0.97	Little differentiation	1
D. capensis	N-S	~0.37 – 1.67	Substantial differentiation	1,2
A. aequidens	N-S	~1.46 – 2.26	Little differentiation	1,3
L. reynaudii	No sig structure, slight IBD	-	3 stocks: Angola, SA west coast, south coast of SA	4

1: Henriques (2012), 2: Richardson (2011), 3: Kruger unpublished data, 4: van der Vyver, submitted

Since high levels of biological and ecological plasticity appear to be a characteristic of *O. vulgaris* (Chapter 1) it is likely that this marked plasticity and thus, adaptability of the species, plays a fundamental role in buffering the evolutionary variation of phenotypic traits despite the divergence of genotypic characteristics. This study therefore highlights the importance of multidisciplinary, integrated approaches to systematic studies, particularly within the genus *Octopus*. The historical systematic approach centered around morphology is no longer appropriate given the advances in the molecular sciences. By utilising molecular and morphological techniques, understanding, recognising their advantages and mitigating for their limitations, one will enhance the level, scope and understanding of *Octopus* systematics.

The increasing recognition of cephalopods as a valuable fisheries resource highlights the need for comprehensive taxonomic investigations and subsequently thorough systematic revision, particularly within the genus *Octopus.* Results from the current study indicate that molecular research is paramount to the advancement of octopus taxonomy and may be considered to be the starting point for further taxonomic investigations within the genus. However, in spite of the pitfalls of morphological data, extensive investigations at the morphological, physiological, behavioural and ecological level should be carried out in order to gain a holistic understanding, and consequently a more accurate representation of the phylogenetic relationship and taxonomic status of any species.

This study made use of mitochondrial markers to investigate *O. vulgaris* population structure, evolutionary history and phylogenetic relationships. Mitochondrial DNA markers are the common markers of choice as their characteristics (discussed in Chapter 3) make it more likely to detect differentiation at a population and species level (Galtier et al. 2009; Park & Moran, 1994; Zhang & Hewitt, 2003). However the limitations of these markers need to be addressed.

Firstly, due to the non-mendelian mode of inheritance associated with mitochondrial markers paternal migrations and introgressions are not accounted for, should such processes occur within *O. vulgaris* (Zhang & Hewitt 2003, Liu & Cordes 2004). However, as highlighted by Rubinoff & Holland (2005), discrepancies in inheritance patterns, between genomes, do not necessarily reflect enigmatic results considering that valid, evolutionary based differences between datasets are not likely to be indicative of false phylogenetic signals. Secondly, the low effective size associated with mtDNA may potentially introduce bias into the study (Ferguson & Danzmann 1998, Zhang & Hewitt 2003). The effective size of mtDNA is considered to be a quarter of that of nuclear loci (Ferguson & Danzmann 1998) and therefore, the extent of molecular diversity may be underestimated and the increased likelihood of the occurrence of missing links in haplotypes could possibly introduce uncertainties into the genealogical analysis (Zhang & Hewitt 2003).

Currently, microsatellites are considered to be the most commonly used of the nuclear markers (Selkoe & Toonen 2006). Unlike mtDNA, microsatellites are not solely matrilineal and have a co-dominant Mendelian mode of inheritance. As such, microsatellites are able to recognise both homozygote and heterozygote genotypes thereby allowing issues regarding structure, phylogenetic relationships and classification to be resolved at both the population and individual level (Wan et al. 2004). Furthermore, the high mutation rates, associated with microsatellite makers, enables recent changes in the population to be easily detected thereby emphasizing the usefulness of such markers within population studies (Hoshino et al. 2012). The isolation and characterisation of multiple microsatellite markers for *O. vulgaris* (Greatorex et al. 2000) has subsequently allowed for the examination and detection of substantial genetic structure on the northwest coast of Africa (Murphy et al. 2002), around the Iberian Peninsula and Canary Islands (Cabranes et al. 2008) and within the Mediterranean Sea (Casu et al. 2002).

The inclusion of multiple genetic markers (mtDNA, nDNA genes and microsatellites) in future studies may therefore provide a more in-depth understanding of population sub-structuring, evolutionary history and phylogenetic relationships within and between species due to differential molecular and biological properties.

