

Molecular and morphological factors contributing to the resolution of the taxonomy of *Octopus vulgaris* Type III

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Statements and declarations

Statement of authorship (Plagiarism declaration)

This dissertation includes work by the author that has been published as described below. No other person's work has been used without due acknowledgement in the main text of the dissertation. The content of this dissertation has not been submitted for the award of any other degree or diploma in any other tertiary institution. I know the meaning of plagiarism and declare that all of the work in the dissertation, save for that which is properly acknowledged, is my own.

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09 February 2024

Date, Signature

Statement of Co-authorship

The following authors contributed to publication of work undertaken as part of this dissertation:

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Chapter 2 is published in Molecular Biology Reports. Alvaro Roura provided the samples which were sequenced in a commercial laboratory. Peter Teske and Arsalan Emami-Khoyi acted as supervisors for this publication. Gareth Fee completed all analyses, wrote the first draft of the manuscript and is the primary author.

Ethical statement

All specimens used during the course of this investigation were purchased from the experimental pot fishery in False Bay and thus no ethical clearance was required.

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Abstract

Cryptic species are common in the marine environment, particularly among invertebrates. The genus *Octopus* Cuiver, 1797 is considered a 'catch all' genus due to the lack of morphological traits available to distinguish closely-related species. In recent years, the *Octopus vulgaris* species complex has received much attention with many cryptic species 'Types' being identified, most of which have now been re- or newly described as separate species. The last remaining Type currently known within this complex which requires taxonomic resolution is the Southern African lineage, *Octopus vulgaris* Type III. This taxon was not included in a recent global morphological assessment of the complex and few specimens were included in phylogenetic studies. Mitochondrial barcodes failed to distinguish *O. vulgaris* Type III from *O. vulgaris sensu stricto* (ss), but nuclear genes did. This dissertation aims to resolve the taxonomy of *O. vulgaris* Type III using both genetic and morphological lines of evidence. Chapter 1 gives a broad background to the cryptic species problem within cephalopods and discusses the significance of resolving the taxonomy of *O. vulgaris* Type III. Chapters 2 and 3 sequence and annotate the first complete mitochondrial genomes (mitogenomes) of *O. vulgaris* ss and *O. vulgaris* Type III respectively. Chapter 2 also includes a phylogenomic assessment and found *O. vulgaris* Type III to be a sister taxon to *O. vulgaris* ss, separated with high statistical support. Chapter 4 presents a detailed morphological assessment of *O. vulgaris* Type III, which successfully delimit it from all other species within the species complex. Finally, Chapter 5 summarises these findings. These results have significant implications, considering the growing octopus fishery in South Africa, as management should consider the population as an isolated species, distinct from *O. vulgaris* ss, which is found in the Mediterranean and Northeast Atlantic.

Chapter 1: General introduction

Species concepts

The definition of what constitutes a species has been widely debated for the last half century (Mayr 1963; De Queiroz 2007; Wilkins 2009). This species concept problem has resulted in disagreements about species boundaries and number of species, depending on the properties applied for species delimitation. De Queiroz (2007) proposed a *Unified Species Concept*, which defines a species as a “separately evolving metapopulation lineage”. This property underlies all other species concepts, and additional properties fundamental to independent concepts are considered secondary criteria supporting the hypothesis of lineage separation. Thus, any one property can be used as evidence of speciation and multiple lines of evidence increase support for the hypothesis of separate species. These secondary criteria can include phylogenetic divergence, morphological differences, reproductive isolation, or separate ecological niches. The identification and description of a species is fundamental to understanding its ecological and evolutionary significance, as well as managing species with commercial value or higher conservation status (Moritz 1994).

Cryptic species

Cryptic species, also referred to as sibling species once formally recognised (Mayr 1963), are morphologically similar or identical natural populations that can be distinguished by other properties (Mayr 1963; Knowlton 1993). Many cryptic species rely on chemical (Knowlton 1993) or auditory (Henry 1994) signals, which do not require corresponding morphological adaptations and thus speciation can occur without detectable changes in morphology (Mayr 1963; Hebert et al. 2004a). Cryptic speciation is often observed in marine environments, as information on the ecology and behaviour of live specimens is relatively difficult to obtain (Knowlton 1993). Commonly, descriptions of marine species are based solely on the morphological assessment of preserved specimens. Cephalopods, and particularly octopuses, have

few hard morphological structures on which to base taxonomy. Resolution among closely-related octopus species is mostly reliant on highly plastic, soft body parts which are prone to distortion and loss of pigmentation upon preservation (Voight 1994, 2001). Thus, cryptic species are common among cephalopods (Pickford and McConnaughey 1949; Leite et al. 2008; Amor et al. 2017; Avendaño et al. 2020).

Cephalopoda

Cephalopods are a fascinating, ancient group of molluscs comprised of nautilus, squids, octopuses and cuttlefish. The last three of these together make up the subclass Coleoidea and are characterised by having reduced and internalised shells, these being completely lost in the case of octopuses (Norman 2000). Their ecology is more comparable to that of teleosts than to that of most other benthic molluscs (Boyle and Rodhouse 2005). At various times in geological history, cephalopods have been among the dominant predators in the oceans (Young et al. 2019), yet the evolutionary relationships among many coleoid lineages is not well understood (Allcock et al. 2015). This is in part due to the lack of hard remains in the fossil record, complex DNA structure, including extensive mitochondrial gene rearrangements, and their great morphological diversity (Akasaki et al. 2006; Stöger and Schrödl 2013; Allcock et al. 2015; Uribe and Zardoya 2017). There are estimated to be over 1000 species of extant cephalopods (Norman 2000), of which over 200 belong to the family Octopodidae d'Orbigny, 1840. However, the taxonomy and phylogeny within this family is far from resolved.

The genus *Octopus* Cuvier, 1798 is considered a 'catch all' genus, due to the small number of morphological traits suitable to distinguish between closely-related species. Generally, they are characterised as having two rows of suckers and an ink sac (Norman et al. 2013). However, this genus is polyphyletic and requires major taxonomic revision. The type species for this genus is the Common octopus, *Octopus vulgaris* Cuvier, 1798, which itself is now understood to be a species complex, comprised of multiple sibling species which are morphologically similar, but with disjunct geographical distributions (Norman et al. 2013; Amor et al. 2017).

The Octopus vulgaris species complex

Octopus vulgaris is presumed to be described from the Mediterranean, although no type specimen was designated (Norman et al. 2013). It was previously assumed that *O. vulgaris* was a cosmopolitan species living in temperate and tropical waters around the globe (Warnke et al. 2004), but today there is substantial support for a species complex comprised of multiple lineages (Norman et al. 2013; Amor et al. 2017). In this complex, *O. vulgaris* sensu stricto (ss) is restricted to the Northeast Atlantic and Mediterranean. Early investigations into this species complex provided evidence for the separation of *O. mimus* in the Eastern Pacific (Söller et al. 2000; Warnke et al. 2000), *O. maya* in the Gulf of Mexico (Pérez-Losada et al. 2002) and *O. insularis* off Northeast Brazil (Leite et al. 2008). Additional lineages hypothesized to be different species based on disjunct geographic distribution and lack of plausible gene flow, but whose taxonomy has remained unresolved, have been referred to as different *O. vulgaris* “Types” (in the sense of a different form of this species) (Norman et al. 2013). The name *O. sinensis* was recently reinstated for the East Asian *O. vulgaris* Type IV (Gleadall 2016). In the West Atlantic, two lineages (Type I and Type II) were identified. The Type II lineage has been redescribed as *O. americanus* (Avendaño et al. 2020), while uncertainty still remains on the identity of the Type I lineage. Closely related are two Australasian species, *O. tetricus* and *O. djinda*, the latter only recently delineated from the former and described (Amor and Hart 2021). Already 25 years ago, Roeleveld (1998) noted that “the South African species needs a new name”. For the present, *Octopus vulgaris* Type III is the last unresolved taxa from the five geographic ‘Types’ in the *O. vulgaris* species complex previously identified by Norman et al. (2013). *Octopus vulgaris* Type III is hypothesized to comprise the population that extends from Tristan Da Cunha in the South Atlantic, along the Southern African coastline of Namibia and South Africa, to Amsterdam Island in the Indian Ocean (Norman et al. 2013; Amor et al. 2017).

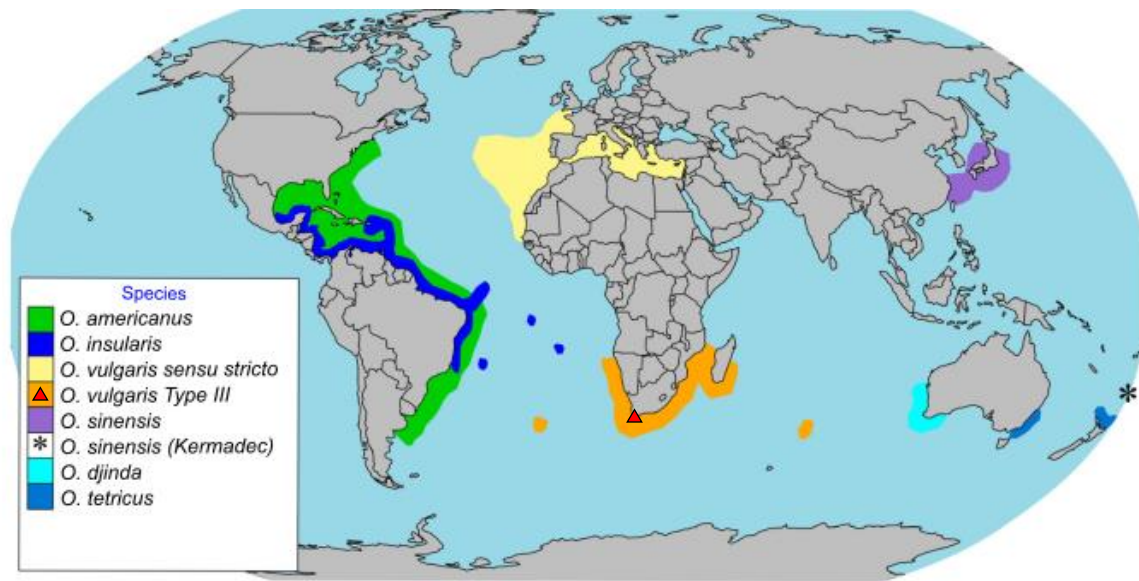


Figure 1.1 Distribution map of species within the *Octopus vulgaris* species complex. *O. vulgaris* Type III is hypothesized to range from Tristan Da Cunha in the South Atlantic, across Southern Africa, to Amsterdam Island in the South Indian (orange). Specimens for this study were sampled from False Bay, South Africa (red triangle). (Figure modified after Avendaño et. al. 2020)

Basic morphological studies (Smale and Buchan 1981; Smith and Griffiths 2002; Oosthuizen and Smale 2003) and early genetic studies using single, or few mitochondrial markers (Oosthuizen et al. 2004; Warnke et al. 2004; Guerra et al. 2010) failed to differentiate between *O. vulgaris* ss and *O. vulgaris* Type III and supported the idea of a cosmopolitan distribution. However, it is not uncommon for mitochondrial sequences to be uninformative for phylogenetic studies if only a portion of the mitogenome is used (Galtier et al. 2009; Morin et al. 2010; Allcock et al. 2015) and these morphological assessments considered only a very limited set of traits. Although the benthic octopuses have planktonic larvae, it is unlikely that dispersal and gene flow can occur between Southern Africa and the North-East Atlantic (Allcock et al. 2015). The Lüderitz upwelling cell and Orange River cone (LUCORC) between approximately 25 and 30°S is known to be a barrier to dispersal of planktonic larvae and organisms from South to North due to surface hydrodynamics and a subsurface thermal barrier (Lett et al. 2007). This could potentially limit the dispersal and gene flow of *O. vulgaris* Type III northwards.

Octopus vulgaris ss and *O. vulgaris* Type III live in shallow coastal waters, but are not present along West equatorial Africa and thus the distributions of these species are widely separated.

Octopus vulgaris Type III

Octopus vulgaris Type III is one of more than 195 cephalopods that inhabit South African waters (Roeleveld 1998). They live in shallow coastal waters up to a depth of 200 m and have a life span of up to 15 months (Smale and Buchan 1981). Their main predators are Cape fur seals, *Arctocephalus pusillus* and endemic cat sharks, including the endangered *Haploblepharus edwardsii* (Lipinski and David 1990; Dainty 2002). Octopuses are generalist predators and play an important role in maintaining local prey populations (Rees and Lumby 1954; Ambrose 1986). In South Africa, the diet of *O. vulgaris* Type III varies along the coast, likely due to a change in prey availability. On the subtropical east coast, their dominant prey is the Brown mussel *Perna perna* (Smale and Buchan 1981). On the warm temperate south-east coast, their prey are mainly teleosts, crustaceans and other octopus (Oosthuizen and Smale 2003), while on the cold temperate south-west coast, their main prey are abalone and crustaceans (Smith 2003). Beyond their direct trophic interactions, benthic octopuses may also play a role as ecosystem engineers (Scheel et al. 2014), modifying their habitat through the construction of dens and the middens of prey. Remains of prey left surrounding the den can provide food for scavenging organisms, while gastropod shells left in the middens provide shelter for hermit crabs (Gilchrist 2003). The dens themselves also provide shelter for associated organisms. For example, in False Bay, a recently-described species of mysid (*Heteromysis octopodis*) has been discovered living in the dens of *O. vulgaris* Type III (Wittmann and Griffiths 2017). This species of *Octopus* is also the star of the Oscar award winning documentary film 'My Octopus Teacher' (Ehrlich and Reed 2020), which has fostered the support for the conservation and welfare of octopus in South Africa and around the world (Cocks, 2021; Craig Foster personal communication), making them somewhat of a flagship species. They have become a favourite among local and international divers and underwater naturalists and thus have socioeconomic value in the tourism industry.



