Phylogenetic and phylogeographic assessment of the temperate octocoral *Eunicella verrucosa*

Volume 1 of 1

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……..

Thesis summary

The three-dimensional structure of gorgonian octocoral, like *Eunicella,* can provide vital habitat for other marine organisms and are thus considered ecosystem engineers. As they are ecosystem engineers, the conservation of *Eunicella* can deliver benefit to other marine organisms and thus their conservation can have a significant impact. Phylogenetic assessment of a species is pivotal in order to inform management efforts and conservation strategies accurately. Unfortunately, phylogenetic assessment of octocoral has been massively hindered by a lack of variation in their mitochondrial genomes. This is highly evident in the *Eunicella* genus, as previous studies have shown no variation in the mitochondrial protein-coding gene *cox1.* In Chapter 2, novel primers were successfully developed and exploited to explore the phylogenetic relationships between three predominant Atlantic-Mediterranean members of *Eunicella* (*E. verrucosa, E. singularis* and *E. cavolini)*. This is one of the first studies to identify variation in the mitochondrial genomes of *Eunicella.* In Chapter 3, complete mitochondrial genomes of 19 *E. verrucosa* individuals were sequenced. Only two *E. verrucosa* genotypes were observed. 17 *E. verrucosa* individuals showed a widespread genotype and two *E. verrucosa* individuals, from Lion Rock, Isle of Scilly, southwest England, showed a unique genotype. Even compared to the known low levels of variation in the mitogenomes of octocoral, extremely low levels of variation were observed between the mitogenomes of *E. verrucosa* and *E. cavolini,* suggesting a recent divergence of the species. Holaxonia-phylogenies were produced by exploiting partial contigs of *mtMutS* and complete mitochondrial protein-coding sequences. These phylogenies support the inclusion of *Eunicella* in the family

Gorgonidae, in line with previous research. The confirmation of *Eunicella spp.* position within the octocoral phylogeny will allow for properly informed conservation efforts. The conservation of *Eunicella spp.* is especially important, as they are ecosystem engineers it is highly probable the conservation of *Eunicella spp.* will have secondary impact on other marine organisms that rely on *Eunicella spp.* for habitat or resources.

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Chapter 1 - General introduction

1.1. The Cnidarian phylum

Phylum Cnidaria has been estimated to include more than 11,000 extant species that are commonly referred to as sea anemones, corals, sea fans, hydroids and jellyfish (Daly et al. 2007). Despite the seemingly simple body plans found within the cnidarians, they are a highly diverse group of animals, in both form and life history strategy, and can be found in all marine environments, where they build reefs, occupy the deep ocean and parasitize other animals (Collins 2009). Organisms within phylum Cnidaria play a vital role within the marine ecosystem. Cnidaria often feed on eggs and larvae of benthic, planktonic and nektonic organisms, where their feeding habits may act to implement some regulation of biodiversity (Boero et al. 2005). Due to their basal position in the metazoan kingdom they are also vital to our understanding of the evolution of the diversity in Metazoa, as well as the development of body plans in all metazoans.

All cnidarians produce the complex extracellular secretory product cnidae, a defining apomorphy (derived characteristic) for the phylum. The widely known nematocysts are one type of cnidae and are most commonly associated with the "stinging cells" of jellyfish. However, nematocysts are the microscopic capsule produced by the cell and not the cell itself (Fautin 2009). Cnidae are produced by all cnidarians and are used to protect themselves and catch prey (Fautin 2009). Nematocysts are one of three categories of cnidae, the other two being ptychocysts and spirocysts, which only occur in the anthozoan subclass Hexacorallia.

The cnidarian phylum along with 3 other phyla (Placozoa, Porifera and Ctenophora), form a group of organisms called the non-bilaterian basal Metazoa. The cnidarian phylum consists of two monophyletic clades Anthozoa and Medusozoa, Figure 1a. These clades can be defined by the mobility of the adult life phase (Kayal et al. 2013). The class Anthozoa, containing corals and sea anemones, is characterized by an adult life phase that shows only the sessile polyp form, Figure 1b. The subphylum Medusozoa, contains the remaining four classes of Cnidaria (Cubozoa, Hydrozoa, Scyphozoa, Staurozoa), housing jellyfish and hydroids. Medusozoa can have a free-living medusa and/or the polyp adult life phase; the medusa stage is believed to be an apomorphy for Medusozoa, as molecular work suggests the sessile polyp adult form is likely the ancestral state (Collins et al. 2006; Daly et al. 2007).

Within phylum Cnidaria taxonomic ranks have different implications due to a mismatch in the hierarchical taxonomic rank. For example, the subphylum Medusozoa is of comparable taxonomic organisation to the class Anthozoa, due to the high density of species found within Anthozoa. The distinction between Anthozoa and Medusozoa is well supported by anatomy and life history evidence (Salvini-Plawen 1978; Bridge et al. 1995; Daly et al. 2007) as well as phylogenetic evidence (Odorico and Miller 1997; Berntson, France, and Mullineaux 1999; Kim, Kim, and Cunningham 1999; Won, Rho, and Song 2001; Collins 2002).

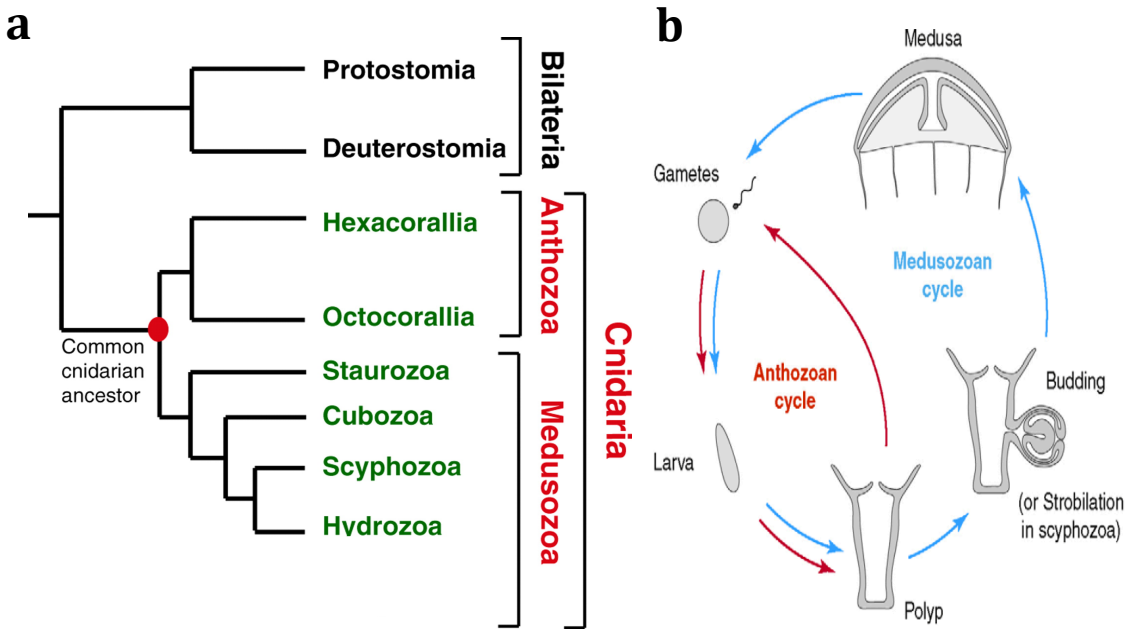

Figure 1 (a) summary of the systematic relationships within the Cnidarian phylum, adapted from Technau and Steele (2011). (b) Adapted from Foret et al. (2010), depicts the general life cycled of Cniadrians. The red arrows show the life cycle of anthozoans where the adult form is a sessile polyp. The blue arrows shows the common Medusozoan life cycle where both polyp and medusa form is seen in the life cycle with the adult life phase often being a free-floating medusa.