Although this study provided evidence for allopatry due to the presence of significant intra-specific genetic structure throughout the Benguela Region, in spite of a lack of morphological variation, inferences regarding the exact location of a biogeographical barrier were not possible. Comprehensive and representative sampling is often regarded as a confounding factor in many marine studies. Adequate sample size and extensive sampling throughout the sampling area are fundamental to draw molecular and morphological conclusions. The present study would have been enhanced with adequate samples across South Africa, Namibia and Angola; however due to time restrictions, financial, logistic and sampling impediments, specimen collection was essentially focused on either extremity of the system. Additional sample collection along the coast of Namibia and the west coast of South Africa would allow for the inferences of the current study to be refined and the positioning of the biogeographical barrier to be more accurately determined.

Considerations for Fisheries Conservation and Management

Marine fisheries are considered to be in a state of crisis, with a large fraction of contemporary fish stocks classified as overexploited (57.4% in 2009), depleted or in a state of recovery (FAO 2012). A concomitant increase in the reliance on cephalopod stocks places these fisheries at an increased risk of over exploitation. The increasing trend in stock overexploitation highlights the continued need for efficient and sustainable management and conservation strategies (FAO 2012). Accurate identification and assessment of the stock itself as well as the fishery as a whole, is therefore essential to the development of stock-specific management frameworks of cephalopods. Understanding the spatial and temporal dynamics of cephalopod fisheries is primarily hindered by the short life span and lack of systematic resolution within this class (Boyle 1990). The rapid turnover of generations is considered to make these species especially vulnerable to the effects of overfishing due to the brief period available to adequately adjust management strategies. In the case of the southern African octopus, this may potentially be exacerbated by the relatively low levels of genetic diversity observed within the current study (as observed in Chapter 3), however such inferences should be made with caution due to the sole reliance upon mtDNA markers. Low levels of molecular diversity renders a species vulnerable to the impacts of anthropogenic activities, such as overexploitation, as a lack of genetic diversity can have major consequences on the health and evolutionary potential of a population or species (Grant & Bowen 1998). Genetic diversity is therefore often regarded as the foundation for adaptation and subsequently survival. It has therefore been suggested that in order to ensure the sustainable harvesting of octopus stocks, management strategies may need to be adjusted on an annual basis to take into account recruitment and population variability (Fernández-Rueda & García-Flórez, 2007; Sauer et al. 2011).

The current study indicates that in spite of a lack of phenotypic differentiation (Chapter 4), *O. vulgaris* is represented by a northern and southern Benguela population (Chapter 3). A study conducted by Oosthuizen (2003) identified four primary precautionary management strategies for *O. vulgaris* specific fisheries: i) restrictive licensing; ii) gear limitation; iii) size limitation; and finally iv) the use of area closures. However, in order to effectively develop such strategies, extensive information on the biology and distribution of the stock, the dynamics and structure of the fishery as well as the interaction between the community, environment and the stock need to be accurately determined. Although this study did provide evidence for stock separation in southern Africa, the lack of extensive sampling, particularly along the South African west coast and Namibia, the distributional limits of each of these populations remains relatively unclear. It is likely that either, or both, of these populations extend across the maritime boundaries between Angola and Namibia or Namibia and South Africa. Should this be the case, sustainable and effective management of *O. vulgaris* populations would therefore require coordination and cooperation between neighbouring countries. The formal establishment of the Benguela Current Convention (BCC), in March of 2013, aims to create a collaborative management approach between Angola, Namibia and South Africa (Department of Environmental Affairs 2013). The BCC was developed in order to achieve "long-term conservation, protection, rehabilitation, enhancement and sustainable use of the Benguela Current Large Marine Ecosystem" (BCLME) (Department of Environmental Affairs 2013), in light of an increase in transboundary issues occurring throughout the region. A regional ecosystem-based approach to ocean management will have positive effects on octopus stocks if assessed and managed effectively.

Currently no information is available on the biology or fishery status of *O. vulgaris* in southern Angola. Nevertheless, the inshore fisheries in this region have grown considerably during the past decade (Potts et al. 2009). A lack of fishery information plays a major role in limiting the effectiveness of stock management strategies and furthermore has the ability to compromise future management and conservation efforts. This is of major concern for the northern Benguela *O. vulgaris* population as the resource is primarily distributed along a very narrow continental shelf. South African *O. vulgaris* is on the other hand, primarily targeted by the subsistence and recreational sectors, in the intertidal zone of the coast, whilst the subtidal component of the population is essentially unexploited (Oosthuizen 2003). This is however likely to change in the near future due to the establishment of the exploratory octopus fishery (DAFF 2011). Research on the South African population has up to now primarily focused on the biology and distribution of the intertidal component of this population. In order for management strategies to be effective, the dynamics and characteristics of the population need to be researched in its entirety. Precautionary management and conservation strategies may be implemented based on the information available for local, as well as global populations, until sufficient research has been conducted to effectively develop a stock-specific management framework. However, precautionary strategies and measures may be undermined by the localized and/or regional life history and behavioural variability of the species, as highlighted in Chapter 1.