Figure 1.2 *Octopus vulgaris* Type III sub-adult in the cold temperate reef of False Bay, South Africa.

Fisheries and the need for taxonomic clarification

The identification and accurate description of cryptic species within the *Octopus vulgaris* species complex is important due to the commercial importance of this and closely-related species worldwide. Global octopus fisheries have been steadily increasing over recent decades, the global catch nearly doubling from 179,042 t in 1980 to 355,239 t in 2014 (Sauer et al. 2021), yet the poor state of octopus taxonomy remains the “largest impediment” to recording accurate catch statistics and developing effective management plans (Norman and Finn 2013). Cephalopod fisheries are predicted to grow in importance as finfish stocks continue to decline due to over-exploitation (Sauer et al. 2021). Some octopus fisheries are already considered over-exploited, such as that for *O. vulgaris* ss in the Mediterranean (Quetglas et al. 2015). Thus, to prevent octopus fisheries from suffering the same fate as numerous severely overfished fin-fish stocks both in South Africa and around the world (Zeller and Pauly 2005; Mann 2013; Vasilakopoulos and Maravelias 2014), it is crucial to build foundational species knowledge for this unknown species of octopus in order to manage the stocks sustainably.

Many of South Africa's fisheries resources are considered over-exploited, with economically-important inshore invertebrate species, such as the Abalone (*Haliotis midae*) and West coast rock lobster (*Jasus lalandii*) considered severely depleted (Mann 2013; DEFF 2020). *Octopus vulgaris* Type III was identified as an under-utilised, yet valuable, alternative to alleviate fishing pressure on those inshore resources (Oosthuizen 2004; John 2022). In 2003, 16 areas were designated for the fishery, yet only three have been regularly fished. False Bay is by far the most active site, catching up to 70 tonnes of octopus per year (John, personal communication). Although the fishery is still in the experimental phase, conversion to a commercial fishery is imminent and the expansion to all 16 designated areas will greatly increase the impact of this fishery on local octopus populations.

Although cephalopods are often considered suitable targets for sustainable fisheries due to their high fecundity, fast growth rates and adaptability (Norman and Finn 2013), they are susceptible to over-exploitation (Rodhouse et al. 2014; Quetglas et al. 2015). When considered on a regional scale, the majority of octopus fisheries are in decline (Norman and Finn 2013) and off the coast of Morocco *O. vulgaris* stocks are considered seriously depleted (Sauer et al. 2021). Therefore, it is vital that management of the *O. vulgaris* Type III fishery is sustainable from the start.

Statement of research problem

Multiple cryptic species have been identified and described within the *Octopus vulgaris* species complex (Söller et al. 2000; Pérez-Losada et al. 2002; Leite et al. 2008; Gleadall 2016; Amor et al. 2017; Avendaño et al. 2020; Amor and Hart 2021). However, the taxonomy of the Southern African lineage, *O. vulgaris* Type III, remains unresolved. This lineage has been hypothesized to be a separate species due to its isolated distribution (Roeleveld 1998; Norman et al. 2013), but early genetic studies considering single mitochondrial markers (Oosthuizen et al. 2004; Warnke et al. 2004; Guerra et al. 2010) and low resolution morphological assessment (Smale and Buchan 1981; Oosthuizen and Smale 2003; Smith and Griffiths 2003) failed to distinguish this lineage from *O. vulgaris* ss. Resolving the taxonomy of this lineage is crucial for the management and conservation of this species considering its ecological and socio-economic value and in light of a growing octopus fishery. Doing so will also provide

further information into the phylogeny and evolutionary history of the *O. vulgaris* species complex.

Dissertation overview

The aim of this dissertation is to resolve the taxonomy of *Octopus vulgaris* Type III from South Africa. Following the unified species concept (De Queiroz 2007), it will consider two separate lines of evidence to investigate whether or not the separation of *O. vulgaris* Type III as an independent species is required.

Firstly, the study considers a phylogeny based on the full mitochondrial genomes (mitogenomes) to overcome the shortcomings of using single genetic markers (Morin et al. 2010), as used in previous studies (Oosthuizen et al. 2004; Warnke et al. 2004). When commencing this investigation, the only publicly available mitogenome for *O. vulgaris* was sequenced from a specimen collected in Japan, and thus likely represents *O. sinensis*. Chapter 2 provides evidence for this misidentification and presents the first complete mitogenome of *O. vulgaris* ss. Chapter 3 sequences and annotates the full mitochondrial genome for *O. vulgaris* Type III and compares it to the mitogenome generated in Chapter 2 and that of other closely-related species using both Maximum likelihood (ML) and Bayesian Inference (BI) methods.

Chapter 4 investigates the morphology of *O. vulgaris* Type III using guidelines set out specifically for the taxonomic description of cephalopods (Roper and Voss 1983). *Octopus vulgaris* Type III was omitted from a global morphological assessment of the *O. vulgaris* species complex (Amor et al. 2017). Using the extensive dataset from Amor et al. (2017), this study conducts a multivariate analysis of up to 25 traits to compare *O. vulgaris* Type III morphology to that of closely related species.

Considering both molecular and morphological findings, Chapter 5 comments on the taxonomic status of *O. vulgaris* Type III and make recommendations for future research. Each chapter is written in the form of a stand-alone paper and so there is some repetition throughout.

Chapter 2: The complete mitochondrial genome of *Octopus vulgaris*

This chapter is published as:

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Introduction

The family Octopodidae d'Orbigny, 1840 consists of over 200 known species, many of which lack detailed taxonomic descriptions (Norman et al. 2013). The taxonomy and phylogenetic relationships within this family have received much attention in recent years, with many cryptic species being identified across the globe (Söller et al. 2000; Leite et al. 2008; Avendaño et al. 2020; Amor and Hart 2021). A taxonomic group which has recently gained particular attention is the *Octopus vulgaris* species complex. Once thought to be a cosmopolitan species in temperate and tropical waters (Warnke et al. 2004), *O. vulgaris sensu stricto* (ss) is now considered to be restricted to the Mediterranean and the northeastern Atlantic, while locations beyond this region are inhabited by morphologically similar, but genetically distinct species (Amor et al. 2017), some of which may still need to be formally described. As a case in point, the scientific name *Octopus sinensis* was recently reinstated for the East Asian common octopus previously referred to as *O. vulgaris*, based on a combination of molecular and morphological differences (Gleadall 2016), while the Western Atlantic lineage was found to contain two more species, *Octopus americanus* (Avendaño et al. 2020) and *Octopus insularis* (Leite et al. 2008).

Octopus vulgaris is the most important commercially-exploited octopus species worldwide, and off northwest Africa, it is the target of the world's largest single-species octopus fishery. In 2010, total octopus catches for the northwest coast of Africa and Europe were 57,982 and 42,945 tonnes, respectively, consisting largely of *O. vulgaris* (Norman and Finn 2013). Currently, the poor state of octopus taxonomy is

the single largest impediment to accurate catch statistics and comprehensive management plans for octopus species worldwide (Norman and Finn 2013).

Mitochondrial DNA is a useful genetic marker for phylogenetic studies, due to its maternal inheritance, conserved gene arrangement and comparatively high mutation rate (Moritz et al. 1987). Mitogenomes have been used to investigate deep phylogenetic relationships across different taxa (Uribe and Zardoya 2017), as well as delineate closely related species (Monsanto et al. 2019).

Within the *O. vulgaris* species complex, two complete mitochondrial genomes are currently available on the public databases: “*O. vulgaris*” (NC_006353.1, Yokobori et al. 2004) and *O. sinensis* (NC_052881.1, Li et al. 2021). However, the original specimen used to create the first publicly available *O. vulgaris* mitogenome (NC_006353.1) was collected at the Tsukiji Fish Market, Tokyo, Japan in the early 2000s (Yokobori et al. 2004), before the scientific name *O. sinensis* was reinstated (Gleadall 2016). Since *O. sinensis* is distributed in the NW Pacific Ocean (from northern Japan to Taiwan), we hypothesized that this record in fact corresponds to *O. sinensis*. This suggests that, despite its commercial and ecological importance, no complete mitogenome has yet been reconstructed for *O. vulgaris* ss. The objective of the current study was to assemble, annotate, and describe the mitochondrial genome of *O. vulgaris* ss collected from its confirmed distribution range within northeastern Atlantic waters, and to examine its phylogenetic placement among closely related species. This study is important to improve our understanding of the taxonomic and phylogenetic relationships within the *O. vulgaris* species complex.

Material and methods

Sample collection, genomic library preparation and sequencing

The tissue sample of *Octopus vulgaris* ss was obtained from a specimen that was commercially caught near the Cies Islands in the Illas Atlánticas de Galicia National Park, Spain, in August 2022. DNA of high molecular weight was extracted from a small piece of muscle tissue using the QIAGEN DNeasy Blood & Tissue kit (Hilden, Germany). A genomic library was constructed from the extracted DNA using the

NOVO kit (Novogene, Beijing, PRC). For this purpose, the DNA was first sheared into smaller fragments, and fragments of size ~ 350 bp were selected for the adaptor ligation step. The quality of the genomic library was checked using a combination of Qubit (Thermo Fisher Scientific, Waltham, USA), qPCR, and the DNA NGS 3K assay (PerkinElmer, Waltham, USA). The quality-checked genomic library was sequenced on a NovaSeq 6000 SP platform (Illumina, San Diego, USA) using the paired-end 250 protocol.

Mitogenome assembly and annotation

The mitochondrial genome was assembled *de novo* using the GetOrganelle v.1.7 assembly pipeline (Jin et al. 2020). The assembly parameters were set to their defaults, except for the kmer values, which were set for a combination of the following: 21, 45, 65, 85 and 105. The assembled mitogenome was then submitted to the MITOS Web Server (Bernt et al. 2013) for annotation. The predicted gene boundaries were manually adjusted in MEGA11 (Tamura et al. 2021) using the mitogenomes of the previously published “*O. vulgaris*” from Japan (NC_006353.1) and *O. sinensis* from China (NC_052881.1) as template references. The nucleotide composition of the complete mitogenome was calculated manually using the formulas AT-skew = $(A-T)/(A+T)$, and GC-skew = $(G-C)/(G+C)$ (Perna and Kocher 1995). The annotated mitogenome was visualised in Chloroplot (Zheng et al. 2020).

Maximum likelihood phylogenomic analysis

To reconstruct the phylogenetic relationships between the mitogenome of European *O. vulgaris* ss and those from other octopus taxa, the assembled mitochondrial genome was blast-searched against the NCBI nucleotide database. The complete mitogenomes of eight closely related species of octopus, as well as an outgroup species, *Tremoctopus violaceus*, were retrieved for phylogenetic analysis. The Ezsplit tool (Cucini et al. 2021) was used to extract the sequences of all 13 protein coding genes (PCG) from the NCBI database, each of which was aligned separately using the codon alignment option in ClustalW with default setting (Thompson et al. 1994).

A maximum likelihood phylogenetic tree was then constructed using IQ-TREE (Nguyen et al. 2015). The most suitable evolutionary model was identified with

protein-coding genes (PCGs), 22 transfer RNAs (tRNA) and two ribosomal RNAs (rRNA) consistent with those reported from other octopus species. The plus strand contained seven PCGs (*atp6*, *atp8*, *cox1*, *cox2*, *cox3*, *nad2*, *nad3*) and eight tRNAs while the minus strand contained six PCGs (*cytb*, *nad1*, *nad4*, *nad4l*, *nad5*, *nad6*), 14 tRNAs and 2 rRNAs. The PCGs start with ATG, except for *nad4*, which has ATA as its start codon. The annotation pipeline also identified a single long intergenic sequence of approximately 645 bp between *trnE(gaa)* and *cox3*, which corresponds to the control region. Several ($n = 21$) shorter intergenic sequences ranging in length from 1 – 52 bp were also found.

The NCBI blast results showed European *Octopus vulgaris* ss to be most closely related to the only two other representatives of the *O. vulgaris* species complex with published mitogenomes. These are *O. sinensis* from China (NC_052881.1) and “*O. vulgaris*” from Japan (NC_006353.1), with 96.33 and 96.25% identity, respectively. Among other publicly-available complete mitochondrial genomes, the next closest matches were *O. bimaculoides* (NC_029723.1) and *O. mimus* (NC_044093.1), each with roughly 85% identity.

The maximum likelihood phylogenetic tree confirmed, with 100% bootstrap support, that the assembled mitogenome is distinct from that of both *O. sinensis* from China and from the Japanese specimen (Yokobori et al. 2004) that has been deposited in the NCBI database as *O. vulgaris* (NC_006353.1) (Figure 2.2). An NCBI blast search of the Japanese specimen showed 99.85% identity to the mitogenome of *O. sinensis* from China (Li et al. 2021), but only 96.25% to that of the European *O. vulgaris* ss generated in this study. This confirms our hypothesis that the mitochondrial sequence from the Japanese specimen represents an additional mitogenome of *O. sinensis*. The present study thus reports the first ever mitogenome of *O. vulgaris* ss.