Class Anthozoa is comprised of two monophyletic clades (subclasses), Octocorallia and Hexacorallia. As mentioned, all members of Anthozoa are exclusively polypoid and may be colonial, clonal or solitary (Daly et al. 2007). Hexacorallia is well known for its reef-building stony corals seen in shallow-water tropical reefs, but also contains sea anemones and the deep-water black coral. They have polyps with tentacle arrangements in multiples of six. The less well-known clade Octocorallia consists of species commonly referred to as blue coral, sea fan and sea pens. They are distinguished from other anthozoans by the presence of polyps bearing eight mesenteries: non-calcareous partitions dividing the gastrovascular cavity of the polyp and joining the pharynx to the body wall (Bayer, Grasshoff, and Verseveldt 1983; McFadden, Sánchez, and France 2010).

1.2. Octocorallia: life history, ecosystem services and threats

1.2.1. Life history

Octocorallia is currently represented by over 3,000 species that can be found in all marine environments (Cairns 2007; Yesson et al. 2012). Despite reaching maximum diversity in the shallow tropical waters of the western Indo-Pacific, where they can occupy 20-50% of the reef substrate, an estimated 75% of octocorals are found below 50 meters depth (Tursch and Tursch 1982; Fabricius 1997; Yesson et al. 2012). However the diversity of octocorals might not be fully recognised as deep-sea species are still being described (Soler-Hurtado and Lopez-Gonzalez 2012); the situation has been further complicated by widespread taxonomic revision, splitting and grouping taxa due to varying degrees of conflicting genetic and morphological data (Saucier, Sajjadi, and France 2017).

With the exception of one species *(Taiaro tauhou)*, all known octocorals are colonial (Bayer and Muzik 1976; McFadden, Sánchez, and France 2010). Hence, the polyps of a single individual are connected via gasto-vascual canals such that polyps have protoplasmic connections (Bayer 1973). Octocorals can reproduce both sexually and asexually (Coma et al. 1995). With the majority of octocorals being internal or external gonochoric brooders (Weinberg and Weinberg 1979; Brazeau and Lasker 1989; Lasker et al. 1996). However, broadcast spawning and hermaphroditism have been reported (Brazeau and Lasker 1989; Lasker et al. 1996).

Like many cnidarians, octocorals are benthic suspension feeders. Octocorals are passive filter feeders due to the relatively high surface area of their tentacles and the fact that they possess relatively basic nematocysts (Gili and Coma 1998). Octocorals feed on detritus, phytoplankton and zooplankton prey (Katharina E. Fabricius and Dommisse 2000; Ribes, Coma, and Rossi 2003).

1.2.2. Environmental benefits

Octocorals are an extremely important component of the benthic biomass and play an import ecological role in the marine realm as 'ecosystem engineers'. Ecosystem engineers are defined as organisms that alter the physical environment (Wright and Jones 2006). The three-dimensional structure of octocorals causes alterations to the physical environment in which they are located, increasing habitat complexity and heterogeneity, providing microhabitats and substrate for other organisms, as well as altering the flow of water and sediment (Buhl-Mortensen et al. 2010). Gorgonian octocorals not only settle in relatively fast flowing water, but orientate themselves perpendicular to the flow of water to maximize the volume of water passing over their polyps and thus they can increase sediment flow (Wainwright and Dillon 1969). Benefits of this increased flow of water and sediment can be passed on to other filter-feeding organisms associated with the colony (Buhl-Mortensen et al. 2010).

They can have a range of facultative and obligatory associated biota including ophiuroids, barnacles, shrimp, anemones, hydroids and molluscs (e.g. Cupido et al. 2012; Herrera, Shank, and Sánchez 2012), with shallow water species showing more obligatory associated biota than deep-sea gorgonians (Buhl-

Mortensen et al. 2010). The increase in habitat complexity brought about by the three-dimensional structure of resident octocorals can have a strong influence on biodiversity (Krieger and Wing 2002; Linares 2007; Ponti et al. 2014), providing essential ecosystem services to the human population**.** Stone (2006) reported that 85% of economically important fish were associated with cold-water corals, mostly gorgonians, in the Aleutian Islands of Alaska. Moreover, Baillon et al. (2012) in a summary of multi-year surveys reported a strong association of commercially important fish larvae (*Sebastes spp*) with octocoral species. This suggested that these octocorals act as nursery grounds for fish larvae and are an essential habitat, requiring specific management in order to maintain the viability of these commercially important fish stocks. Although hard to calculate in monetary terms, the evidence presented here suggests octocorals provide a clear ecosystem service; as ecosystem engineers they provide a disproportionate benefit to the human population via facilitating commercial fisheries as well as the ecosystem as a whole.

1.2.3. Economic benefits

Octocorals have evolved an array of secondary metabolites used in ecological functions such as anti-predator defence, interspecific space competition and anti-fouling (Sammarco and Coll 1992). For example, a lipid extracted from the Caribbean gorgonian coral *Erythropodium caribaeorum* has been shown to reduce predation by reef fish in both laboratory and field experiments (Pawlik, Burch, and Fenical 1987; Fenical and Pawlik 1991). The gorgonian octocoral, *Leptogorgia virgulata*, produces secondary metabolites with strong anti-fouling properties, which can inhibit settlement of the barnacle *Amphibalanus amphitrite*. There are also potential benefits of allelochemicals, metabolites

released by an organism that has a detrimental effect on other organisms, which could be used in producing a non-polluting antifoulant, as an alternative to the globally banned biocide TBT (Gerhart, Rittschof, and Mayo 1988). Eight out of twenty-nine Indian Ocean octocoral species showed high rates of settlement inhibition (Raveendran, Limna Mol, and Parameswaran 2011), thus showing potential as natural product-derived antifoulants (NPAs).

Prospecting the potential of octocoral secondary metabolites is of high interest to the pharmaceutical industry. For example, the compounds, 9, 11-secosterols, isolated from *Eunicella cavolini* have been shown to have anti-proliferation activity to adenocarcinoma cells, strongly inhibiting the growth of human prostate and breast adenocarcinoma cells (Ioannou et al. 2009). *Pseudopterosin elisabethae* octocorals are harvested in The Bahamas for use in the cosmetics industry due to the anti-inflammatory properties of the pseodoterosin class of compounds they produce (Goffredo and Lasker 2008). With the increased interest in marine bioprospecting, the great economic potential of octocorals is obvious. Erwin, López-Legentil and Schuhmann (2010) predicts a $563 billion – $5.69 trillion economic value to novel anti-cancer drugs from marine organisms, quantifying the potential and importance of marine bioprospecting as an ecosystem service. The majority of novel marine bioprospecting compounds have come from soft-bodied invertebrates, such a Porifera and Cnidaria, including Octocorallia, making the research and conservation of Octocorallia of keen interest to both researchers in academia and industry.

1.2.4. Threats

Despite their potential ecological and economic benefits, octocoral populations are threatened globally by anthropogenic impacts, either from direct harvesting and mechanical damage or indirectly through climate change. Due to the sessile nature of octocorals, they are particularly vulnerable to bottom trawling fishing gear (Watling and Auster 2006). The impact of bottom trawling fishing gear is prevalent in both shallow and deep offshore-waters, such that local inshore conservation efforts have focused on banning trawling in certain areas due to the damage fishing gear has on particular octocoral species (Hall-Spencer et al. 2007; Atrill et al. 2011). Despite there being less research into the impact of fishing on octocorals compared to their sister taxa Hexacorallia, where evidence suggests the impact of bottom trawling fishing gear is long-lasting (Althaus et al. 2009), there is mounting evidence to show gorgonians are a prominent component of by-catch and potentially they are as vulnerable as hexacorals to mechanical damage by bottom trawling (Watling and Norse 1998; Stone 2006; Edinger, Wareham, and Haedrich 2007; Bo et al. 2014). Gorgonians are not only directly impacted by fishing gear but are also frequently entangled in lost fishing gear (Sheehan et al. 2017). Bo et al. (2014**)** showed the impact of lost fishing debris in up to 62% of video frames, from ROV surveys in the Mediterranean.