Restrictive licensing, gear limitations, and size restrictions are often undermined by poor enforcement and reduced compliance (Holland & Brazee 1996). However, the use of marine protected areas (MPAs) is often regarded as an effective method for maintaining the diversity and evolutionary potential of coastal species, thereby reducing the likelihood of a collapse of the fisheries (Holland & Brazee, 1996; Teske et al 2010; von der Heyden, 2009). At present a number of MPAs are distributed throughout the South African coastline, with a total of 57 being reported in the year 2000 (Turpie et al. 2000). However, the majority of these MPAs have been established on an impromptu, sporadic basis and are therefore regarded as ineffective (Turpie et al. 2000). To date no MPAs have been formally established in Angola. Nevertheless, a number of areas serve to act as informal MPAs due to the inaccessible and uninhabited nature of certain stretches of the coastline, including but not limited to Baía dos Tigres and the Flamingo River mouth (Henriques 2012, Richardson 2011). In particular, Baía dos Tigres has been identified as an area of high importance for a number of marine species, including *O. vulgaris*, therefore increasing the motivation for the establishment of a marine reserve within this region in the future (Henriques 2012, De Beer & Potts 2013). In order for marine reserves to be effective a comprehensive understanding of the spatial dynamics of *O. vulgaris*, the impacts of local oceanographic features as well as the factors affecting recruitment variability need to be determined (Botsford et al 2003; Sauer et al., 2011). However, as with conventional fisheries management techniques, the effectiveness of MPAs can often be undermined by a lack of enforcement and poor compliance by resource users.

Alternatively, a number of authors have suggested that the use of seasonal area closures, during the recruitment phases, may have significant positive impacts for octopus populations thereby reducing the risk of overexploitation (Faraj & Bez, 2007; Fernández-Rueda & García-Flórez, 2007; Sauer et al. 2011). Temporary closures have had positive impacts on the abundance and size of octopus (Harris 2006). Although compliance and enforcement remain issues regarding their effectiveness, previous cases have shown that compliance from fishers can reduce these negative impacts. The *Octopus cyanea* fishery on the south-west coast of Madagascar is a prime example of an effective temporarily closed fishery. Octopus is of high economic value along the south-west coast of Madagascar and is often regarded as the primary marine commercial commodity (Harris 2006). Collaboration and cooperation between the Andavadoaka village fishermen, Blue Ventures Conservation, and other key stakeholders, led to the initialization of a trial closure of approximately 15% of local octopus fishing grounds between November 2004 and June 2005 (Harris 2006). The reopening of this no-take zone (NTZ) led to fishermen catching larger octopus more frequently and subsequently resulted in fishers receiving higher remuneration per octopus (Harris 2006). The increase in octopus yield, and consequently the economic benefits, as a result of the establishment of the NTZ led to the development of a community appreciation for this management strategy (Harris 2006). This support was highlighted by the increase in the number and range of NTZ’s established in subsequent years. The increased cooperation and communication between fishers and local organisations has substantial positive impacts on the levels of enforcement and compliance to such regulations (Harris 2006). Furthermore, due to an increased understanding of the benefits of marine conservation, the community has expressed their support for the development and establishment of more permanent conservation sites (Harris 2006).

The success and continued support of the implementation of temporary area closures in south-west Madagascar highlights the potential of this management strategy for the conservation of *Octopus spp.* Community support can be seen to be a fundamental component in the success of conservation and management efforts. The use of NTZ may therefore be a critical stepping stone, in gaining community compliance and support in the development of broader-scale management incentives, such as the establishment of MPAs.