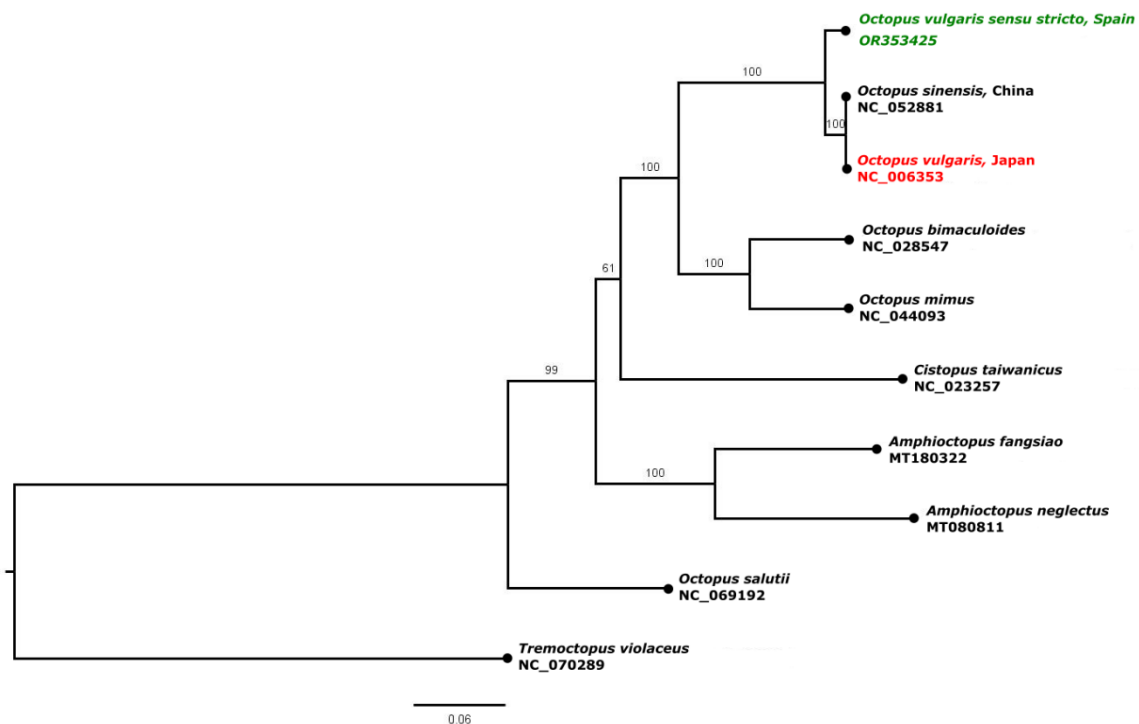


Figure 2.2 A maximum likelihood phylogenetic tree reconstructed using protein coding genes under the TVM+F+I+G4 model, with a proportion of invariable sites of 0.51 and a gamma distribution shape parameter (α) of 1.54. The new mitogenome produced from the European specimen is shown in green, and that of the misidentified specimen that represents a second specimen of *Octopus sinensis* is shown in red. Bootstrap support values are shown to the left of the nodes.

Conclusion

Recent advances in DNA sequencing technology and high throughput computation have made it possible to assemble large numbers of mitogenomes. However, this information can only be useful in resolving taxonomic uncertainties when the specimens were correctly identified (Teske 2021); to this end, existing records need to be revisited to reflect the latest developments in how species are classified.

The phylogenetic relationships within the family Octopodidae are an ongoing topic of investigation. Mitogenomic evidence in this study shows that the current record of an “*O. vulgaris*” mitogenome in the NCBI database represents mitochondrial sequences from *O. sinensis*. Overall, our findings contribute towards improving our understanding of octopus phylogeny and taxonomy, which can ultimately inform fisheries management and improve the accuracy of catch statistics. The generation of additional mitogenomes from the broader geographical region inhabited by

species within the *O. vulgaris* species complex will further enhance this field of knowledge.

Chapter 3: Complete mitochondrial genome of *Octopus vulgaris* Type III and its phylogenetic relationship within the *Octopus vulgaris* species complex

Introduction

Cryptic species are ubiquitous in marine invertebrate yet are often overlooked due to inadequate information (Knowlton 1993). Cryptic species are common in the Cephalopoda, particularly in the benthic octopuses (Norman et al. 2013). The genus *Octopus* Cuvier, 1797 is currently considered a 'catch-all' genus due to the subtlety of morphological traits available to distinguish closely related taxa. However, the genus is known to be polyphyletic and in need of taxonomic revision (Norman et al. 2013). The type species for this genus is *Octopus vulgaris* Cuvier, 1797, which itself is now understood to be a species complex comprising of geographically isolated, closely-related species. As early as 1998, it was speculated that the South African lineage of *O. vulgaris* is a unique species in need of a new name (Roeleveld 1998). More recent studies have supported this idea, and it is hypothesized that the population that extends from Tristan da Cunha Island in the South Atlantic, across the Southern African coastline of Namibia and South Africa, to Amsterdam Island in the Indian Ocean is a distinct species "Type III" within the *O. vulgaris* species complex (Norman et al. 2013; Amor et al. 2017). Of the six hypothesized species Types within the *O. vulgaris* species complex, as identified by Norman et al. (2013), *O. vulgaris* Type III is the least studied and the only taxon which has not yet been redescribed.

Octopus vulgaris was originally described by Cuvier in the Mediterranean in 1797 and, based on indistinguishable morphology, it was subsequently 'found' to occur in temperate and tropical waters throughout the world (Warnke et al. 2004). Initial genetic studies based on a single, or few, mitochondrial markers supported the hypothesis of a cosmopolitan species as populations from South Africa, Brazil, the Mediterranean, Senegal, Taiwan, Tristan Da Cunha and Venezuela formed a monophyletic clade (Oosthuizen et al. 2004; Warnke et al. 2004). However, more recent studies using a wider range of genetic markers and incorporating detailed

morphological assessments have supported the hypothesis of geographically distinct species (Norman et al. 2013; Amor et al. 2014, 2017, 2019), with many of these having already been formally described (*O. insularis* Leite et al. 2008; *O. sinensis* Gleadall et al. 2016; *O. americanus* Avendano et al. 2020; *O. djinda* Amor and Hart 2021). Although previous genetic studies did not support the separation of *O. vulgaris* Type III, it is not uncommon for mitochondrial sequences to be uninformative for phylogenetic studies if only a portion of the genome is used (DeFilippis and Moore 2000; Galtier et al. 2009; Xavier et al. 2015), these limitations can be overcome by using whole genomes to provide greater power in phylogenetic inferences (Morin et al. 2010).

Animal mitochondrial genomes are circular in shape and small (~16k base pairs) relative to the physically separate nuclear genomes. Conserved across most animal mitogenomes are the same 37 genes: two ribosomal RNA genes, 22 transport RNA genes and 13 protein coding genes which function in ATP synthesis (Moritz et al. 1987; Boore 1999). There are generally few intergenic sequences apart from a single large non-coding region (referred to as the “D-loop”) which contains controlling elements for replication and transcription (Boore 1999). Mitochondrial DNA is useful for phylogenetic studies due to its maternal inheritance, conserved gene arrangement and content, yet comparatively high mutation rate at the nucleotide sequence level (Avice 1986; Moritz et al. 1987).

Molluscs break the rules when it comes to mitochondrial genomes, as they vary greatly in size and contain deviations due to extensive gene rearrangements, additional genes, or nucleotide compositional skew (Ghiselli et al. 2021). These deviations are inconsistent among molluscan classes, thereby complicating investigations on deep phylogenetic relationships and thus the early origin and evolution of molluscs (Stöger and Schrödl 2013). Cephalopods are the only molluscan class to be resolved as monophyletic, yet uncertainty remains at various levels. The Nautiloidea and Decapodiformes are prone to frequent gene rearrangements, while the Octopodiformes retain the ancestral molluscan gene order and standard metazoan structure (Stöger and Schrödl 2013; Uribe and Zardoya 2017). With the improvements of molecular analysis and the increasing availability of

genetic data, many cryptic species of cephalopods are being identified across the globe.

Although individual mitochondrial genes failed to differentiate between species within the *O. vulgaris* species complex, no focused comparison has been made considering the full mitochondrial genomes. The aims of this study are to 1) annotate and describe the complete mitochondrial genome of *O. vulgaris* Type III and 2) determine the phylogenetic placement of this taxon within the species complex, with the hypothesis that *O. vulgaris* Type III is a unique species requiring a new name.

Methods

Sample collection, genomic library preparation and sequencing

Tissue samples were taken from two specimens of *Octopus vulgaris* Type III specimens, one collected in False Bay, South Africa and one from Amsterdam Island in the South Indian Ocean. DNA of high molecular weight was extracted from a small piece of muscle tissue using the standard protocol of QIAGEN DNeasy Blood & Tissue kit (Hilden, Germany). A genomic library was constructed from the extracted DNA using the NOVO kit (Novogene, Beijing, PRC). For this purpose, the DNA was first sheared into smaller fragments, and fragments of size ~ 350 bp were selected for the adaptor ligation step. The quality of the generated genomic library was checked using a combination of Qubit (Thermo Fisher Scientific, Waltham, USA), qPCR, and the DNA NGS 3K assay (PerkinElmer, Waltham, USA). The quality-checked genomic library was sequenced on a NovaSeq 6000 SP platform (Illumina, San Diego, USA) using the paired-end 250 protocol following manufacturer's instruction.

Mitogenome assembly and annotation

The mitochondrial genomes were assembled de novo using the GetOrganelle v.1.7 assembly pipeline (Jin et al. 2020). The assembly parameters were set to their defaults, except for the kmer values, which were set for a combination of the following: 21, 45, 65, 85 and 105. The assembled mitogenomes were then submitted to the MITOS Web Server (Bernt et al. 2013; Donath et al. 2019) for annotation. The predicted gene boundaries were manually adjusted in MEGA11 (Tamura et al. 2021)

using the mitogenomes of the previously published *O. sinensis* from China (NC_052881.1; Li et al. 2021) as a template reference. The nucleotide composition of the complete mitogenome was calculated manually using the formulas $AT\text{-skew} = (A-T)/(A+T)$, and $GC\text{-skew} = (G-C)/(G+C)$ (Perna and Kocher 1995). The annotated mitogenomes were visualised in Chloroplot (Zheng et al. 2020). Since both mitogenomes were extremely similar, only that of the South African specimen is discussed here as representative of the species. Details and figures of the Amsterdam Island specimen are included in the appendix.

Phylogenomic analysis

The phylogenetic relationships of *O. vulgaris* Type III was assessed using both bayesian inference (BI) and maximum likelihood (ML) methods. First, all available complete mitochondrial genomes from species within the *O. vulgaris* species complex and an outgroup were retrieved from NCBI after conducting a blast search (Altschul et al. 1990). This included *O. vulgaris* ss (OR353425.1) and two sequences for *O. sinensis* (NC052881.1 and NC006353.1). The sequence with accession number NC006353.1 is listed on NCBI as *O. vulgaris*, but Fee et al. (2024) showed this mitogenome to be incorrectly labelled and provided conclusive evidence that this sequence comes from an *O. sinensis* specimen. In both analyses, *Octopus bimaculoides* (NC_029723.1) and *O. mimus* (NC044093.1) were used as an outgroup. The Ezsplit tool (Cucini et al. 2021) was used to extract the sequences of all 13 protein coding genes (PCG) from the NCBI database, each of which was aligned separately using the MAFFT sequence alignment tool (Kato et al. 2018) before being trimmed and concatenated into a single continuous sequence.

A BI phylogenetic tree was reconstructed using BEAST2 v.2.5 (Bouckaert et al. 2019). For each gene, the best substitution model was predicted using the model averaging method implemented in the package bModelTest (Bouckaert and Drummond 2017). The phylogenetic reconstruction analysis was run for 100 million iterations in length following an initial burnin of 500 thousand iterations. A maximum clade credibility tree using median heights and a 30% burnin was then constructed in TreeAnnotator (Bouckaert et al. 2019), and the resulting tree was visualised in Figtree v.1.4.3 (Rambaut 2016).

A ML phylogenetic tree was constructed using IQ-TREE (Nguyen et al. 2015). The most suitable evolutionary model was identified with ModelFinder (Kalyaanamoorthy et al. 2017). Branch support was assessed using the ultrafast bootstrap analysis (Hoang et al. 2018) based on 1000 bootstrap alignment replicates. All other parameters were set to their default values. The resulting tree was visualised in Figtree v.1.4.3 (Rambaut 2016).

A pairwise distance analysis was performed on the concatenated sequence of protein coding genes using the Tamura-nei model (Tamura and Nei 1993) in Mega11 (Tamura et al. 2021) with 1000 bootstraps.

Results

The sequencing runs produced 12.1 million paired-end sequences, each 250bp in length with an average Phred quality score of 36. *De novo* assembly of the sequences produced a single circular contig 15672 base pairs in length with an average coverage of $\gg 100$, and a GC content of 25% (Figure 3.1). Base frequencies are A = 41%, C = 17.4%, G = 7.6% and T = 34% and the genome has a positive AT skew (0.093) and negative GC skew (-0.392). The MITOS annotation pipeline identified 13 protein-coding genes (PCGs), 22 transfer RNAs (tRNA) and two ribosomal RNAs (rRNA) consistent with those reported from other octopus species. The plus strand contained seven PCGs (atp6, atp8, cox1, cox2, cox3, nad2, nad3) and eight tRNAs while the minus strand contained 6 PCGs (cytb, nad1, nad4, nad4l, nad5, nad6), 14 tRNAs and 2 rRNAs. All PCGs start with ATG, except for nad4 and nad4l, which both start with ATA. The annotation pipeline also identified a single long intergenic sequence of 649 bp between trnE(gaa) and cox3, which corresponds to the putative control region. Several (n = 21) shorter intergenic sequences ranging in length from 1 – 81 bp were also found.