Coral bleaching, the expulsion of endosymbiotic *Symbiodinium spp* found within coral polyps, is well documented in Hexacorallia but has also been recorded in Octocorollia (Weil, and Yoshioka 2010). This highlights the potential sensitivity of octocorals to the effects of climate change and, specifically, increased sea surface temperatures. Moreover, disease outbreaks closely linked with

temperature anomalies have been documented to caused mass mortality of multiple Mediterranean octocoral species including *Paramuricea clavata, Eunicella singularis* and *Eunicella cavolini* (Cerrano et al. 2000; Martin, Bonnefont, and Chancerelle 2002; Cupido et al. 2012). These mass mortality events occurred during 1999 and 2003 within the Ligurian Sea, most likely by an opportunistic bacterial or fungal disease. Between 2003-2006 *Vibro spp* bacteria caused another disease outbreak in the shallow water gorgonian *Eunicella verrucosa* in the southwest of England; to date, however, there is a paucity of scientific evidence for the aetiology of the disease outbreak. Nonetheless, Cerrano et al. (2000) observed reduced reproductive output in *Paramuricea calvata* populations after the Ligurian Sea disease outbreak in 2003, while a study into the population recovery of *P. clavata* (Linares et al. 2008) showed that a significant skew in sex ratio occurred, leading to a male-dominated population from an originally approximately equal sex ratio. Such a finding highlights the potential long-term impact that increased incidence of disease outbreaks, due to increasing sea surface temperatures, could have on octocoral populations, which may then have knock-on effects to the biodiversity they support. Anthropogenic impacts on octocorals are made even more alarming by their slow-growth rates and long lifespans, as it appears that population recovery post-disturbance will be slow (Sheehan et al. 2017).

1.3. Mitochondrial DNA

Over the last four decades, mitochondrial DNA (mtDNA) has been the marker of choice for phylogenetic and phylogeographic studies. Despite recent advances in techniques that use nuclear DNA as a marker, such as RADseq, mtDNA is still highly popular marker due to the technical ease of its use in genetic

research (Galtier et al. 2009). Firstly, mtDNA is easy to amplify, is present in multiple copies per cell, and genes are not duplicated (Galtier et al. 2009). Moreover, certain evolutionary and biological properties of mtDNA simplify analysis, such as it being non-recombining, its near-neutrality and clock-like rate of base substitutions (Rubinoff and Holland 2005; Birky 2001; Galtier et al. 2009).

Despite high levels of base substitutions that occur in metazoan mitochondrial genomes, some general patterns are evident such as the structure, average size, and gene content, which is often highly conserved (Boore 1999). A typical mitochondrial genome is roughly 16 kb long but can range from 14–17 kb, variation in length often being due to variation of the single large non-coding control region (Shadel and Clayton 1997). Metazoan mtDNA usually encodes 13 proteins, 2 rRNAs (for mitochondrial ribosomal subunits) and 22 tRNAs (Boore 1999). However, within the basal metazoans there seems to be a concentration of exceptions to these outlined generalisations. For example, Medusozoa are unique among metazoans as they have linearised mitochondrial genomes; this is a molecular synapomorphy for the sub-phylum (Bridge et al. 1992). Throughout Anthozoa, mitochondrial genomes contain a severely reduced complement of tRNA genes, encoding only 1-2 tRNA genes, 20-21 less than compared to other metazoans (Beagley, Okimoto, and Wolstenholme 1998; Brugler and France 2007; Sinniger and Pawlowski 2009; Poliseno et al. 2017).

Potentially the most unique mitochondrial genomes can be seen in the anthozoan sub-class Octocorallia. Octocoral mitochondrial genomes are usually

18-19 kb long and contain a unique open reading frame, *mtMutS*, of roughly 2950 bp (Poliseno et al. 2017; McFadden, Sánchez, and France 2010; Brugler and France 2008). Since *mtMuts* was first observed in 1995 in the mitochondrial genome of *Renilla kolikeri* and *Sarcophyton glacum* (Anthozoa: Octocorallia), it has since been observed in all octocoral species studied; conversely, it has not been observed in the mitochondrial genome of any other organism and is a defining apomorphy for the clade (Pont-Kingdon et al. 1995; Beaton, Roger, and Cavalier-Smith 1998; Bilewitch and Degnan 2011). This unique open reading frame (ORF) encodes for a mismatch repair protein MutS. All lines of evidence suggest this ORF encodes for a functional mismatch repair gene, such that *mtMutS* is actively transcribed and the gene encodes for a protein that has the necessary components for DNA mismatch repair (Bilewitch and Degnan 2011). These two factors coincide with observed reduced rates of sequence variation in octocoral mitochondrial genomes when compared to their sister taxa Hexacorallia, as well as other metazoans (France et al. 1996). This reduced rate of variation can have serious implications for the phylogenetic analysis of octocoral.

The octocoral unique open reading frame, *mtMutS*, was previously referred to as *mtMSH1* based on a 19.7% similarity of its translated sequence to the yeast nuclear Mutational Suppressor Homolog 1 (Pont-Kingdon et al. 1995). However, recent compelling evidence shows that *mtMutS* has no immediate common ancestor with any eukaryotic MutS Homologs 1 (MSH1) families and is likely of non-eukaryotic origin (Bilewitch and Degnan 2011). Phylogenetic analysis shows strong clustering of all octocoral *MutS* sequences with the *MutS7* lineage, forming a clade with epsilonproteobacteria *MutS* and viral *MutS*;

in turn, all share Domains I, III and V of *MutS*, plus a C-terminal unique to the *MutS7* lineage. This evidence is persuasive and suggests this is potentially the first case of horizontal gene transfer into a metazoan mitochondrial genome (Bilewitch and Degnan 2011).

1.4. Octocoral systematics

1.4.1 Monophyly

The monophyly of Octocorallia has long been recognized and is generally well-supported by both morphological and molecular data (McFadden, Sánchez, and France 2010). The sub-class can be distinguished by the ubiquitous symmetry shown by octocoral polyps, which bear eight tentacles and eight internal mesenteries, a diagnostic apomorphy for the sub-class. Other features are often used to identify Octocorallia, for example, skeletons containing calcareous sclerites, polyps having pinnules, and lateral extensions on the tentacles (Daly et al.2007). However, these features are not true diagnostic apomorphies as, despite these features being widespread in Octocorallia, a few taxa lack some of these characteristics (Daly et al. 2007). For example, such exceptions as pinnule-less polyps described by Alderslade and McFadden (2007) or the order Heliporacea, which possess no sclerites but instead lays down a crystalline aragonite skeleton (Bayer 1973).

Early phylogenetic studies of phylum Cnidaria, which used 16S, 18S and 28S-partial rDNA sequence data, all support morphology-based characterisation and define Octocorallia as monophyletic (Berntson, France, and Mullineaux 1999; Bridge et al. 1995; Chen et al. 1995). Moreover, the above studies also support the sister relationship of Octocorallia with Hexacorallia. More recently, Figueroa

and Baco (2014) conducted a phylogenetic study using 83 mitochondrial genomes and were able to demonstrate the monophyly of Octocorallia. However, arguably the most compelling evidence for the monophyly of Octocorallia is the presence of a unique ORF, *mtMutS*, which, as outlined above, has been found in all octocoral mitochondrial genomes studied and in no other metazoan mitochondrial genome (Culligan et al. 2000; Bilewitch and Degnan 2011).