Implications of marine invasions

The identification of potentially introduced *Octopus* species (Chapter 3) in both South Africa and Angola highlights our lack of knowledge on the state of non-indigenous species (NIS) in the region. The translocation and introduction of NIS poses a major threat, locally and globally, to the environmental and economic health of marine ecosystems (Blackburn et al., 2011; Drake & Lodge, 2004; Ruiz et al. 1997). In spite of an increased level of interest in invasion biology and an improved understanding of the process by which marine invasion occurs, the rate of introduction and establishment continues to rise (Bax et al. 2003; Blackburn et al. 2011; Carlton, 1996; Ruiz et al., 1997). In order to fully understand the translocation, introduction and establishment of NIS, the mechanisms facilitating species transfer, the rate and magnitude at which translocation occurs and the implications of subsequent invasion and establishment need to be determined (Ruiz et al. 1997). Mechanisms enabling the transfer of marine organisms are seen to predominantly be associated with anthropogenic activities; however, shifts in distributional patterns can occur as a consequence of natural events. Anthropogenic dispersal vectors of NIS may include: i) the transfer of hull fouling communities via transoceanic shipping; ii) the transfer and deliberate release of aquaculture species; iii) intentional release of aquarium related species, and finally, perhaps the greatest concern; iv) the movement of organisms through the discharge of ballast waters (Blackburn et al. 2011; Drake & Lodge, 2004; Grosholz & Ruiz, 1996; Streftaris et al. 2005).

The capacity of the shipping industry has undergone extreme growth with the onset of globalization (Robinson et al. 2005). In order to meet the global demand, ships have to be bigger, faster and undergo trips more frequently (Robinson et al. 2005). The growth and development of the seaborne trade appears to correspond to an increase in the abundance, diversity and survival of NIS, and consequently a global increase in species invasions (Carlton, 1996; Robinson et al. 2005).

With particular reference to this study, the rise in commercial relations between Asia (and more specifically China) and sub-Saharan Africa has promoted an increase in the transportation of marine organisms between these two regions. It is likely that the three individuals (EC6, EC11 and EC15) that grouped with the Indo-Pacific clade were translocated through ballast water from Asia. Teske et al. (2007) previously identified a second lineage of *Octopus* near the port of Durban, the major port of sub-Saharan Africa, which was assumed to be an introduced species. Although it is yet to be verified, should the individuals from the current study be of the same descent of those identified by Teske et al. (2007) it is likely that the species has established itself in the coastal region of South Africa and has successfully dispersed from the point of introduction. Alternatively, additional species may have been introduced into South African waters. Both possibilities are of major concern due to the potential environmental, economic and social implications associated with the introduction of non-native species. Further research is therefore required in order to determine the number of non-indigenous octopus species and the invasion success along the coastline of South Africa. It is important to note that determining the extent of invasions of octopus species may be challenging due to the cryptic nature, morphological plasticity and lack of systematic resolution associated with these cephalopods, as is highlighted by the current study (Feral 2002). Molecular approaches however, as this study has demonstrated, have the potential to resolve issues regarding both cryptic and cryptogenic species and are therefore fundamental to the study of octopus invasions (Feral 2002).

Besides establishing the extent of octopus invasions, predicting its effects is challenging as the implications of invasions are generally species and community specific (Ruiz et al. 1997, Streftaris & Zenetos 2006). Inferences regarding the interactions and effects of non-native species therefore cannot always be drawn from knowledge of the species within its native range. A prime example of this is highlighted by Grosholz & Ruiz (1996), who demonstrated the variability in several aspects of the green crab, *Carcinus maenas*, between native and non-native ranges. *C. maenas* is native to Europe, but the species has been translocated through ballast water exchange to a number of countries around the world, of which South Africa is one. The study conducted by Grosholz & Ruiz (1996) compared five characteristics between a native and three non-native sites. The study revealed that *C. maenas* attained greater sizes, grew quicker and made use of different habitat types in its non-native range (Grosholz & Ruiz 1996)

Potential environmental impacts associated with the introduction of NIS include the modification of community structure and function, alteration of nutrient dynamics and a loss of native biota (Ruiz et al. 1997). This is due to a combination of multiple direct and indirect effects such as predation by the NIS, competition for habitat and food with native biota and through the introduction of parasites and diseases (Briggs, 2007; Ruiz et al., 1997; Streftaris & Zenetos, 2005). Increased octopus abundance has been negatively correlated with species richness (Ambrose 1986) and a number of authors have highlighted the consequences of increased octopus abundance on commercially important crustacean populations (Boyle, 1990; Groeneveld et al. 2006; Rees & Lumby, 1954). An example of this is the drastic increase of *O. vulgaris* within the English Channel which led to the regional decimation of crab and lobster populations (Rees & Lumby 1954, Garstang 2009). It is therefore likely that the introduction of non-indigenous octopus would threaten the diversity of their potential prey along the coastline of South Africa.