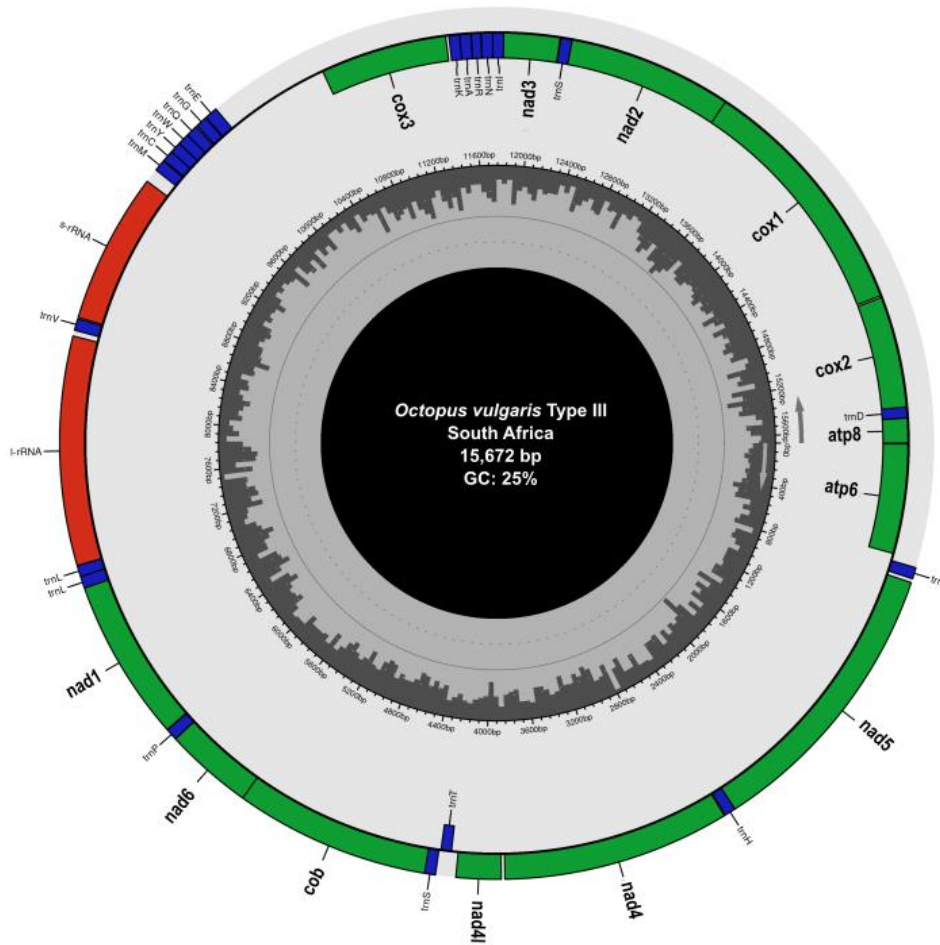


Figure 3.1 Graphic representation of the South African *Octopus vulgaris* Type III mitogenome, indicating the location of tRNAs (blue), rRNAs (red), and protein coding genes (green). Grey bars in inner circle represent GC content.

The NCBI blast results showed *Octopus vulgaris* Type III from South Africa to be most closely related to *O. vulgaris* ss with 98.76 and 98.68% identity (OR353425.1 and OY720585.1 respectively). *Octopus sinensis* shared 96.3 and 96.2% identity (NC052881.1 and NC006353.1), while the outgroup taxa *O. mimus* (NC044093.1) and *O. bimaculoides* (NC029723.1) were 85 and 85.2% respectively. This is reflected in both the BI (Figure 3.2) and ML (Figure 3.3) phylogenetic reconstructions where the *O. vulgaris* Type III mitogenomes generated in this study forms a monophyletic clade sister to *O. vulgaris* ss. These reconstructed phylogenies are statistically supported by high bootstrap (ML) and posterior probability (BI) values. Based on the Tamura-nei model, the two *O. vulgaris* Type III mitogenomes from South Africa and

Amsterdam Island had a genetic distance of 0.096% and an average genetic distance of 1.06% and 2.91% to *O. vulgaris* ss and *O. sinensis*, respectively (Table 3.2).

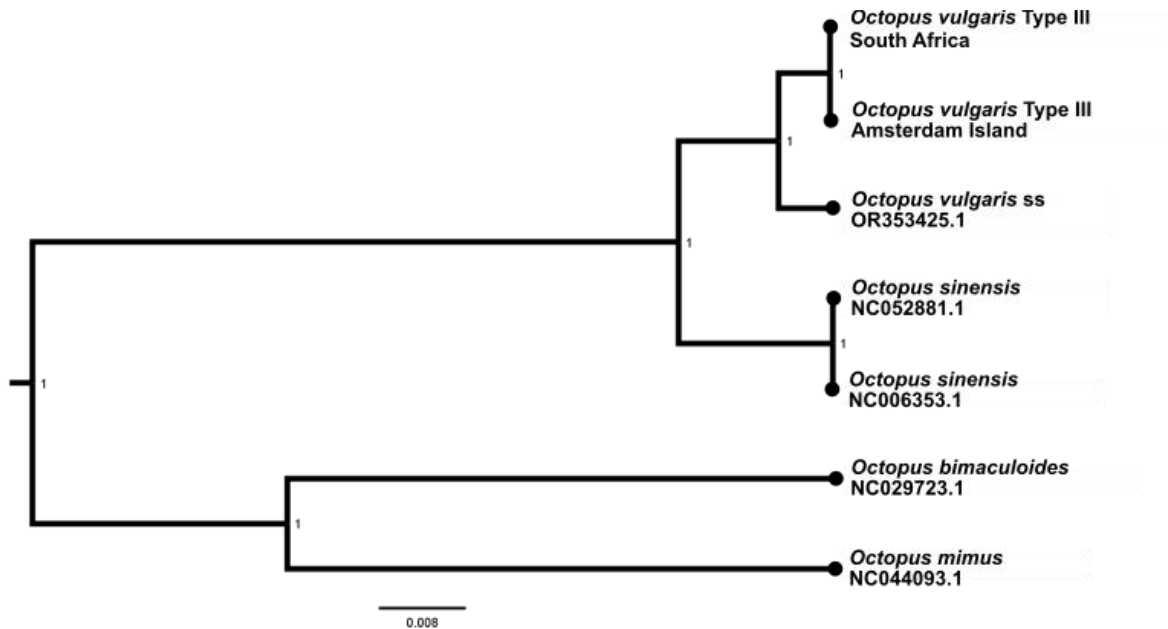


Figure 3.2 A maximum clade probability consensus phylogenetic tree (BI) reconstructed using a concatenated sequence of protein coding genes. Posterior probabilities are shown at each node.

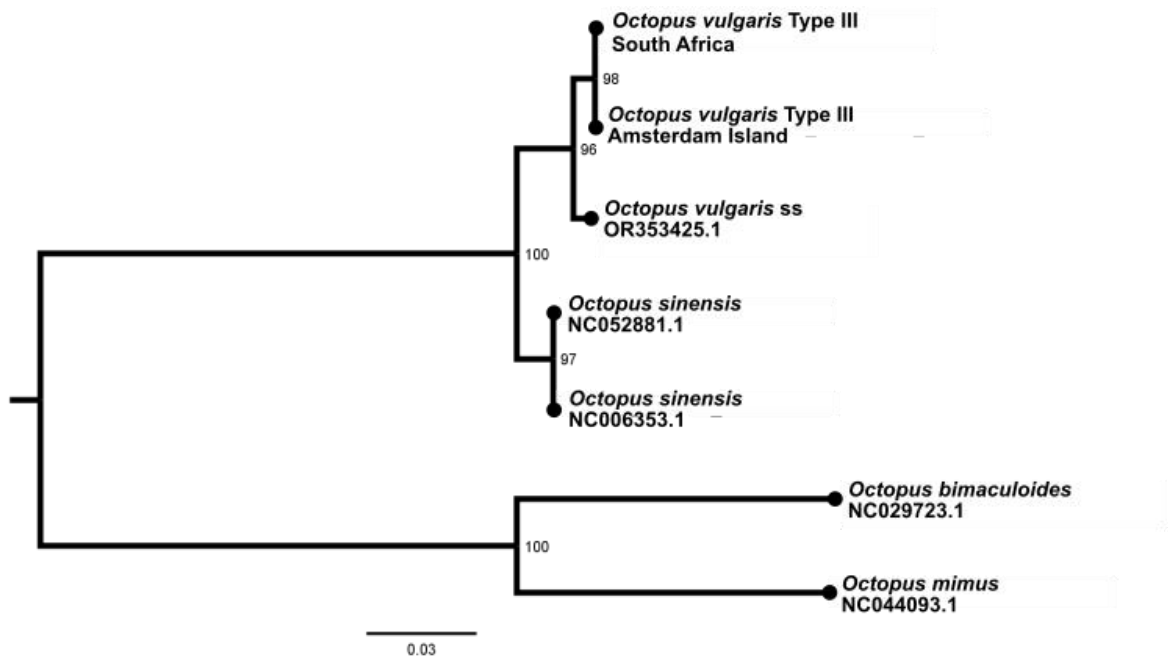


Figure 3.3 A maximum likelihood phylogenetic tree reconstructed using protein coding genes under the TPM2 + F + I model, with a proportion of invariable sites of 0.72. Bootstrap support values are shown at each node.

Table 3.1 Genetic distance based on the concatenated protein coding gene sequences from the mitochondrial genome of octopus in the *Octopus vulgaris* species complex and close relatives. Results are based on the Tamaura nei model (bottom left diagonal) with standard error estimates (top right diagonal).

	<i>O. vulgaris</i> ss OR353425.1	<i>O. vulgaris</i> Type III SA	<i>O. vulgaris</i> Type III AI	<i>O. sinensis</i> NC052881.1	<i>O. sinensis</i> NC006353.1	<i>O. mimus</i> NC044093.1	<i>O.</i> <i>bimaculoides</i> NC029723.1
<i>O. vulgaris</i> ss OR353425.1		0.000991	0.000955	0.001582	0.001583	0.003928	0.003929
<i>O. vulgaris</i> Type III SA	0.010965		0.000308	0.001681	0.001679	0.003914	0.003896
<i>O. vulgaris</i> Type III AI	0.010323	0.000985		0.001687	0.001686	0.003912	0.003914
<i>O. sinensis</i> NC052881.1	0.027415	0.029144	0.028855		0.000180	0.003893	0.003940
<i>O. sinensis</i> NC006353.1	0.027607	0.029336	0.029047	0.000358		0.003885	0.003933
<i>O. mimus</i> NC044093.1	0.146580	0.146078	0.146432	0.145580	0.145587		0.003402
<i>O.</i> <i>bimaculoides</i> NC029723.1	0.143162	0.143302	0.143892	0.143925	0.143931	0.102860	

Discussion

Recognition and the accurate classification of cryptic species has ecological and evolutionary implications, not only in theory, but also in understanding and managing marine resources and environments (Knowlton 1993). Mitochondrial genomes are useful for investigating population structure, gene flow, hybridisation, biogeography and phylogenetic relationships (Moritz et al. 1987), including identifying and delineating closely related species (Morin et al. 2010). In this study, the complete mitochondrial genome of *Octopus vulgaris* Type III was *de-novo* sequenced, annotated, described, and compared to the mitochondrial genome of closely related species. The structure and features of the complete mitochondrial genome of *O. vulgaris* Type III is consistent with other *Octopodidae*. Phylogenetic reconstruction placed the two *O. vulgaris* Type III samples as a sister clade to *O. vulgaris* ss, providing further evidence for its distinction as a separate species.

The first genetic studies of the *O. vulgaris* failed to distinguish between cryptic species and recognised a single cosmopolitan species (Warnke et al. 2004). Later investigations considering further genetic, as well as morphological and ecological evidence, successfully identified multiple species within the *O. vulgaris* species complex, most of which have since been redescribed (Leite et al. 2008; Gleadall

2016; Avendaño et al. 2020; Amor and Hart 2021). Phylogenetic analyses based on individual mitochondrial genetic markers (COI, COIII or 16S RNA) failed to distinguish between *O. vulgaris* ss and *O. vulgaris* Type III (Warnke et al. 2004; Oosthuizen et al. 2004), while another study found two separate lineages living in South African waters (Teske et al. 2007). On the other hand, ddRADseq of nuclear DNA confidently supports the distinctiveness of *O. vulgaris* Type III and even finds it more closely related to the Australasian species *O. tetricus*, *O. sinensis* and *O. djinda* than to *O. vulgaris* ss (Amor et al. 2019). Utilising complete mitochondrial genomes can sometimes provide higher power in phylogenetic analyses for closely related species, compared to single or few mitochondrial markers (DeFilippis and Moore 2000; Galtier et al. 2009; Morin et al. 2010). Our analyses based on the full mitochondrial genome showed *O. vulgaris* Type III to be more closely related to *O. vulgaris* ss than to *O. sinensis*.

Amor et al. (2019) suggested that the discrepancies between the nuclear and mitochondrial genomes could be a result of isolation of populations followed by secondary contact and hybridisation. *Octopus vulgaris* ss and *O. vulgaris* Type III have allopatric distributions in the northern and southern hemispheres respectively. Although the larvae of octopus are planktonic with large dispersal capabilities, there is a discontinuation of their populations along equatorial Africa. The upwelling of the Benguela Current is known to be a barrier to dispersal in the north of Namibia (Lett et al. 2007), the edge of the distribution range of *O. vulgaris* Type III. Due to this barrier and the large geographic separation between species, natural gene flow is unlikely between the two populations. However, there could have been a time in the past when gene flow occurred between these populations. More recently, accidental introductions could have occurred through the transportation of animals on ships, including in ballast water (Williams et al. 1988; Bello et al. 2020).