1.4.2. Ordinal, sub-ordinal and family level relationships

Despite the fact octocorals have been of interest to naturalists for centuries, Octocorallia is arguably the least well-resolved higher taxonomic group within Cnidaria, with classification into taxonomic groups at the ordinal level or below remaining problematic. There are a few characteristics of Octocorallia taxa that can make them hard to classify. Firstly, there is a general paucity of defining morphological and molecular synapomorphies (Daly et al. 2007; McFadden, Sánchez, and France 2010). Moreover, plasticity in morphological features is observed, but a lack of understanding as to what degree the environment influences the phenotype of an individual makes it hard to delineate species or ectomorphs (Berntson et al. 2001). Finally, there is also a general lack of fossil records for these soft-bodied animals, making mapping the evolution of any diagnostic feature particularly challenging (Berntson et al. 2001; Carlos Prada, Schizas, and Yoshioka 2008).

In general, the morphological features used to distinguish octocorals are colony growth form, features of skeletal axis (if present), the shape and arrangement of sclerites, microscopic calcite crystals embedded in coenenchymal tissue and

polyps, as well as other features specific to certain clades, such as the arrangement of secondary polyps around the rachis only seen in the sea pen families. From a morphological point of view, the lack of synapomorphies is due to the relatively simple body plans of octocorals, and the significance of any given feature is often highly debated due to the plasticity in these features (Berntson et al. 2001).

Throughout the 20th century, Hickson (1930) taxonomic classification system was the most widely used for classifying taxa within the Anthozoa. Largely based on colony growth form, he produced six orders (O. Pennatulacea, O. Helioporacea, O. Gorgonacea, O. Stolonifera, O. Telestacea, O. Alcyonacea)**.** Currently, Bayer (1981) 3-order classification system is the most widely used by taxonomists and retains two of Hickson (1930) orders Helioporacea and Pennatulacea, as they are defined by clear morphological synapomorphies. The remaining four orders (Gorgonacea, Stolonifera, Telestacea, Alcyonacea) were collapsed into a single large order, O. Alcyonacea, due to the recognition that these groups graded into each other morphologically.

Helioporacea contains only two extant families that are unique among octocorals as they lay down a solid skeletal matrix of crystalline aragonite, a convergent feature with taxa of Scleractinia (Hexacorallia) (Bayer and Muzik 1977). Pennatulacea, often referred to as sea pens, contains 14 extant families that have a level of colony architecture that surpasses any complexity seen in Anthozoa. Pennatulacea is characterised by having primary axil polyps (oozooids), which differentiate into a barren stalk (penduncle) and inflates,

thereby anchoring the colony in soft sediment, and a distal rachis where secondary polyps are arranged (Bayer 1961, 1973). Families within Pennatulacea are distinguished via the arrangement of secondary polyps around the rachis (Daly et al. 2007).

Unlike the other two orders, Alcyonacea lacks any defining synapomorphies. This large order consists of 31 families and contains groups often referred to as soft corals and gorgonians. For taxonomic convenience order Alcyonacea is currently divided into two suborders (Calcaxonia, Holaxonia) and four sub-ordinal groups (Scleraxonia, Stolonifera, Alcyoniina, Protoalcyonaria). These sub-ordinal groups are known to represent grades of colony morphology and skeletal composition, but are not recognised as true clades (Daly et al. 2007; McFadden, Sánchez, and France 2010). The major features used to distinguish orders, within Alcyonacea are the overall colony growth form, the presence of a supporting skeletal axis and details of axial composition (Daly et al. 2007; McFadden, Sánchez, and France 2010). The Alcyoniina, Stolonifera and Protoalcyonaria groups include families that lack a skeletal axis or axial-like layer. The final three groups informally referred to as gorgonians produce a skeletal axis or axial like layer composed of calcite and a proteinaceous material called gorgonin.

Within Aclyonacea, the gorgonian groups Calcaxonia and Holaxonia are defined by clear morphological apomorphies and are thus recognised as true suborders (Grasshoff 1999; Daly et al. 2007). As defined by Daly et al. (2007) the axis of both Calcaxonia and Holaxonia consists of gorgonin, however, they differ by the amount of non-sclerite calcite and the composition of their central

core. Calcaxonia possesses a large amount of non-sclerite calcite and a solid cross-chambered core, while Holaxonia have small amounts of non-sclerite calcite and a hollow cross-chambered central core (Daly et al. 2007).

Early molecular studies that included sufficient taxa to allow assessment of the relationships within Octocorallia did not reproduce the six sub-ordinal groups of Alcyonacea (France et al. 1996; Berntson et al. 2001). Subsequently, Sánchez, Lasker, and Taylor (2003) reanalysed data from these previous studies, along with the addition of a secondary structure analysis for 16S rDNA sequences, and produced highly similar results to the original studies. Despite the fact these partial 16S and complete 18S rDNA studies did not corroborate the taxonomic classification of Bayer (1981), they did produce general clades that are still reproduced in modern octocoral molecular phylogenies (McFadden et al. 2006; Figueroa and Baco 2014).

Indeed, the study by McFadden et al. (2006) is to date still renowned as the most extensive octocoral phylogenetic study available and produced well-supported clades similar to those seen in the earlier molecular studies; Figure 2 shows the phylogeny produced by McFadden et al. (2006) based of the partial contigs of *ND2* and *mtMutS,* totaling 1429 bp. They sampled a total of 115 taxa representing 29 of the 45 families. McFadden et al. (2006) produced two major clades, Clade 1 (Holaxonia-Alcyoniina) and Clade 2 (Calcaxonia-Pennatulacea); as well as a third smaller clade, Clade 3 (Anthosmastus-Corallium), whose relationship to the two major clades was unresolved (McFadden et al. 2006). Clade 1 consists of the majority of soft corals (sub-ordinal group Alcyoniina), as well as the gorgonian suborder Holaxonia, along

with the majority of representatives from Scleraxonia and Stolonifera. Clade 2 is comprised of all sea pens (Pennatualcea), which resolved as a monophyletic clade, and it also includes O. Helioporacea and the gorgonian suborder Calcaxonia.

More recently, the study by Figueroa and Baco (2014) showed the increased resolution that complete mitochondrial genomes can provide in phylogenetic studies. Figueroa and Baco (2014) used 34 complete octocoral mitochondrial genomes and produced clades largely congruent to McFadden et al 2006. With the increase in sequence data and resolution, they were able to resolve the position of the deeper nodes, such as the *Anthomastus-Corallium* clade, showing its sister relationship to the Pennatulacea-Calcaxonia clade, which are unresolved in McFadden et al. (2006). Unfortunately, for Figueroa and Baco (2014), only 34 complete octocoral mitogenomes, were available for analysis so, despite the increase in resolution, the lack of sufficient taxa limited the study of relationships within Octocorallia. Poliseno et al. (2017) calculated that only 1% of known octocoral species had publically available mitochondrial genomes. Thus, finding a cheap and scalable method to sequence mitochondrial genomes would vastly improve our knowledge of the relationships within subclass Octocorallia

Figure 3 Maximum likelihood phylogram from McFadden et al. (2006) that utilises partial contigs of the mitochondrial protein coding genes *mtMutS* and *nad2* from 103 Octocorallia. Circled numbers on the phylogram show the clades discussed in text (1) Holaxonia-Alcyoniina, (2) Calcaxonia-Pennatualcea and (3) *Anthomastus-Corallium.*

In summary, there is a general lack of resolution in the molecular markers used to date; this makes it hard to draw any conclusions on the taxonomic and evolutionary relationships between and within the clades of Octocorallia. A lack of resolution in these markers at the deeper nodes and a lack of fossil records has implications for mapping the evolution of morphological characteristics, making it almost impossible to map out the evolution of diagnostic morphological features. Complete mitochondrial genomes have shown some promise in increasing resolution in octocoral phylogenetic studies. Of the studies reviewed above, that by McFadden et al. (2006) is the only one to include sufficient taxa to be able to make any assumptions about family-level relationships within Octocorallia. Unfortunately, even this study is characterised by varying degrees of polyphyly of the morphologically-defined taxonomic groupings; this disparity between morphological and molecular data appears to be widespread throughout Octocorallia (McFadden et al. 2010).