Two species, the west coast rock lobster (*Jasus lalandii*) as well as the South African Abalone (*Haliotis midae*) are under serious threat due to overexploitation (Bester-van der Merwe et al. 2011). The introduction of non-indigenous octopus has the potential to place these stocks under additional pressure as a variety of abalone and lobster species have been found to be incorporated in the diet of *Octopus* spp. around the globe (Groeneveld et al. 2006; Harrington et al. 2006). The collapse of shell fish fisheries in South Africa will not only result in severe changes in the function and structure of marine ecosystems but may also have social and economic drawbacks. NIS threaten the social and economic welfare of communities and nations worldwide (Bax et al. 2003; Ruiz et al. 1997). Detrimental socio-economic effects, due to species invasions, are generally associated with a decrease in revenue and subsequently a decline in livelihoods through the interference with, in this case, fisheries on which industry and local communities depend (Bax et al. 2003; Ruiz et al. 1997). However, NIS also potentially constitute a threat to human health and well-being through the introduction of parasites and diseases as well as through a decline in food security due to an increase in competition for resources (Ruiz et al. 1997).

Adaptability to Climate Change

Climate change is a worldwide phenomenon of increasing concern as it jeopardizes oceanic ecosystems and threatens marine biodiversity, thereby exacerbating current marine stressors (Cheung et al. 2009; Hoegh-Guldberg & Bruno, 2010; Robinson et al. 2009). Predictions of the impending effects of global climate change on marine species are a vital component in the development of adaptive fisheries management and conservation strategies. Inferences regarding the response of oceanic organisms to climate change are often built on the foundation of species-specific physiological optima and critical limitations (Grillo 2011). However, the evolutionary response of a species to historical climatic events may also prove useful in forecasting the effects of future anthropogenic induced climate change.

Reconstruction of the evolutionary history of *O. vulgaris*, in Chapter 3, indicated that climatic variability during the Pleistocene had significant effects on the abundance and distribution of the species throughout the Benguela region. A decline in SST, coupled with a substantial decrease in sea level are likely to have played a major role in the observed contemporary divergence between the South African and southern Angolan populations (Chapter 3). Subsequent to the northern and southern Benguela divergence, population specific evolutionary history analyses suggested that expansion or growth of these genetically distinct populations may have resulted from further climatic fluctuations. It is therefore likely that future environmental changes will similarly have significant effects on the population demography of *O. vulgaris* within the Benguela region.

Climate change predictions suggest that global sea levels will continue to rise at accelerated rates as a consequence of the melting of polar ice caps, oceanic warming and subsequent thermal expansion (Overpeck et al. 2006, SAEON 2011). Sea level rise may result in the expansion of populations as a result of the widening of the continental shelf off the south-western and southern coast of Africa. However, research indicates that climate change is likely to have severe implications on the frequency, timing and intensity of upwelling regimes (Bakun 1990). The intensification of upwelling cells in eastern boundary systems, such as the Benguela Current, is likely to occur due to strengthening of along shore winds as a result of the increase in continental thermal lows adjacent to these systems (Bakun 1990, SAEON 2011). Lett et al (2007) concluded that a combination of surface hydrodynamic characteristics from upwelling would result in offshore advection, and the subsurface thermal barrier would restrict or limit dispersal of ichthyoplankton across this region.

The intensification of the system may however have negative implications on the recruitment success of the species, as offshore advection is likely to increase (Lett et al. 2007). The strengthening of offshore advection would therefore remove *O. vulgaris* larvae from the productive continental shelf waters of the system, decreasing the chance of larval survival. Offshore advection is found to limit the recruitment success of a number of marine fish, particularly those that make use of the inshore environment as nurseries for eggs, larvae and juveniles, such as the Pacific hake, *Merluccinus productus* (Bailey 1981). Thus, the intensification of upwelling in the Benguela will most likely maintain and even strengthen the observed contemporary patterns of *O. vulgaris* population structure.