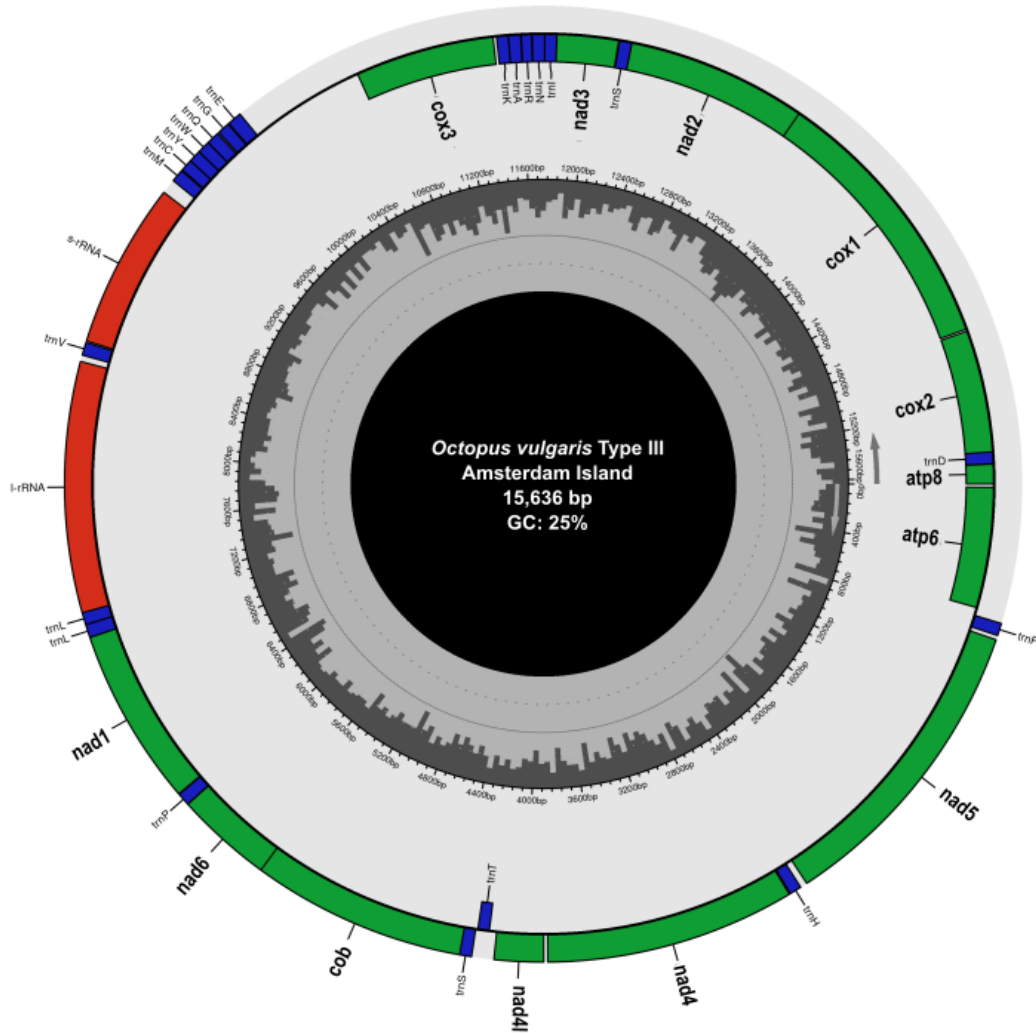
Low amounts of inter-specific genetic distance can be a result of hybridisation or recent divergence of lineages (Hebert et al. 2004b). Amor et al. (2019) calculated that the *O. vulgaris* species complex formed within the last 2.5 million years ago, with the split between the *O. vulgaris* ss and *O. vulgaris* Type III lineages around 1 – 1.5 million years ago. *Octopus vulgaris* Type III had an average genetic distance of 1.06%

to *O. vulgaris* ss and 2.91% to *O. sinensis*. Genetic divergence of greater than 2% generally corroborates accepted species boundaries (Roux et al. 2016) and is widely used as indication of possible speciation. However, many species have been delimited on much lower levels of genetic divergence, for example, large white-headed gulls (Crochet et al. 2003) and Killer Whales (Morin et al. 2010), and even as low as 0.075% in a pair of fairywren species (Roux et al. 2016). Hebert et al. (2004b) proposed that an interspecific threshold for genetic distance be set at 10X the mean intraspecific variation of the group under study. Within the *O. vulgaris* species complex, the intraspecific distance for *O. vulgaris* Type III and *O. sinensis* was 0.096% and 0.036% respectively which is an order of magnitude less than the interspecific distance between *O. vulgaris* Type III and any other species.

One limitation of using full mitochondrial genomes for phylogenomic studies is the cost of sequencing, and thus few data are currently available for comparison. This study contributed two of only five full mitochondrial genomes from three different species within the *O. vulgaris* species complex. As sequencing technology advances and becomes cheaper, additional sequences can strengthen the inferences made in this study and can be utilised in studies investigating deeper evolutionary relationships within the cephalopods and molluscs.

Conclusion

Despite multiple studies investigating the *Octopus vulgaris* species complex, the phylogeny and taxonomy within this group is still not fully resolved. A question remains on the identity of the Southern African lineage, *Octopus vulgaris* Type III. This study found only a small amount of genetic sequence divergence between the full mitochondrial genomes of *O. vulgaris* Type III and *O. vulgaris* ss, however this distance was an order of magnitude larger than intraspecific distance within the group which can be considered evidence of speciation.



Appendix 3.1. Graphic representation of the South African *Octopus vulgaris* Type III mitogenome, indicating the location of tRNAs (blue), rRNAs (red), and protein coding genes (green). Grey bars in inner circle represent GC content.

Chapter 4: Morphological assessment of *Octopus vulgaris*

Type III

Introduction

Morphological difference has long been the dominant criterion of species definitions (Mayr 1963). However, today, multiple species concepts exist which consider biological (reproductive), genetic or ecological criteria to be the defining characteristic of a species (De Queiroz 2007). Indeed, it is now common for species to be defined using evidence from several species concepts. For example, taxonomy is still underpinned by morphology-based descriptions and the assignment of reference specimens for comparison. However, cryptic species – defined as morphologically similar, but genetically or otherwise distinguishable taxa (Mayr 1963; Knowlton 1993) – by definition, require complimentary evidence for accurate identification (e.g. geographic, genetic, etc). These subtle morphological differences are often unknown or overlooked until additional evidence is obtained (Riziki 1951; Hebert et al. 2004a; Leite et al. 2008). Cryptic species is relatively common and it is not limited to understudied organisms (Bickford et al. 2007). In fact, the world's most studied and economically important octopus, *Octopus vulgaris*, was recently found to be composed of several discrete taxa (Amor et al. 2017).

Cephalopods are a group of marine molluscs consisting of nautilus, squids, cuttlefish, and octopuses. The last three of these together make up the subclass Coleoidea and are characterised by having reduced and internalised shells, these being completely lost in the case of octopuses (Norman 2000). The benthic octopuses (family Octopodidae) contains over 200 species (Norman and Hochberg 2005), many of which are placed in the genus *Octopus* Cuvier, 1798. This genus is characterised by a muscular mantle and arms, saccular mantle with a wide opening, two rows of suckers on each arm, hectocotylized third right arm in males, terminal organ with diverticulum in males, functional ink sac, well-developed anal flaps, absence of water pouches on the oral surface of webs and a benthic adult life history (Norman and Sweeney 1997; Norman et al. 2013). The characteristic soft bodies of

octopuses are morphologically plastic (Voight 1994) with few hard structures, making them susceptible to distortion upon preservation (Voight 2001). This, along with limited number of morphological traits available to distinguish between morphologically similar species (Pérez-Losada et al. 2002), has complicated species-level taxonomy. Thus, this genus is strewn with cryptic taxa (Norman and Hochberg 2005). However, detailed morphological assessments regularly reveal distinct characters (Pickford and McConnaughey 1949; Leite et al. 2008; Amor et al. 2017) which corroborate genetic or ecological evidence for separation. For example, *O. bimaculoides* was first discovered to be separate to *O. bimaculatus* based on different parasites carried (Pickford and McConnaughey 1949). Later ecological and morphological differences were documented to support their distinction as separate species. Similarly, *O. mimus* was once synonymous with *O. vulgaris*, but on closer inspection was morphologically distinguished based on the number of suckers on the hectocotylyzed arm, a smaller calamus and the presence of faint paired ocelli (eye spots) between the eyes and base of arms two and three in fresh specimens, a trait which *O. vulgaris* lacks (Guerra et al. 1999).

Octopus vulgaris is presumed to be described from the Mediterranean, although no type specimen was designated (Norman et al. 2013). It has long been assumed that *O. vulgaris* was a cosmopolitan species living in temperate and tropical waters around the globe (Warnke et al. 2004), but today there is substantial support for a species complex comprised of multiple lineages (Norman et al. 2013; Amor et al. 2017). In this complex, *O. vulgaris* sensu stricto (ss) is considered to be restricted to the Northeast Atlantic and Mediterranean, while the other geographically separated lineages were assigned species 'Types' (Norman et al. 2013). Many of these 'Types' have already been formally described as unique species (*O. sinensis* Gleadall 2016; *O. americanus* Avendaño et al 2020; *O. djinda* Amor and Hart 2021).

When the cosmopolitan distribution of *O. vulgaris* was first questioned, it was speculated that the South African taxon "needs a new name" (Roeleveld 1998). However, early population biology studies of this taxon, which included a morphological assessment, failed to distinguish it from *O. vulgaris* ss from the Mediterranean, likely because only a limited set of traits (body mass, total length,

mantle length, head width and gonad mass) were examined (Smale and Buchan 1981; Smith and Griffiths 2002; Oosthuizen and Smale 2003). Many other traits could, however, be examined. For example, the sexual traits of male octopuses are important characters for morphology-based species discrimination (Amor et al. 2017), none of which have been considered in previous studies on the South African lineage (*O. vulgaris* Type III).

The South African taxon was first considered as a separate species 'Type' based on its geographic isolation and lack of plausible gene flow (Norman et al. 2013). More recently, molecular evidence considering nuclear genes (Amor et al. 2019), as well as the full mitochondrial genome (Chapter 3, this thesis) provided support for its distinction. Amor et al. (2017) found that detailed morphological assessment of the *O. vulgaris* species complex reflected the phylogenetic relationships obtained using genetic evidence, but *O. vulgaris* Type III morphology was not included in this global assessment. Thus, to date, a detailed morphological study on the South African taxon is lacking. The aim of this study is to assess the morphology of *O. vulgaris* Type III and compare it to other species in the *O. vulgaris* complex, with the hypothesis that *O. vulgaris* Type III is a morphologically distinct taxon in need of a new name.

Methods

Sample collection and preparation

Fresh *Octopus vulgaris* Type III specimens were purchased from the experimental octopus fishery operating in False Bay, South Africa. The fishery uses un-baited pots permanently placed at 4 – 30 m depth (John 2022). On board, octopus are euthanised in an ice slurry and transported on ice to the processing factory. A 5 x 5 mm sample of muscle tissue was taken from the second or third left arm of each specimen and stored in 96% ethanol for later genetic analysis. Whole specimens were then frozen for storage.

Prior to fixation, specimens were thawed in a seawater bath over several hours. Once completely defrosted, formalin (formaldehyde 37% w/w) was added to the seawater baths to a final concentration of 10% (3.7% formaldehyde), ensuring the solution

entered the mantle cavity, following the procedure of Roper and Sweeny (1983). Specimens were treated in formalin for 7 – 10 days before being transferred to a series of three seawater baths, lasting 4 – 12 h each, to remove residual formalin. Whole specimens were then preserved and stored in 70% ethanol and measured. Morphological data for *O. vulgaris* ss, *O. sinensis*, *O. americanus*, *O. tetricus*, *O. djinda* and *O. insularis* were obtained from Amor et al. (2017) supplementary materials to be used for comparative analysis.

Morphological and meristic measurements

Morphological traits were measured following Roper and Voss (1983) and Huffard and Hochberg (2005). Total preserved weight (TWt) was recorded via digital scale to the nearest gram. Total length (TL), paleal aperture (PA), web diameter (WD) and arm lengths (AL) were recorded using a non-stretch cord to the nearest 1 mm. Small structures such as Ligula (LL), calamus (CL), spermatophores and eggs were measured to the nearest 0.01 mm with an ocular micrometre in a binocular microscope (WILD Heerbrugg). Dorsal mantle length (MLd), ventral mantle length (MLv), mantle width (MW), head width (HW), funnel length (FL), free funnel length (FFL), arm width (AW), enlarged (SDe) and non-enlarged (SDn) sucker diameters, and gill lengths (GL) were recorded using digital callipers to the nearest 0.1 mm. Sucker counts included all suckers on each arm and were recorded with the aid of the dissecting microscope. Arm length and sucker counts were excluded where significant damage to the arm was evident. Males were classified as mature by the presence of spermatophores in the Needham's sac. Females were categorised according to Mangold (1987): immature – ovary small and white; maturing – ovary is larger and eggs off-white; mature – loose eggs present in the ovisac; spent – ovisac flaccid.