1.4.3. Intrageneric and intraspecific relationships

Galtier et al. (2009) called into question how rigorously all animal mitochondrial DNA follows the general assumptions held about metazoan mitochondrial DNA (i.e. faster rates of nucleotide substitution than nuclear DNA, clock-like evolution and near-neutrality) and its use as an evolutionary and taxonomic marker. Such exceptions to these general assumptions are highly prevalent in octocorals where severely reduced rates of variation within mitochondrial genomes are observed; estimates suggest anthozoan mitochondrial genomes evolve 50-100 times slower than other metazoan mitochondrial genomes (Shearer et al. 2002); studies on scleractinian corals suggest their mitochondrial genomes may evolve five-times slower than their nuclear genomes (Chen et al. 2009). This lack of

base substitution has acted to severely hinder the use of molecular phylogenetic tools for exploring intra- and intergeneric relationships in octocorals. As mentioned previously, the reduced rates of variation observed within octocoral mitochondrial genomes are likely due to the presence of the mismatch repair gene *mtMutS* within the mitogenome of Octocorallia (Bilewitch and Degnan 2011).

The study by France et al. (1996) showed reduced rates of variation in octocorals even compared to their sister taxa (Hexacorallia). Hexacorallia are also known to have low levels of mtDNA sequence variation; pairwise comparison of 16S rDNA intrageneric genetic distances between genera of octocorals and hexacorals, showed values of 2.7–6.3% and 16.1–26.3%, respectively. Reduced rates of sequence variation have been observed in almost all octocoral mitochondrial markers, analysed to date. For example, the mitochondrial cytochrome oxidase I gene (*cox1*) has been proposed as a universal genetic barcode, a means by which to identify known species based on the DNA sequence of *cox1* (Hebert, Ratnasingham, and Jeremy 2003). However, one study of Mediterranean octocorals found identical *cox1* sequence (547 bp) between different *Eunicella* species (*Eunicella singularis* and *Eunicella cavolinii*), showing a lack of variation of this marker at both intergeneric and intrageneric levels (Calderón, Garrabou, and Aurelle 2006).

Paradoxically the mismatch repair gene *mtMutS* has been shown to have elevated levels of variation compared to other mitochondrial protein-coding sequences (France and Hoover 2002; van der Ham, Brugler, and France 2009). Estimates suggest it has roughly twice the variation of other mitochondrial

protein-coding regions, hence it is often the focal marker used in genus-level and species-level phylogenetic studies of octocorals (Mcfadden et al. 2011; McFadden, Sánchez, and France 2010). Currently, partial contigs of *mtMutS* have been applied to a wide range of octocoral taxa, such as Caribbean holaxonians (Wirshing et al. 2005), deep-sea gorgonians (France 2007) and Indo-Pacific alcyoniids (Mcfadden et al. 2009).

The only single-copy nuclear gene that has been applied to multiple taxa in octocoral is *SRP54* (Concepcion et al. 2008). It has been shown to have intraspecific and interspecific variation an order of magnitude above mitochondrial protein-coding genes (Concepcion et al. 2008). Unfortunately, it has proven difficult to amplify in many octocoral species, and has not been widely applied (McFadden, Sánchez, and France 2010).

Genetic barcoding is also problematic in Octocorallia due to this lack of variation (Mcfadden et al. 2011). van der Ham, Brugler and France, (2009) suggested no single mitochondrial gene region has sufficient variation to distinguish species unequivocally and thus suggested the need for species-specific barcodes. Recent work by Poliseno et al. (2017) has shown the power of mitogenomic sequencing in identifying areas of high variation for intrageneric biogeographic studies. Firstly, they used complete mitogenomes to show nucleotide diversity of ~2.6% between *Leptogorgia* taxa, far greater than the 1% nucleotide diversity threshold proposed by McFadden et al. (2011) for *mtMutS* based species delimitation. Moreover, they (Poliseno et al. 2017) were able to identify the intergenic region between *nad5* and *nad4* as having the highest levels of variation between the *Leptogorgia* species studied. The identification of areas of

highest variation allows for the proposal of species-specific genetic barcodes that would, in theory, provide a lower cost mitochondrial marker with the most informative sites possible.

In summary, studies into the intrageneric relationships in Octocorallia have been severely hampered by the lack of variation in their mitochondrial genomes. This lack of variation is likely due to the presence of a functional mismatch repair gene, *mtMutS*, found in their mitogenomes. The increased variation found in *mtMutS* sequence has been used to studied intrageneric relationships within Octocorallia, with varying levels of success. A study by Poliseno et al. (2017) showed the potential for mitogenomes to identify areas of greatest mtDNA variation in their study species and allowed identification of candidate areas to target for genetic barcoding.

1.5. Study species

This study will investigate the phylogenetic relationships within European members of the octocoral genus *Eunicella,* with a focus on *Eunicella verrucosa. E. verrucosa* (pink sea fan) is a Holaxonia octocoral in the Gorgonidae family. It is thought to be gonochoristic and to reproduce sexually by broadcast spawning with external fertilization (Munro 2004). Typically, colonies are approximately 30 cm tall when fully-grown, but have been documented as reaching up to 75cm (Wood 2013). They are found at depths of between 10 –150 m on rocky substrates in areas of moderate to high water currents (Hayward and Ryland 1995). They are native to the northeast Atlantic and parts of the Mediterranean Sea, where they range from northwest Ireland down to western Africa, as well as the western Mediterranean (Sheehan et al. 2017).

Eunicella cavolini and *Eunicella singularis* are both Mediterranean species and at some locations can be found in sympatry with *E. verrucosa.* Previous genetic work, by Calderón, Garrabou and Aurelle (2006) on these three species has shown no variation at all across 576 bp of the *cox1* mitochondrial gene, they were also unable to distinguish between these three *Eunicella* members with the nuclear marker *ITS2*.

1.6. Research aims

This research aimed to use mitochondrial gene data to assess the phylogenetic relationships between three species of *Eunicella*: *E. verrucosa*, *E. cavolini* and *E. singularis.* Finding a mitochondrial marker with sufficient variation to differentiate these three species would be of use to researchers after Calderón, Garrabou and Aurelle (2006) found no sequence variation in the *cox1* gene between these *Eunicella* species. Moreover, by analysing a large number of *E. verrucosa* samples from across the species range, we aimed to elucidate any phylogeographic patterns discernable using mitochondrial DNA sequences.

The aim of Chapter 2 was to describe the use of newly developed primers that target areas of the *Eunicella sp.* mitochondrial genome that are suspected to have the highest levels of variation. The markers were also combined with sequence data available in GenBank to explore their utility in discerning relationships throughout the Holaxonian suborder.

Chapter 3 focussed on elucidating intraspecific variation in *Eunicella verrucosa,* as well as, intrageneric variation between *Eunicella* species. This was achieved

by sequencing complete mitochondrial genomes in a relatively large number of *E. verrucosa* individuals from across the species range. Again, sequences from GenBank were used to assess the utility of complete mitochondrial genomes for determining relationships in suborder Holaxonia.

The study aimed to assess the phylogenetic relationships between *E. verrucosa, E. cavolini* and *E. singularis,* as well as, their relationships to other Holaxonia octocoral. Phylogenetic studies like this are vital to properly informing conservation efforts. As ecosystem engineers the conservation of *Eunicella sp.* is especially important, as their conservation can pass on benefits to other marine species and thus conservation of these gorgonian octocorals can have a disproportionately large benefit (Boogert, Paterson, and Laland 2006). Moreover, understanding the phylogenetic relationships in basal metazoans, like Octocorallia, is required to understand the evolution and development of all Metazoans.