Temperature is often regarded as one of the major determinants of distributional patterns due to the fundamental role that this environmental parameter has on the biological processes of marine organisms (Hoegh-Guldberg & Bruno 2010). Moderate fluctuations in environmental temperatures can have significant effects on the metabolic rates of marine fauna, indirectly affect the life history characteristics of a species, the growth rates of populations and the structure and functioning of surrounding ecosystem processes (Wood & McDonald 1997, Clark 2006, Hoegh-Guldberg & Bruno 2010). Increasing oceanic temperatures may therefore have implications for *O. vulgaris*. However, as thermal limitations of the species remain relatively unclear, inferences regarding the effects should be made with caution. The results from this study suggest that both northern and southern Benguela populations experienced a phase of population growth which coincided with warmer paleoclimatological phases. This, in conjunction with the information on the octopus “plague” in the English Channel which is thought to have been driven by the preference for warmer waters (Rees 1950), suggests that *O. vulgaris* populations are likely to expand in the future with increasing oceanic temperatures. However, should upwelling along the south-western coast of Africa strengthen within the future and subsequently result in a significant decline in SST, *O. vulgaris* populations may undertake substantial distributional shifts, thereby further isolating northern and southern populations. Furthermore, an increase in upwelling intensity is likely to have a greater impact on the Angolan population, due to the northerly displacement of the confluence between the warm Angola Current and the cold, equator-ward flow of the Benguela.

The ABFZ is considered a global hotspot for climate change, with an increase of approximately 1.0ºC being recorded within the offshore region during the past 20 years (Fidel & O'Toole 2007) and an 0.8ºC increase per decade in the coastal zone since 1982 (Potts et al submitted). It is therefore likely that the northern Benguela *O. vulgaris* stock will be exposed to warming sooner and more intensely, making this an ideal hotspot for researching the potential changes for the species. The low genetic diversity levels observed for *O. vulgaris* throughout the Benguela region suggests that climatic fluctuations associated with the effects of climate change may have major implications for their status and distribution. However, predictions should be made with caution due to the complex nature of the relationship between physical, chemical and biological processes affected by this global phenomenon and would require some resolution on the thermal tolerance of the species at all life history phases.

Concluding remarks and future research considerations

The findings of the present study provided insight into the effects of the Benguela Current on the molecular and morphological characteristics of *O. vulgaris*. Additional research on the following topics may provide further knowledge on extent of variation, the mechanisms behind local adaptation as well as aid in the effective management and conservation of *O. vulgaris* throughout the Benguela Current region, and is highlighted below:

1) Future research should aim to obtain samples between Baía dos Tigres, in southern Angola, and Cape Point, in South Africa, in order to gain a more in depth understanding of the positioning of the geographical disjunction between the identified Angolan and South African populations. This is vital for the development of sustainable, trans-boundary management practices as well as for the establishment of reliable predictions regarding species-specific responses to global climate change.
2) Furthermore, the inclusion of alternative neutral and adaptive markers may provide further insight into the magnitude of *O. vulgaris* population sub-structuring as well as the factors responsible for the observed local adaptation.

3) Future studies should include comparative biological and ecological research (eg. De Beer and Potts 2013) in order to further understand the observed variability and similarities between populations across the Benguela Current system.
4) Additionally, extensive research on the thermal limitations of all life history stages of this species may provide valuable insight into the consequences of climatic changes, such as ocean warming.

Appendix

Table 3.8: Abbreviation, sequence origin and accession number for *cytb* sequences obtained from GenBank for phylogenetic studies.