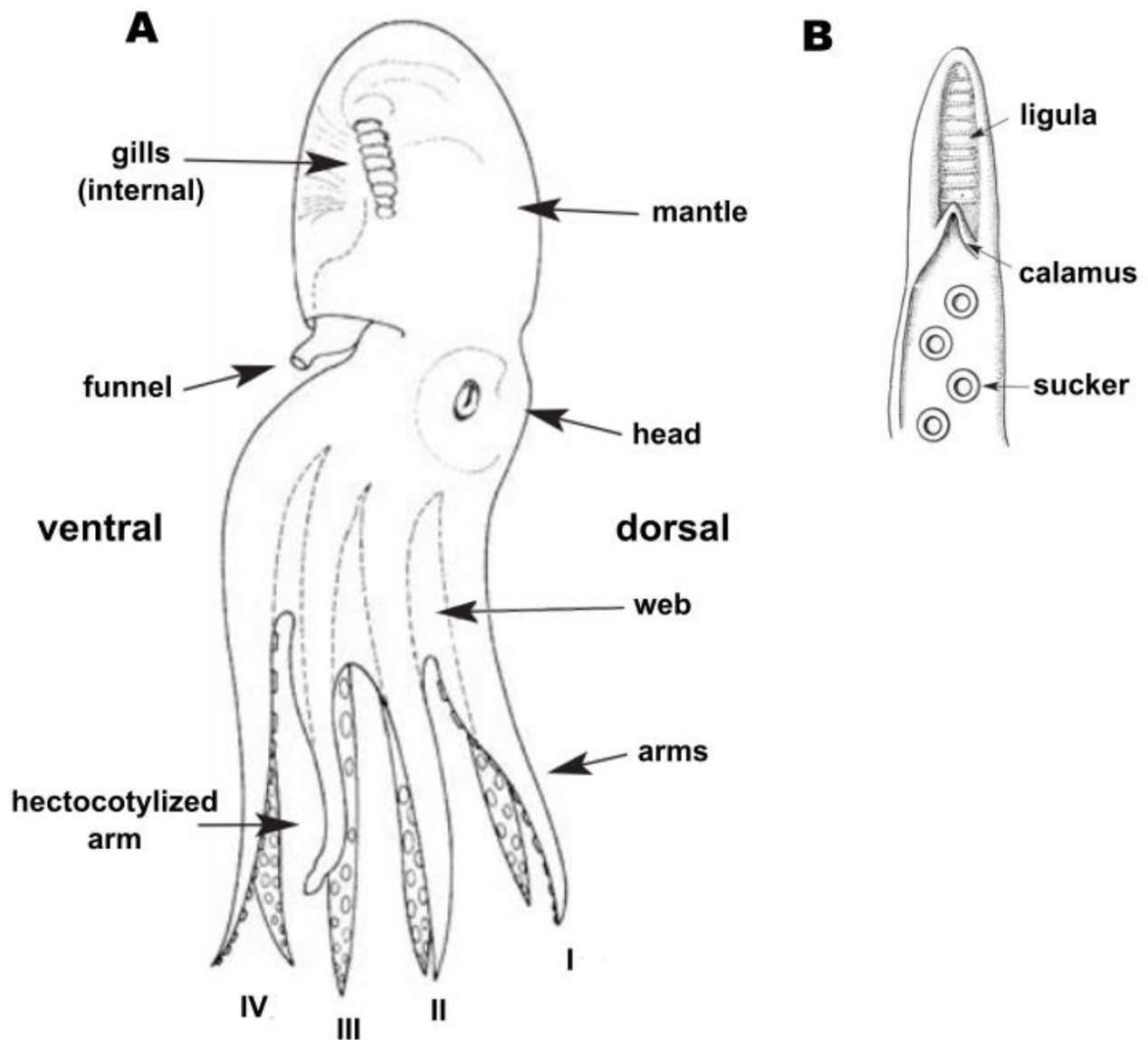


Figure 4.1 A) Schematic diagram of an Octopus and B) the hectocotylized third right arm of males, highlighting some of the morphological features discussed. Roman numerals refer to arm pairs. (Figure modified after Jereb et al. 2014)

Statistical analysis

Following the methods of Amor et al. (2017), analyses were performed on males and females separately to allow inclusion of male-specific reproductive characters (SDe, LL, CL, TOL and DL). Missing data points were replaced with the mean of that trait across all samples of the particular species, as the multivariate analyses do not allow for missing data points. All morphometric and meristic traits were transformed to indices to account for differences attributed to variation in overall size (Table 4.1).

Sucker counts were divided by the respective arm length, FFL was divided by FL, SDn and SDe were divided by AW, LL was divided by ALR3, CL was divided by LL and DL was divided by TOL. All remaining traits were divided by the MLd (a proxy for body size). MLd was then removed as a trait for the analyses to allow investigation of size free trait variation.

Table 4.1 Abbreviation and definition of the trait and formula of the indices used in comparative analyses.

Abbrev.	Trait description	Index
MLvi	Ventral mantle length	MLv / MLd
MWi	Width of mantle at its widest point	MW / MLd
HWi	Width of head at level of the eyes	HW / MLd
FLi	Length of funnel from inside mantle to tip of funnel	FL / MLd
FFLi	Length of 'free' funnel from back of head to tip of funnel	FFL / FL
WDi	Web depth from beak to mid-point of deepest web sector	WD / MLd
ALi	Arm length, recorded for each arm L1–4, R1–4	AL / MLd
AWi	Width of stoutest arm at its stoutest point	AW / MLd
SDni	Diameter of largest non-enlarged sucker on any arm	SDn / AW
SDei	Diameter of largest enlarged sucker on any arm (Male only)	SDe / AW
SCi	Sucker count on arms L3 and R3	SC / respective arm length
GLi	Gill length, both L and R	GL / MLd
LLi	Ligula length (Male only)	LL / ALR3
CLi	Calamus length (Male only)	CL / LL
TOLi	Terminal organ length (Male only)	TOL / MLd
DLi	Diverticulum length (Male only)	DL / TOL

All analyses were performed using *PRIMER E+ v7* (Clarke and Gorley 2015) and *PERMANOVA+* (Anderson et al. 2008). Indices were log-transformed and normalised using the 'normalise variables' function to allow for comparisons of traits despite differing scales of measurement. Draftsman plots were used to check for outliers or obvious errors in the data, which were then corrected or removed. Collinearity of morphological trait indices was investigated via principal component analysis (PCA) vector plots, Draftsman plots and Spearman rank correlation matrices. Highly correlated variables ($R^2 \geq 80\%$) were considered redundant and removed. Variables with R^2 values of 75 – 80% were removed if they also showed high correlation in the PCA space. The colour and symbol of each individual in the PCA plot reflect their (hypothesized) species identity.

A resemblance matrix based on Euclidean distance was calculated. Difference in traits among sampled individuals and species groups were analysed via both main test and pairwise permutational multivariate ANOVA (PERMANOVA). The effect of within-clade multivariate dispersion (the significance of within-clade variation contributing to between-clade differences) was investigated via permutational distance-based tests for homogeneity of multivariate dispersion (PERMDISP). Trait contributions to variation within and between species were investigated via similarity percentages (SIMPER) analysis. A canonical analysis of principal components (CAP) was performed to evaluate the discriminative power of traits and the cross validation of group (species) assignment estimates.

Results

Description of morphology

A total of 14 specimens (seven males and seven females) were examined ranging from 432 – 2814 g total weight. Male dorsal mantle length ranged from 104.9 – 171.3 mm. Sucker counts ranged from 200 – 291 on normal arms and 147 – 162 on the hectocotylyzed arm (third right arm of males), which was on average 16% shorter than the corresponding left arm. Males had one or two enlarged suckers on arm pairs two and three on the level of the 13th to 15th proximal suckers. Ligula length ranged

from 3.6 – 5.68 mm while the calamus ranged from 1.12 – 2.16 mm in length. Female dorsal mantle length ranged from 90.1 – 177.8 mm, with sucker counts ranging from 217 – 312. All male specimens were mature, while females had relatively small and white, or slightly larger white ovaries and were thus considered immature or maturing (Mangold 1987). Gill length of both sexes ranged from 36.6 – 67.1 mm with 7 – 10 lamellae per demibranch.

Analyses of male specimens

Male arm lengths L2, L4, R2 and R4 were highly correlated, as were both gill lengths (GLL and GLR) and sucker counts (SCL3 and SCR3). Removing arm lengths L2, L4 and R2 as redundant, while retaining R4, reduced all collinearity. Sucker count data were more complete for the SCR3, also corresponding to the hectocotylized arm in males, and were thus retained, while SCL3 was considered redundant and removed. Gill length data were more complete on the right side, thus GLL was considered redundant and removed. PERMDISP analysis showed a significant difference in dispersion among the seven species ($F = 4.633$, $df = 6$, $P = 0.0005$), which is also evident in the PCA (Figure 4.2A). *Octopus vulgaris* Type III was significantly less dispersed ($P < 0.05$) than other species, except for *O. tetricus* and *O. djinda*. The main-effect PERMANOVA showed significant morphological difference among the seven species groups (Pseudo- $F = 4.919$, $df = 6$, $P = 0.0001$).

Pairwise tests comparing the South African taxon to all other species showed that male *O. vulgaris* Type III morphology was always significantly different ($P \leq 0.05$) (Table 4.2). *Octopus vulgaris* Type III and *O. americanus* were primarily distinguished on differences in gill length (GLR) and LL. MLv was the primary distinguishing trait between *O. vulgaris* Type III and both *O. vulgaris* ss and *O. sinensis*, with FL and WD a secondary trait for each respectively. ALR1 and HW distinguished *O. vulgaris* Type III from *O. tetricus*, SCR3 and CL distinguished it from *O. insularis*, and DL and TOL differentiated it from *O. djinda*.

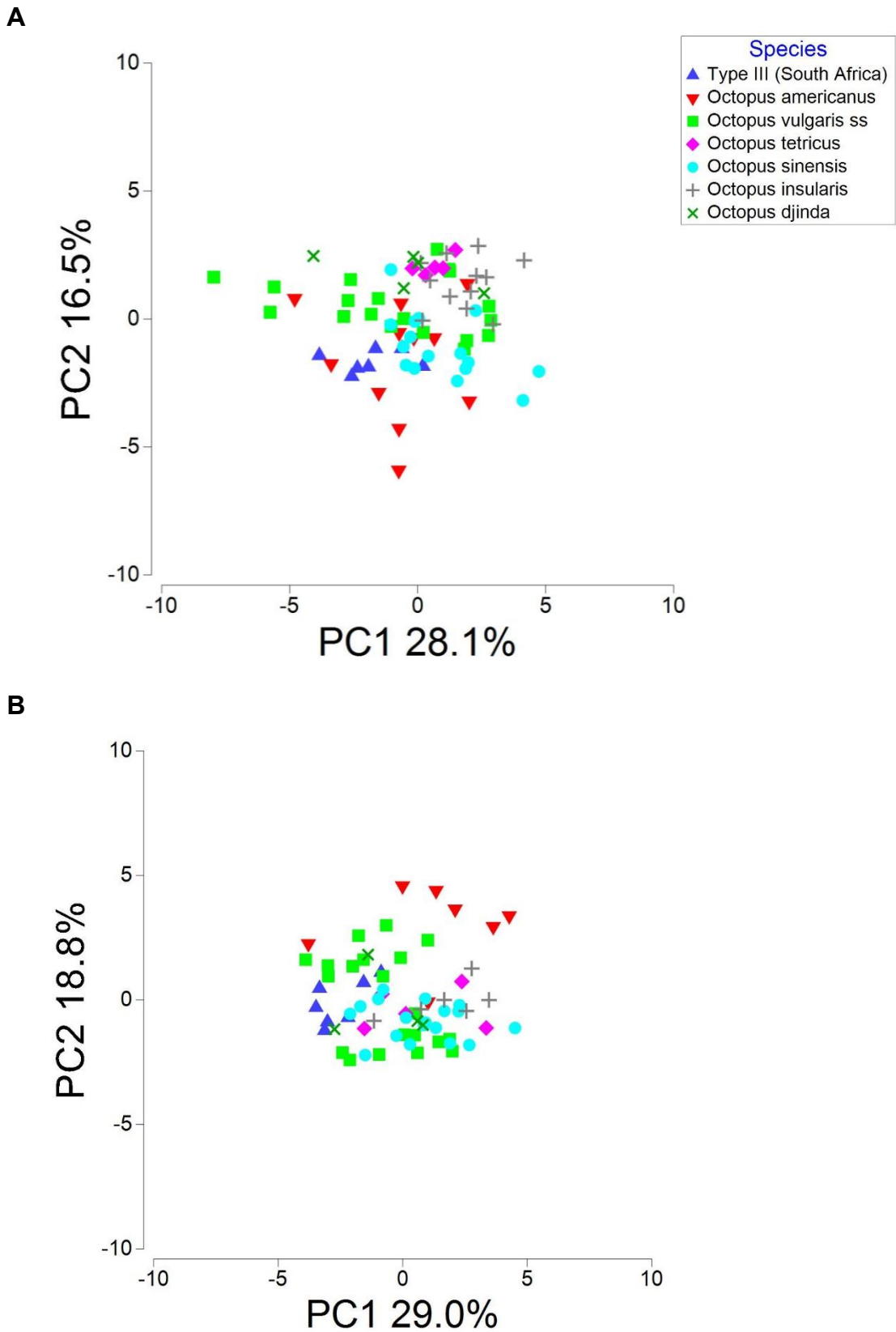


Figure 4.2 Principal component biplot of male (A) and female (B) octopus individuals grouped by species. Analysis is based upon 20 and 15 morphological traits, respectively (redundant variables removed).

The principal component biplot (Figure 4.2A) showed *O. vulgaris* Type III to have lower levels of morphological variability in comparison to other species, particularly *O. vulgaris* ss and *O. americanus*, as depicted by the spread of the data points. *Octopus vulgaris* Type III appeared to occupy the space within the variability of *O. americanus*, although this was less clear in the 3-dimensional plot (Appendix 4.1), where divergence was driven by GLR. There was a partial overlap between *O. vulgaris* Type III and *O. sinensis*.

Of the 75 male individuals analysed, 60 (80%) were correctly assigned to their *a priori* group via CAP analysis (Table 4.2). *Octopus vulgaris* Type III was classified with 100% accuracy. *Octopus vulgaris* ss and *O. sinensis* each had one individual misclassified as *O. vulgaris* Type III, with 84.2% and 68.8% accuracy for the respective groups.

Table 4.2. Pairwise comparison of male specimens of *Octopus* species based on 20 morphological traits. Lower left diagonal represents PERMANOVA results. Upper right diagonal represents results of SIMPER analysis showing traits that contribute the most (>15% cumulative) to variation between groups. Far right column represents the percentage of individuals correctly assigned to their *a priori* group via canonical analysis of principle components (CAP).