Chapter 2 - Phylogenetic assessment of European members of the octocoral genus *Eunicella*

2.1. Abstract

Phylogenetic research into the relationships within Octocorallia has been hindered by the reduced variation observed within their mitochondrial genomes. In one study the octocoral genus *Eunicella* has shown no variation across the sequence of the gene *cox1*, hindering any inference of relationships between members of the genus. In this study, we explored the phylogenetic relationships between three Atlantic-Mediterranean members of *Eunicella*; *E. verrucosa, E. cavolini* and *E. singularis* by applying novel primers, which target partial contig of the protein-coding gene *mtMutS* and the *nad5-nad4* intergenic region. We utilised the mitochondrial genomes of *Eunicella* to explore the relationships within the genus, as well as, their relationships to other Holaxonia octocoral. The phylogenetic analysis utilising partial *mtMutS* sequences resolved all members of *Eunicella* as monophyletic and confirmed the placement of *Eunicella* within the family *Gorgonidae.* Variation was observed in the *nad5-nad4* intergenic region between *E. verrucosa and E. cavolini*. This is the first study to show variation in mitochondrial sequences between these two individual species. The variation found in *nad5-nad4* intergenic region demonstrates the region's potential candidate for a genus-specific character-based barcode, a crucial tool for molecular ecologists**.** Studies into the phylogenetic relationships of *Eunicella* are pivotal to inform management and conservation strategies for the genus properly; conservation of ecosystem engineers like *Eunicella* can have a disproportionately large impact passing on benefit to other marine organisms.

2.2. Introduction

The octocoral genus *Eunicella* has five members that inhabit the coastal waters of Europe, with *Eunicella verrucosa, Eunicella cavolini* and *Eunicella singularis* being the most abundant by far (Grasshoff 1992). As mentioned in Chapter 1, the three-dimensional structure of gorgonian octocorals, such as *Eunicella*, can provide important habitat for other marine organisms and are thus consider ecosystem engineers (Buhl-Mortensen et al. 2010; Baillon et al. 2012). However, due to their sedentary nature, they are vulnerable to destructive fishing methods, such as bottom trawling fishing, and population recovery is slow due to their slow growth rates (Bo et al. 2014; Althaus et al. 2009; Sheehan et al. 2017). Moreover, these European members of *Eunicella* have been affected by mass mortality events within both the Mediterranean Sea and the Atlantic Ocean, most likely due to thermal anomalies (Garrabou et al. 2009; Cerrano et al. 2000; Martin, Bonnefont, and Chancerelle 2002; Cupido et al. 2012).

Eunicella verrucosa (pink sea fan) is generally the most common of these five coastal octocoral species and can be found within both the Mediterranean Sea and the Atlantic Ocean (Holland, Jenkins and Stevens, 2017). The pink sea fan is listed as vulnerable on the IUCN Red List (World Conservation Monitoring Centre 1996); however, IUCN rules state listings are outdated after 10 year, so this 23-year-old listing is not considered accurate and needs updating (Rondinini et al. 2014). *E. verrucosa* is most abundant within the costal waters of Britain but is still considered nationally rare due to its range being limited to the south west coast of England (Hiscock et al. 2010). It is listed as a priority species under the UK Biodiversity Action plan and a species of principal

importance in England under the NERC Act 2004. Although the pink sea fan is also found within the Mediterranean Sea, the yellow sea fan (*Eunicella cavolini*) and the white sea fan (*Eunicella singularis)* are most abundant within this region.

Throughout Octocorallia, there is a general lack of definitive morphological characteristics by which to reliably distinguish species; *Eunicella* is no exception to this (McFadden et al. 2006). Within *Eunicella* a long un-branching colony architecture and presence of symbionts can be used to distinguish *E. singularis* from *E. verrucosa* and *E. cavolini* (Grasshoff 1992). *Eunicella cavolini* and *Eunicella verrucosa* are near morphologically identical-looking organisms. Grasshoff (1992) defined diagnostic taxonomic characteristics for the *Eunicella* genus, these features can be seen in Table 1, with the major difference being the protuberance of the polyps in *E. verrucosa* and a general lack of these in *E. cavolini* (Grasshoff 1992; Carpine and Grasshoff 1975). However, subtle differences in their colony architecture and sclerites are still apparent.

Table 1 Outlines the diagnostic morphological features of members of the octocoral genus *Eunicella,* as outlined by Grasshoff (1992).

Species	Diagnostic characteristics
Eunicella verrucosa	Pink or white in colour, densely ramified with short branches, protuberant polyps, sclerites are balloon clubs with large spiny edges, no symbionts.
Eunicella cavolini	Yellow to light-red, densely branched, polyps with little or few protuberant, sclerite are clubs widely circular with fine humps in exterior surface, no symbionts.
Eunicella singularis	Long straight branches, polyps a little protuberant, sclerites clubs narrowly circular with exterior face completely flat, photosynthetic symbionts present.

To further complicate this, plasticity in morphological characteristics has been observed in Octocorallia and the *Eunicella* genus (Gori et al. 2011; Carlos Prada, Schizas, and Yoshioka 2008). Observations show an increased variation in colony growth form and changes in colour at depth for *Eunicella singularis*, as well as asymbiotic *E. singularis* individuals being observed at depths (Gori et al. 2011). Despite its importance, there is a lack of published data that elucidates the role of the environment in shaping morphological characteristics across *Eunicella* and Octocorallia as a whole.

From a genetic point of view, the genus *Eunicella* also lacks molecular markers with sufficient resolution to delineate individual species; no variant sites have been found between these three *Eunicella* species within the mitochondrial protein-coding genes COI and mtMutS (Calderón, Garrabou, and Aurelle 2006; Gori et al. 2012). The lack of variation seen within these mitochondrial markers is most likely due to the unique mitochondrial mismatch repair gene, mtMutS. As stated in Chapter 1, this actively transcribed protein-coding gene contains all the mechanisms required for mismatch repair and coincides with octocoral mitochondrial variation rates 50-100 times lower than other metazoans (France et al. 1996; Shearer et al. 2002). The nuclear markers ITS 1 and ITS 2 do not have high enough resolution to delineate the three *Eunicella* species (Calderón, Garrabou, and Aurelle 2006; Costantini et al. 2016). It has been suggested that incomplete concerted evolution could have caused this lack of variation between the three species in the multi-copy nuclear markers (Costantini et al. 2016; Calderón, Garrabou, and Aurelle 2006).

The accurate assessment of species boundaries is pivotal in order to advance the understanding of the proper phylogeographical and/or phylogenetic relationships within a species or group of species, which is required for the successful conservation of an organism (Knowlton 2000; Eytan et al. 2009). Molecular markers with sufficient resolution to delineate species are essential for the phylogenetic assessment of species. This is especially important for a genus such as *Eunicella*, where morphologically similar individuals living in sympatry show variation in morphological characteristics due to environmental factors (Hillis 1987).

The aim of this study was to investigate the phylogeographic relationships within *Eunicella verrucosa*, as well as the wider phylogenetic relationship in the *Eunicella genus,* specifically between *Eunicella verrucosa, Eunicella cavolini* and *Eunicella singularis*. A proper phylogeographic assessment of *E. verrucosa* is essential for the proper conservation of this IUCN red-list species. We aim to investigate these phylogenetic relationships via the use of newly designed molecular markers.