Species	Abbreviation	Origin	Accession #
Octopus australis	O_aust	Port Stephens, NSW	AJ628181
Octopus aculeatus	O_acul	Orpheus Island, QLD	AJ628177
Octopus aegina	O_aegiI	Viet Nam	FJ360547
Octopus aegina	O_aegiII	Viet Nam	FJ360548
Octopus aegina	O_aegiIII	Viet Nam	FJ360549
Octopus bunurong	O_bunu	St. Leonards Pier, VIC	AJ628183
Octopus californicus	O_cali	Santa Barbara, CA	HM572209
Octopus defilippi	O_defiI	Mediterranean Sea	GQ153825
Octopus defilippi	O_defiII	Mediterranean Sea	GQ153826
Octopus kagoshimensis	O_kago	One Tree Island, QLD	AJ628190
Octopus macropus	O_macrI	Mediterranean sea	FJ360553
Octopus macropus	O_macrII	Mediterranean sea	FJ360554
Octopus maya	O_mayaI	Mexico	FJ360556
Octopus maya	O_mayaII	Mexico	FJ360557
Octopus minor	O_mino	Weihai, Shandong Province, China	HQ638215
Octopus ocellate	O_ocel	Queen Victoria Market, VIC	AJ628197
Octopus oculifer	O_ocul	Galapagos Islands	AJ628198
Octopus tetricus	O_tetrI	Australia:New South Wales	AJ628203
Octopus tetricus	O_tetrII	Australia:Western Australia	AJ628201
Octopus tetricus	O_tetrIII	Australia:Western Australia	AJ628202
Octopus tetricus	O_tetrIV	Australia:New South Wales	AJ628200
Octopus vulgaris	O_vulgIV	Portugal	EF423015
Octopus vulgaris	O_vulgV	Portugal	EF423016
Octopus vulgaris	O_vulgVI	South Africa	EF423030
Octopus vulgaris	O_vulgVII	Spain	EF423022
Octopus vulgaris	O_vulgVIII	Spain	EF423026
Loligo subulata	Loligo	English Channel	EF423114

Table 3.9: Abbreviation, sequence origin and accession number for *COI* sequences obtained from GenBank for phylogenetic studies.

Species	Abbreviation	Origin	Accession #
Amphioctopus aegina	A_aegiI	China	JX456265
Amphioctopus aegina	A_aegiII	China	JX456267
Enteroctopus dofleini	E_dofl	Japan	AB191272
Octopus aegina	O_aegiIII	Taiwan	JN790684
Octopus areolatus	O_areo	Japan	AB191274
Octopus californicus	O_cali	Santa Barbara,CA	AF377968
Octopus conispadiceus	O_coni	Japan: Hokkaido, Kushiro	AB430533
Octopus cyanea	O_cyanI	Japan: Okinawa, Nakagusuku	AB430535
Octopus cyanea	O_cyanII	Japan: Tokyo, Ogasawara Island	AB430534
Octopus cyanea	O_cyanIII	Japan	AB191280
Octopus fusiformis	O_fusi	China	JX456272
Octopus hongkongensis	O_hongI	Japan:Kouchi, Mimase	AB430538
Octopus hongkongensis	O_hongII	Japan: Kanagawa, Misaki	AB430537
Octopus incella	O_ince	Japan: Okinawa, Motobu, Hamamoto	AB430542
Octopus joubini	O_joub	Carolina Supplies	AY377732
Octopus laqueus	O_laqu	Japan: Okinawa, Kadena, Mizugama	AB430543
Octopus longispadiceus	O_long	Japan: Hyogo, Kasumi, Shirosaki	AB430539.
Octopus mimus	O_mimuI	North Chile/Peru	GU355926
Octopus mimus	O_mimuII	North Chile/Peru	GU355925
Octopus mimus	O_mimuIII	North Chile/Peru	GU355924
Octopus minor	O_minoI	China	HQ846119
Octopus minor	O_minoII	China	HQ846118
Octopus minor	O_minoIII	China	HQ846117
Octopus nanhaiensis	O_nanh	Sanya, China	HQ846121
Octopus ocellatus	O_ocel	China: Yellow Sea	AF346854
Octopus oliveri	O_oliv	Japan: Tokyo, Ogasawara Island	AB430532
Octopus oshimai	O_oshi	China	JX456268
Octopus parvus	O_parv	Japan: Kanagawa, Misaki	AB430544
Octopus sasakii	O_sasa	Japan	AB191277
Octopus tankahkeei	O_tank	China	JX456264
Octopus tehuelchus	O_tehuI	San Matías Gulf, Argentina	GU355936
Octopus tehuelchus	O_tehuII	San Matías Gulf, Argentina	GU355935
Octopus tehuelchus	O_tehuIII	San Matías Gulf, Argentina	GU355934
Octopus variabilis	O_vari	China	JX456263
Octopus vulgaris	O_vulgI	Eastern Mediterranean	HQ908436
Octopus vulgaris	O_vulgII	Japan: Kanagawa, Misaki	AB430547
Octopus vulgaris	O_vulgIII	Japan: Hyougo, Akashi, Futami	AB430546
Octopus wolfi	O_wolf	Japan: Okinawa, Kadena, Mizugama	AB430545
Loligo subulata	Loligo	Plymouth, United Kingdom	AF075387

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