	<i>O. vulgaris</i> Type III	<i>O.</i> <i>americanus</i>	<i>O. vulgaris</i> ss	<i>O. tetricus</i>	<i>O. sinensis</i>	<i>O.</i> <i>insularis</i>	<i>O. djinda</i>	Correct (%)
<i>Octopus vulgaris</i> Type III		GLR / LL	MLv / FL	ALR1 / HW	MLv / WD	SCR3 / CL	DL	100
<i>Octopus americanus</i>	0.0001		SDn / AW	ALR1 / AW	GLR / LL	SCR3 / SDe	DL / SDn	81.8
<i>Octopus vulgaris</i> ss	0.0155	0.0023		ALR1 / ALL3	ALR4 / WD	ALR3 / ALL3	DL / MW	84.2
<i>Octopus tetricus</i>	0.0023	0.0003	0.0399		AW / HW	AW / CL	ALR1	80
<i>Octopus sinensis</i>	0.0008	0.0001	0.0042	0.0003		TOL / CL	TOL / DL	68.8
<i>Octopus insularis</i>	0.0001	0.0001	0.0002	0.0005	0.0001		ALR3 / MW	66.7
<i>Octopus djinda</i>	0.001	0.0024	0.0952	0.008	0.0003	0.0048		100

Analyses of female specimens

Female arm lengths L1, L3, L4, R1, R2 and R3 were each highly correlated with one or more other arm length. By considering L1, L3, L4 and R2 redundant, while retaining the remaining arms, collinearity was reduced. Sucker counts R3 and L3 were highly correlated, data were more complete on the left side, thus SCR3 was removed as redundant. Dispersion of *O. vulgaris* Type III was significantly lower ($P <$

0.05) than all other groups (Figure 4.2B). A significant morphological difference was found among the seven species groups (Pseudo-F = 4.8081, $df = 6$, $P = 0.0001$). Pairwise comparison showed that *O. vulgaris* Type III morphology differed significantly ($P < 0.005$) from all other groups (Table 4.3). Female *O. vulgaris* Type III were primarily distinguished from *O. americanus* based on differences in GLR and AW. ALL2 was an important trait of *O. vulgaris* Type III, distinguishing it from all remaining species along with MLv for *O. vulgaris* ss and *O. sinensis*, FL for *O. tetricus* and *O. djinda*, and ALR3 for *O. insularis*.

Visualisation of the principal component biplot (Figure 4.2B) showed *Octopus vulgaris* Type III females were characterised by negative PC1 loadings and had the least morphological variability relative to other species. *Octopus vulgaris* ss appeared split in two distinct groups characterised by low and high PC2 loadings respectively. *Octopus vulgaris* Type III occupied the space between these two *O. vulgaris* ss groups, along with a few *O. sinensis* individuals.

Only 41 of the 68 (60%) female individuals analysed were correctly assigned to their *a priori* groups via CAP analysis (Table 4.3). One *O. vulgaris* Type III individual was mis-classified as *O. sinensis*, while the other six individuals were correctly classified (86%). One of seven and one of 18 *O. americanus* and *O. sinensis* individuals respectively were mis-classified as *O. vulgaris* Type III.

Table 4.3 Pairwise comparison of female specimens of *Octopus* species based on 15 morphological traits. Lower left diagonal represents PERMANOVA results. Upper right diagonal represents results of SIMPER analysis showing traits that contribute the most (>15% cumulative) to variation between groups. Far right column represents the percentage of individuals correctly assigned to their *a priori* group via canonical analysis of principle components (CAP).

	<i>O. vulgaris</i> Type III	<i>O.</i> <i>americanus</i>	<i>O. vulgaris</i> ss	<i>O. tetricus</i>	<i>O. sinensis</i>	<i>O.</i> <i>insularis</i>	<i>O. djinda</i>	Correct (%)
<i>Octopus vulgaris</i> Type III		GLR / AW	MLv / ALL2	FL	ALL2 / MLv	ALL2	ALL2 / FL	85.7
<i>Octopus americanus</i>	0.0017		SDn	SDn	SDn	SCL3	AW	71.4
<i>Octopus vulgaris</i> ss	0.0002	0.0001		HW / FL	MW / FFL	SCL3	FFL / MLv	66.7
<i>Octopus tetricus</i>	0.0017	0.0126	0.0595		FL	FFL / FL	HW	20
<i>Octopus sinensis</i>	0.0001	0.0001	0.0127	0.2399		SCL3 / GLL	HW / WD	44.4
<i>Octopus insularis</i>	0.0013	0.0028	0.0001	0.0336	0.0001		AW / WD	83.3
<i>Octopus djinda</i>	0.0037	0.0158	0.1555	0.2368	0.0301	0.0409		50

Discussion

For the first time, the morphology of the South African *Octopus vulgaris* Type III has been assessed in detail and compared to that of its closest relatives. Importantly, significant morphological differences were observed between this taxon and *O. vulgaris* ss from the Mediterranean and Northeast Atlantic. Some previous phylogenetic studies placed *O. vulgaris* ss and *O. vulgaris* Type III into a single clade (Oosthuizen et al. 2004; Warnke et al. 2004), while others separate the two lineages (Amor et al. 2019; Chapter 3, this dissertation). Thus, the comparison between *O. vulgaris* ss and *O. vulgaris* Type III is of particular interest. Indeed, hypothesis-based classification of individuals using their morphology placed 100% of South African males into their correct group, one of only two taxa with 100% classification success among the six accepted species within the *O. vulgaris* species complex. Superficially, *O. vulgaris* Type III displays very similar morphology to other taxa within the species complex and thus it is no surprise that it was previously mistaken as part of a cosmopolitan species. Octopus individuals in the *O. vulgaris* species complex have been shown to display great variability in morphological traits, often overlapping between different species, but also diverging between different populations of the same species as is the case with *O. vulgaris* ss. Despite this, statistical morphological differences have been found to corroborate the genetic and geographic separation of species within this group (Amor et al. 2017). The results of this study show that the morphology of *O. vulgaris* Type III reflects its phylogenetic distinction from *O. vulgaris* ss, as shown by genetic evidence (Amor et al. 2019; Chapter 3 this dissertation).

Detailed morphological assessments and comparisons of octopus taxa have shown diagnosable differences between species. *Octopus insularis* was a cryptic species to *O. vulgaris*, but is now known to be part of a sister species complex, having diverged from the *O. vulgaris* group roughly 30 mya (Lima et al. 2020) as compared to the 2.5 mya divergence of the *O. vulgaris* species complex (Amor et al. 2019). *Octopus insularis* has relatively shorter arms with deeper webs than species of the *O. vulgaris* species complex and has a distinct radula (Leite et al. 2008). In the Western Atlantic, *O. insularis* and *O. americanus* occur in sympatry, but can be easily distinguished in the field by body pattern components, such as the colouring on the ventral surface of

the arms, as well as different deimatic displays, and habitat preferences (O'Brien et al. 2021). *Octopus americanus* was distinguished from *O. vulgaris* ss based on the number of gill lamellae per demibranch, position of enlarged suckers in mature males and diameter of enlarged suckers (Avendaño et al. 2020). *Octopus sinensis* was distinguished from *O. vulgaris* ss based on shorter arms with fewer suckers, but more suckers on the hectocotylyzed third right arm (Gleadall 2016). Difference in size and position of enlarged suckers of males was also noted. The Australian octopuses, *O. tetricus* and *O. djinda* are distinguished based on the number of suckers on the hectocotylyzed third right arm, with the latter displaying more (Amor et al. 2014; Amor and Hart 2021).

Multivariate morphological analyses of both sexes were successful in distinguishing *O. vulgaris* Type III from all other species within the *O. vulgaris* species complex. Discriminatory power of male traits was more powerful than females, as previously shown for the *O. vulgaris* species complex (Amor et al. 2017). This greater discriminatory power of males can be attributed to the sex-specific reproductive traits available for morphological comparison. The modified third right arm of male octopuses, referred to as the hectocotylyzed arm, is generally shorter than the other arms and contains a spermatophore groove along its length, leading into the ligula at the tip, which is used to pass spermatophores to the female in sexual reproduction (Norman et al. 2013). The sucker count on this hectocotylyzed arm (HASC in some literature, SCR3 in this study) is a powerful discriminatory trait for octopods (Toll 1988; Amor et al. 2014), as is the size and shape of the ligula. Males also have enlarged suckers on the second and third arms on both sides which varies in size and location between some closely related species. Males of *O. vulgaris* Type III have 147–162 suckers on the hectocotylyzed arm, which is significantly more than *O. insularis* (117–142) and significantly less than *O. djinda* (177–218), both distinguishing traits of these two species (Leite et al. 2008; Amor and Hart 2021). *Octopus vulgaris* ss has the largest SCR3 range (122–183) which was not significantly different to *O. vulgaris* Type III. The presence of enlarged suckers in males is associated with gonadic maturity and sexual recognition during mating (Voight 1991). Male *O. vulgaris* Type III had only one or two enlarged suckers located at the 13–15th proximal suckers. This is similar to *O. sinensis* (Gleadall 2016) but

different to *O. americanus* (Avendaño et al. 2020) with two enlarged suckers (7–8th) and *O. vulgaris* ss (Norman et al. 2013) with two or three enlarged suckers (15th–18th). A smaller ligula discriminated *O. vulgaris* Type III from *O. americanus* and although the shape of the ligula can also be a discriminatory factor among species, investigation of this was beyond the scope of this project.

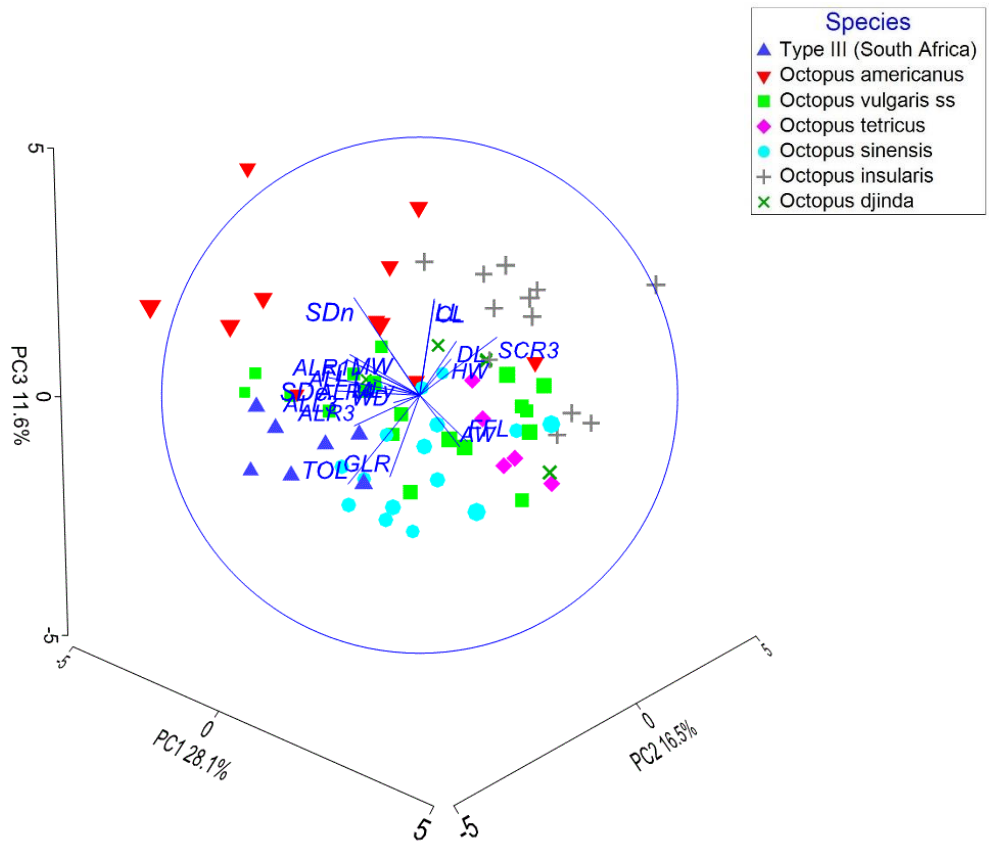
Significant PERMANOVA results can be attributed to a difference in dispersion and/or location in a multivariate space (Anderson et al. 2008). *Octopus vulgaris* Type III had a significantly lower dispersion than all other species groups, most likely due to sampling range. While other species were sampled from multiple locations, some spanning large geographic ranges (>3000 km), sampling of *O. vulgaris* Type III was limited to False Bay, as it contains the only consistently operational fishery along the coast of South Africa (DEFF 2020; John 2022). Alternatively, this dispersion could represent lower levels of intraspecific variation in the *O. vulgaris* Type III lineage or local adaptation to specific environments, as is the case with *O. vulgaris* ss (Amor et al. 2017). Additional sampling throughout the distribution range of this species can provide further insight to the morphological variability of *O. vulgaris* Type III in the different biotopes/ecosystems they inhabit. Meanwhile, the PCA showed that *O. vulgaris* Type III occupies a distinct location on the multivariate space, showing that the significant PERMANOVA result is likely a combination of variation in dispersion and a difference in traits analysed.