Due to the lack of variation seen in previously used mitochondrial markers, a new set of primer pairs was created to target regions suspected to have the highest levels of variation. The mtMutS gene has been reported to have twice the variation of other mitochondrial protein-coding genes, however, currently available markers only target the first ~750 bp (Mcfadden et al. 2011)**.** Here we use two newly developed primer pairs designed to target the remaining 2,000 bp of this protein-coding gene. Primers were also created to target the intergenic region (IGR) between the mitochondrial protein-coding genes NAD5

and NAD4. This roughly 1,000 bp IGR was selected following on from work by Poliseno et al. (2017) which identified this IGR as having the greatest variation in the mitogenomes of *Leptogorgia spp.,* a genus of octocoral in the same family (Gorgoniidae) as *Eunicella.* (Poliseno et al. 2017)

2.3. Material and Methods

2.3.1. Sample collection

Eunicella verrucosa samples were collected from northwest Ireland to Portugal and the northwest of the Mediterranean Sea (Table 3), representing most of their known range. Samples were collected via SCUBA at depths of between 5 and 30 metres, by removing 3 cm of an individual's terminal branch. Collection has occurred since 2007 to present. To avoid sampling clones, samples were taken from colonies spaced 1 m apart (as explained in Holland, Jenkins and Stevens 2017). Once samples were removed from a colony they were placed in a mesh bag, brought to the surface and rapidly immersed in 95-100% ethanol. Ethanol was changed 24 - 48 hours after initial immersion to remove any excreted secondary metabolites that might affect downstream analysis. Samples of *Eunicella cavolini* and *Eunicella singularis* were also collected from areas across the Mediterranean Sea via the method outlined above.

2.3.2. DNA extraction

DNA extraction was carried out using a modified salting-out protocol that was originally designed for the extraction of DNA from crustacean exoskeleton (Li et al. 2011)**.** The slightly longer modified salting-out extraction protocol was chosen after poor quality DNA was obtained when using a commercial DNA extraction kit. The modified salting-out protocol was carried out using 15 - 20

polyps cut away from the hard central axis of a roughly 1 - 2 cm long section of branch from the sea fan. The polyps were added to 35 µl of 1*M* DTT (dithiothreitol), 42 µl 0.5 *M* EDTA, 10 µl proteinase K and 350 µl 1% SDS cell lysis buffer (100mM Tris-Cl; 50 mM EDTA; 1% SDS) and left to incubate at 65 °C for 4 hours. After the incubation 140 µl 7.5 M ammonium acetate was added and samples incubated at 4°C for 10 minutes before centrifuge at 12,000 g for 10 minutes. Supernatant was then added to a new tube. This stage was then repeated by adding 140 µl 7.5 M ammonium acetate to the new test tube and incubated at 4°C for 10 minutes before centrifuge at 12,000 g for 10 minutes and removing supernatant to another new test tube. 700 µl cold isopropanol was added and samples mixed by gentle inversion before being centrifuge at 8000 g for 5 minutes. Supernatant was discarded and DNA pellet left undisturbed. The DNA pellet was then washed with 400 µl of 70% ethanol, pellet is then left to completely dry before being re-dissolved in 100 µl of nuclease-free water. The purity and concentration of the extracted DNA were quantified using a NanoDrop One (Thermo Scientific, Wilmington, DE, USA).

2.3.3. Primer design and DNA amplification

New primers were designed to target an area of the mitochondrial genome suspected to have the highest levels of variation, detailed below. The first group of three primer pairs were designed to target the increased variation seen in the mitochondrial gene encoding for mtMutS (van der Ham, Brugler, and France 2009; Mcfadden et al. 2011). A primer pair was also designed to capture any variation in the intergenic region between NAD4 and NAD5. Poliseno (et al. 2017) identified the intergenic region between *nad5* and *nad4* to show the highest levels of variation in *Leptogorgia,* a genus of octocoral in the same

family as *Eunicella* (Gorgonidae*).* Moreover, this intergenic region is the largest intergenic region seen in a published *Eunicella cavolini* mitogenome (Poliseno et al. 2017)**.** Primers were designed using published sequences of the complete *Eunicella cavolini* mitochondrial genome (NCBI Accession: KY559408.1).

All PCR reactions were performed in 25 µL reactions with: 0.375 µL of each the forward and reverse primer, 0.25 µL BSA (NEB), 12.5 µL HotStart Taq Master Mix (Qiagen), 9.0 µL RNase free water and 2.5 µL extracted DNA. A 60˚C to 55˚C touch down protocol was used for the primer pairs MutS3 and NAD5-IGR-NAD4 and a 60˚C to 50˚C touch down protocol was used for MutS2 primer pairs. The PCR thermal cycle for the 60˚C to 55˚C touch down were as follows: an initial denaturing stage 94˚C for 5 minutes; followed by 35 cycles of 30 seconds denaturing at 94˚C, 30 seconds annealing at 60˚C (decreasing by 0.5˚C every cycle for 10 cycles then remaining at 55˚C for the remaining 25 cycle), and finally 30 second extension at 72˚C; after these 35 cycles there is a final extension for 5 minutes at 72˚C. The thermal cycle for 60˚C to 50˚C touchdown is identical except the 0.5˚C decrease in annealing temperature will continue for 20 cycles, and subsequently remain at 50 ˚C for the final 15 cycles.

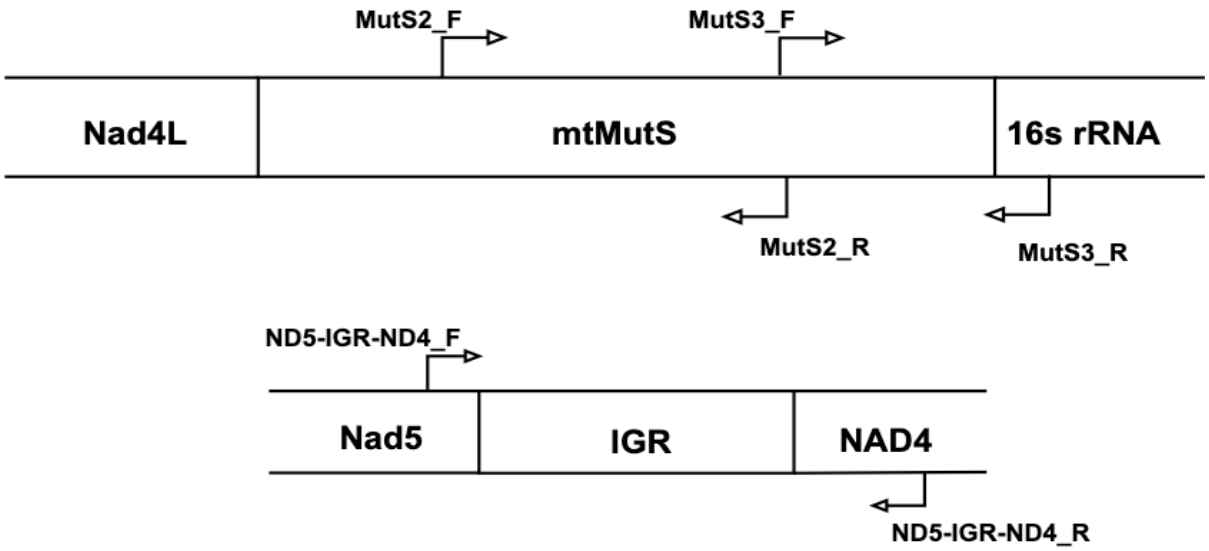

Figure 3 The diagram visualises the segments of the octocoral mitochondrial genome the newly developed primer pairs target.

The MutS1 primer pairs were tested multiple times by gradient PCR to find the optimal annealing temperature, however, none were successful. The MutS1 primer pairs were not investigated further after a lack of variation found in the returned sequences for other primer pairs.

A 5 µL aliquot of each amplified PCR product was viewed using gel electrophoresis on a 1% agarose gel in order to check successful amplification and the rough size of each amplicon. A 20 µL aliquot was then purified using a Qiagen Minielute PCR purification kit (Qiagen GmbH, Germany) following the manufacturer's instructions. If there was presence of any ghost band on the initial gel, the remaining 20 µL was also separated by gel electrophoresis on a 1% agarose gel and only the target band was extracted and purified by Qiagen Minielute gel extraction kit (Qiagen GmbH, Germany). Purified amplicon DNA concentration was measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, USA). Sanger sequencing was completed

externally by a commercial sequencing facility EUROFINS® (Eurofins Genomics, United Kingdom).