It is important to note limitations of this investigation. Only quantitative morphological measurements and meristic counts were assessed, following the methods of Amor et al. (2017). Measurements and counts of eggs were not possible as none of the females sampled were fully mature. Slight distortion of the arms of some specimens occurred during initial freezing after collection from the fishery, although this did not affect evaluation of these traits. Some characters, such as the chromatophore pattern and shape of the funnel organ, could not be clearly observed due to preservation of specimens (as noted by Díaz-Santana-Iturrios et al. 2019). These traits, as well as other descriptive characters which are important for the taxonomic description of cephalopods (Roper and Voss 1983), were beyond the scope of the present study and thus require further investigation. Measurements and counts of gamete will

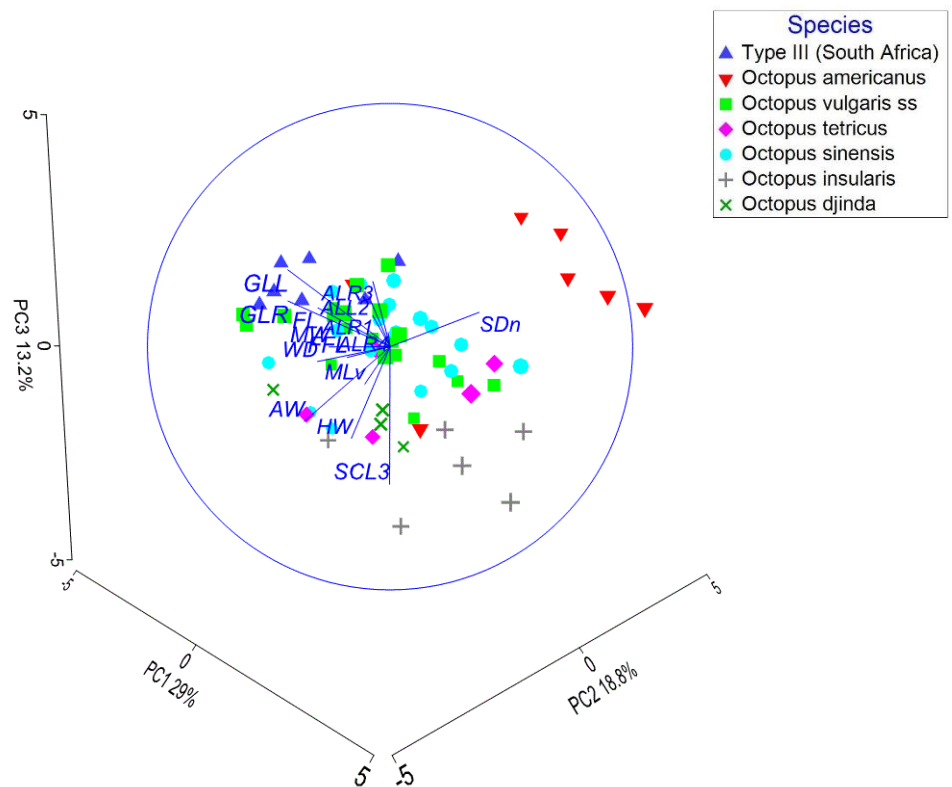
require the analysis of additional, mature specimens. Chromatophore pattern and funnel organ shape can also be assessed on these specimens prior to preservation.

Conclusion

Much current conceptualisation of species still rests on the morphological criterion for speciation. The aim of this study was to assess the morphology of *O. vulgaris* Type III and compare it to the rest of the *O. vulgaris* species complex using multivariate statistics. *Octopus vulgaris* Type III differed morphologically from all other recognised species within the *O. vulgaris* species complex. Importantly, it differed from *O. vulgaris* ss both statistically and on the number and location of enlarged suckers in males. The hypothesis that *O. vulgaris* Type III is a morphologically distinct species is supported and thus a taxonomic revision and new name for this taxon is recommended.



Appendix 4.1. Three-dimensional principal component biplot of male octopus individuals grouped by species. Analysis is based upon 20 morphological traits as shown in vector overlays (redundant variables removed).



Appendix 4.2. Three-dimensional principal component biplot of female octopus individuals grouped by species. Analysis is based upon 15 morphological traits as shown in vector overlays (redundant variables removed).

Appendix 4.3. Collinearity analysis based on Spearman's rank correlations for all morphological traits. High correlations ($R^2 \geq 80\%$) highlighted in **Bold**. Lower left diagonal represents results for males. Upper right diagonal represents results for females.

	MLV	MW	HW	FL	FFL	WD	ALL1	ALL2	ALL3	ALL4	ALR1	ALR2	ALR3	ALR4	AW	SDn	SDe	SCL3	SCR3	GLL	GLR	LL	CL	TOL	DL	
MLV																										
MW	5.81																									
HW	39.66	48.82																								
FL	17.15	13.58	36.05																							
FFL	2.35	-8.48	11.57	-36.95																						
WD	32.24	39.08	37.87	26.05	-6.69																					
ALL1	36.84	37.14	33.27	46.46	-21.41	48.74																				
ALL2	33.10	24.03	16.10	38.03	-16.62	42.60	69.08																			
ALL3	23.68	26.03	6.63	43.14	-24.40	37.16	62.81	79.61																		
ALL4	35.04	30.98	40.19	36.38	-13.51	55.02	73.40	77.72	71.40																	
ALR1	31.95	30.58	16.07	47.72	-27.36	46.22	63.70	74.33	74.67	68.81																
ALR2	31.55	31.24	25.77	42.43	-17.32	45.69	76.78	76.09	71.30	84.19	65.54															
ALR3	30.54	16.83	25.96	37.32	-14.03	51.21	67.01	67.01	68.11	75.67	59.39	79.58														
ALR4	26.98	28.56	43.99	33.74	-18.69	57.92	64.57	66.17	70.94	85.64	62.19	76.00	77.29													
AW	19.29	7.47	60.56	-8.57	9.40	35.15	23.17	20.75	14.24	35.73	8.22	33.73	39.99	45.42												
SDn	11.29	4.64	-39.50	30.23	-12.46	-10.19	6.37	11.02	8.86	5.33	14.99	5.98	-3.09	-5.00	-72.08											
SDe	-5.00	19.46	-35.93	28.86	-11.05	-7.03	12.43	17.86	30.45	11.83	21.18	22.44	16.99	8.52	-51.84	44.12										
SCL3	5.33	-7.89	34.26	-15.71	20.86	-2.08	-16.86	-33.15	-53.54	-20.16	-18.29	-30.60	-33.16	-25.73	11.32	-6.38	-45.26									
SCR3	-2.34	-0.30	36.06	-17.75	17.23	-7.16	-24.73	-31.17	-44.53	-19.05	-18.40	-38.22	-50.51	-21.26	12.07	-13.34	-38.56	79.40								
GLL	41.93	14.87	5.09	36.50	19.25	20.74	20.73	13.06	15.78	11.25	9.88	19.89	29.74	8.16	20.38	-15.05	7.64	3.06	-0.24							
GLR	45.30	17.63	-8.73	31.63	13.18	13.61	19.28	16.80	20.81	12.95	11.90	21.21	21.20	2.83	8.42	-4.11	16.83	-0.98	-2.88	83.72						
LL	-2.07	17.01	4.67	2.31	-7.48	-14.49	-5.22	-10.87	-15.93	-13.94	-8.90	-10.67	-40.45	-16.12	-16.86	0.52	-5.40	5.35	27.49	-20.89	-20.06					
CL	-0.54	17.88	42.63	10.72	-8.17	1.53	18.99	13.26	8.61	22.69	19.06	11.46	0.82	10.56	4.56	1.73	-12.41	21.96	30.38	-19.56	-25.74	21.80				
TOL	14.10	3.81	-28.83	22.04	-0.95	5.52	14.46	22.97	31.98	12.92	20.21	25.56	31.08	5.86	-7.07	7.64	41.37	-42.72	-40.77	48.26	51.00	-18.85	-19.32			
DL	7.98	1.04	35.46	-3.55	6.72	17.99	12.88	5.05	-6.34	1.76	11.83	-9.94	-2.13	10.22	10.83	-5.17	-23.39	42.43	37.92	-7.23	-7.99	-12.47	20.66	-47.83		

Chapter 5: Synthesis and conclusion

Dissertation overview

Octopus vulgaris is the most scientifically researched and economically important octopus in the world (Norman et al. 2013). Previously described as a cosmopolitan species (Mangold 1983), it has now been shown to consist of a complex of morphologically similar but genetically distinct and geographically disjunct species (Norman et al. 2013; Amor et al. 2017). Of the five proposed independent species 'Types', all but the Southern African lineage, *Octopus vulgaris* Type III have been fully redescribed. The *Unified Species Concept* defined by De Queiroz (2007) defines a species as a "separately evolving metapopulation lineage" and considers additional properties as supporting evidence for the hypothesis of speciation. Subscribing to this species concept, in this dissertation, both molecular and morphological properties of *O. vulgaris* Type III were investigated which supported the hypothesis that this taxon is a unique species, in need of a new name.

The main enquiry into the delimitation of *Octopus vulgaris* Type III is its relationship to the European lineage, *O. vulgaris* ss, as previous studies have shown conflicting results (Warnke et al. 2004; Amor et al. 2019). Thus, Chapter 2 was a prerequisite to the phylogenomic investigation as a complete mitogenome from *O. vulgaris* ss was needed with which to compare that of *O. vulgaris* Type III. Chapter 2 thus reconstructed the full mitogenome of *O. vulgaris* ss from its confirmed range in the Northeast Atlantic and provided evidence that the existing record of this species on the NCBI database was sequenced from an *O. sinensis* specimen. Thus, it presents the first mitochondrial genome of the most commercially important octopus which can be used to better understand the evolution and phylogenetic relationships of this species complex.

Chapter 3 returns to the focal taxon of this dissertation, *O. vulgaris* Type III. Following the same methods as Chapter 2, the mitogenome of two specimens of the Type III lineage were sequenced, annotated, and described. Both bayesian inference and maximum likelihood methods placed this taxon as sister to *O. vulgaris* ss with high

statistical support, indicating its distinctiveness. Intraspecific genetic distance between the two *O. vulgaris* Type III mitogenomes was an order of magnitude less than the interspecific distance to *O. vulgaris* ss or any other species, which is further evidence of species separation (Hebert et al. 2004b).

As the definition states, cryptic species are morphologically very similar or indistinguishable (Mayr 1963). However, on close inspection, diagnostic features to delineate closely related species regularly become apparent. A previous study showed multivariate comparison of morphological traits to corroborate molecularly defined clades within the *O. vulgaris* species complex (Amor et al. 2017). Chapter 4 conducts the first detailed morphological assessment of *O. vulgaris* Type III.

Multivariate statistics successfully distinguished *O. vulgaris* Type III from all other taxa, with the analysis of male traits proving more powerful than that of females. *Octopus vulgaris* Type III is distinguished from *O. vulgaris* ss based on the number and location of enlarged suckers in males, a sexual trait which has been used to delineate species in the past (Gleadall 2016).

Implications

The accurate identification and description of cryptic species not only helps us better understand the full diversity of life on Earth, but also has significant implications for the conservation and effective management of species (Norman and Finn 2013). The importance of octopus as a fisheries resource, already a US\$1.07 billion global industry (FAO 2012), is predicted to grow in the future as an alternative to diminishing fin-fish stocks (Sauer et al. 2021). Greater taxonomic resolution and understanding of local populations is required for effective management of octopuses as fishery resources. Currently, multiple species of octopus from around the world are harvested and sold under the name *O. vulgaris*, including the South African taxon. With the planned expansion of the South African octopus fishery from an experimental phase, operating to a large extent out of False Bay alone, to a commercial fishery at up to 16 locations along the coast (DEFF 2020; John 2022), it is important to recognise and manage this taxon as a local species distinct from *O. vulgaris* ss.

Limitations

Although full mitogenomes, as utilised in this dissertation, are more powerful for investigating the phylogeny of closely related species (DeFilippis and Moore 2000; Galtier et al. 2009; Morin et al. 2010), the high cost of sequencing means only few full mitogenome resources are available for comparison. This dissertation reconstructed three of only five mitogenomes available within the *Octopus vulgaris* species complex, representing a total of three species. Additional sequences from these and other species will improve phylogenomic inferences made. However, cheaper options may exist. Amor et al. (2019) found ddRADseq of the nuclear genome to provide greater taxonomic resolution within the *O. vulgaris* species complex and thus may be a more useful tool considering the high cost of sequencing.

Variation in both morphology (Amor et al. 2017) and genetics (Murphy et al. 2002) have been shown for regional populations of *Octopus vulgaris* ss across the distribution of this species. The current hypothesized distribution for *O. vulgaris* Type III ranges from Tristan Da Cunha in the South Atlantic Ocean to Amsterdam Island in the South Indian Ocean, including the entire coastline of Southern Africa from Luderitz in Namibia to Durban, South Africa, and Madagascar (Norman et al. 2013). Although species level distinguishing features were observed, all *O. vulgaris* Type III specimens used in this dissertation, apart from the single genetic sample from Amsterdam Island, were collected in False Bay, South Africa, through the experimental fishery, as this is the only consistently operational fishery in the country.

Future research

South Africa has three major biogeographic marine provinces, each with a distinct environment and composition of species. These being the warm subtropical East coast, the warm temperate South coast and cool temperate West coast (Branch et al. 2016). Spatial structuring of breeding stocks of the commercially important sardine, *Sardinops sagax*, has been shown to occur between the West and East coasts of South Africa (Sakamoto et al. 2020; de Moor et al. 2022). As the South African octopus fishery expands along the country's coastline, it is important to determine regional variability in the population and adaptation to local environments. This is

particularly interesting considering Teske et al. (2007) found what appears to be a second lineage on the east coast near Durban. Population genetic studies, as well as morphological assessments, including specimens throughout the species distribution will help understand spatial variation in populations and better inform management of the species.

Once new species have been identified and described, their distinctiveness is often enhanced through examination of behavioural and ecological characteristics (Mayr 1963). Little research has been conducted on the biology and ecology of *O. vulgaris* Type III (Smale and Buchan 1981; Smith and Griffiths 2002; Oosthuizen and Smale 2003; Smith 2003; Fee et al. 2023) and much of the broader knowledge of this taxon is drawn from studies conducted on what are now known to be other species elsewhere in the world. Local studies on their behavioural ecology such as activity and migration patterns, habitat use, and reproductive cycles will improve foundational knowledge on this local species.

Conclusion

The naming of a species is a hypothesis about its evolutionary relationships and distinguishability. As further information comes to light, hypotheses may be corroborated or refuted. The *Unified Species Concept* (De Queiros 2007) defines a species as a “separately evolving metapopulation lineage” and considers distinguishing properties as lines of evidence to support the species hypothesis. *Octopus vulgaris* Type III was hypothesized to be a separate species based on geographic isolation and lack of plausible gene flow (Norman et al. 2013). This dissertation, provides further genetic and morphological evidence for its separation and concludes that this taxon is a unique species which requires a new name and taxonomic description.

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