2.3.4. Phylogenetic analysis

For each individual, the software Geneious 6.1.8 was used to examine the forward and reverse sequences; sequence quality was visualised and sequences trimmed where needed. Consensus sequences for each individual were produced after assessment of the pairwise alignment performed in Geneious 6.1.8. The two MutS sequences (forward and reverse) for an individual were then assembled to produce a "MutS contig" for each individual. In Geneious 6.1.8 sequences for each contig were aligned using the ClustalW alignment software, with default settings. Due to a disparity in the number of individuals sequenced, each contig (MutS vs IGR) were analysed separately.

For each contig the Geneious 6.1.8 plugin PhyML (Guindon & Gascuel, 2003) with 100 bootstrap replicates was used to construct maximum-likelihood trees. The Geneious 6.1.8 plugin MrBayes (Ronquist and Huelsenbeck 2003) was used to produce Bayesian inference trees; four Monte-Carlo chains were selected at a chain length of 1,100,000 with sampling frequency set at 200, burn-in length set at 100,000, and a random number seed setting. The Consensus Tree Builder in Geneious 6.1.8 was used to build consensus trees for both Maximum-Likelihood and Bayesian reconstructions after removing the initial 25% burn-in and the support threshold set at 50%. Appropriate nucleotide substitution models were selected using MEGA 7.0.26 (Kumar, Stecher & Tamura 2015).

For the analysis of the MutS contig, all available Holaxonia octocoral complete mitochondrial genomes were obtained from GenBank and sequences corresponding to the MutS contig were extracted. The octocoral *Renilla muelleria*, a member of the order Pennatulacea (sea pens), was chosen as the outgroup for this analysis. For the NAD5-IGR-NAD4 contig analysis the complete mitogenome of *Eunicella albicans* was obtained from GenBank and NAD5-IGR-NAD4 sequence was extracted and used as the outgroup in this analysis.

Table 2 List of specimens used in the analysis of the mtMutS contig.

Species	Family	Genbank accession no	Sequence from
***Eunicella verrucosa* (Bla01)**	Gorgoniidae	n/a	This study
***Eunicella verrucosa* (Lun20)**	Gorgoniidae	n/a	This study
***Eunicella verrucosa* (Men16)**	Gorgoniidae	n/a	This study
***Eunicella verrucosa* (Por2_02)**	Gorgoniidae	n/a	This study
***Eunicella verrucosa* (EV_MAI03)**	Gorgoniidae	n/a	This study
***Eunicella verrucosa* (EV_MAI12)**	Gorgoniidae	n/a	This study
***Eunicella verrucosa* (Cul1)**	Gorgoniidae	n/a	This study
***Eunicella verrucosa* (Cul2)**	Gorgoniidae	n/a	This study
Leptogorgia sarmentosa	Gorgoniidae	NC_035670.1	Genbank
Leptogorgia alba	Gorgoniidae	NC_035669.1	Genbank
Pacifigorgia cairnsi	Gorgoniidae	NC_035668.1	Genbank
Eunicella cavolini	Gorgoniidae	NC_035667.1	Genbank
Eunicella albicans	Gorgoniidae	NC_035666.1	Genbank
Eugorgia mutabilis	Gorgoniidae	NC_035665.1	Genbank
Leptogorgia gaini	Gorgoniidae	NC_035664.1	Genbank
Leptogorgia capverdensis	Gorgoniidae	NC_035663.1	Genbank
Paramuricea macrospina	Paramuriceidae	NC_034750.1	Genbank

Paramuricea clavata	Paramuriceidae	NC_034749.1	Genbank
Muricea purpurea	Plexauridae	NC_029698.1	Genbank
Muricea crassa	Plexauridae	NC_029697.1	Genbank
Calicogorgia granulosa	Acanthogorgiidae	NC_023345.1	Genbank
Euplexaura crassa	Plexauridae	NC_020458.1	Genbank
Echinogorgia complexa	Plexauridae	NC_020457.1	Genbank
Pseudopterogorgia bipinnata	Gorgoniidae	NC_008157.1	Genbank
***Renilla muelleri* (O. Pennatulacea)**	Renillidae	NC_018378.1	Genbank

^Where applicable GenBank accession numbers shown. For the *Eunicella* individuals' sequenced in this study, letters within the brackets indicates the individual's name. *Renilla muelleri* a member of the sea pen suborder Pennatulacea was used as the out-group in subsequent analysis.

Table 3 List of specimens used in the analysis of the NAD5-IGR-NAD4 contig.

Individuals ID code	Species	Geographic region	GenBank acession no.	Sequence from
Thu05	*Eunicella verrucosa*	Ireland	n/a	This study
Thu10	*Eunicella verrucosa*	Ireland	n/a	This study
Bla01	*Eunicella verrucosa*	Ireland	n/a	This study
Bla05	*Eunicella verrucosa*	Ireland	n/a	This study
Lun20	*Eunicella verrucosa*	Britain	n/a	This study
Lio09	*Eunicella verrucosa*	Britain	n/a	This study
Eten04	*Eunicella verrucosa*	Britain	n/a	This study
Eten11	*Eunicella verrucosa*	Britain	n/a	This study
Men16	*Eunicella verrucosa*	France	n/a	This study
Faro03	*Eunicella verrucosa*	Portugal	n/a	This study
Por2_02	*Eunicella verrucosa*	Portugal	n/a	This study
EV_MAI03	*Eunicella verrucosa*	Mediterranean	n/a	This study
EV_MAI12	*Eunicella verrucosa*	Mediterranean	n/a	This study

Eunicella albicans	*Eunicella albicans*	n/a	NC_035666.1	Genbank
Cp_56	*Eunicella singularis*	Mediterranean	n/a	This study
Mp_70	*Eunicella singularis*	Mediterranean	n/a	This study
Faro1_10	*Eunicella cavolini*	Portugal	n/a	This study

Where applicable GenBank accession numbers shown. For the Eunicella individuals' sequenced in this study geographic region column indicates where the sample was collected. NAD5-IGR-NAD4 sequence was extracted from Eunicella albicans mitogenome and subsequent used as the out-group in the analysis.

2.4. Results

A total of 17 sequences were used in the analysis of the NAD5-IGR-NAD4 contig; 16 were sequenced in this study and the outgroup (*E. albicans*) NAD5-IGR-NAD4 contig was extracted from previously published mitogenome. Full details of all sequences used can be seen in Table 3 (NAD5-IGR-NAD4). The HKY model was selected for the analysis of IGR contigs with a fixed transition transversion ratio of 1.47. Both maximum-likelihood (ML) and Bayesian inference trees separated out *E. verrucosa, E. singularis* and *E cavolini*. Both ML and Bayesian inference showed very strong consensus support (100) for the *E. singularis* and *E. verrucosa* clade. Within this clade the *E.* verrucosa clade had 70.7 and 96.7 consensus support for ML and Bayesian inference, respectively (Fig. 4). Strong consensus support (>90) with the Bayesian inference tree was observed for all nodes. Within species, sequences were identical for the NAD5-NAD4 intergenic region, and are shown as unresolved polytomies (Fig. 4).

For the analysis of the mtMutS contig, 25 sequences were used; 8 were sequenced in this study and 17 were extracted from published Holaxonia octocoral mitogenomes, Table 2**.** The HKY+G substitution model was selected

with 5 gamma categories, a gamma distribution parameter fixed at 0.53 and a fixed transition / transversion ratio of 2.59, as outlined using the MEGA7 substitution model test. The mtMutS phylogenetic tree presents consensus support values for both the maximum-likelihood and Bayesian inference methods, Figure 6 visualises *mtMutS* phylogram and cladogram in Figure 7. High consensus support is observed for the majority of nodes. Within *Eunicella*, *E. cavolini* groups within the *E. verrucosa* clade, with two individuals from Valencia (Cul1 and Cul2) being more distantly related to the other *E. verrucosa* individuals than is *E. cavolini*. Unfortunately, amplification of *E. singularis* mtMutS contigs was not successful; thus, no assessment can be made on their relationship to the other *Eunicella* individuals.

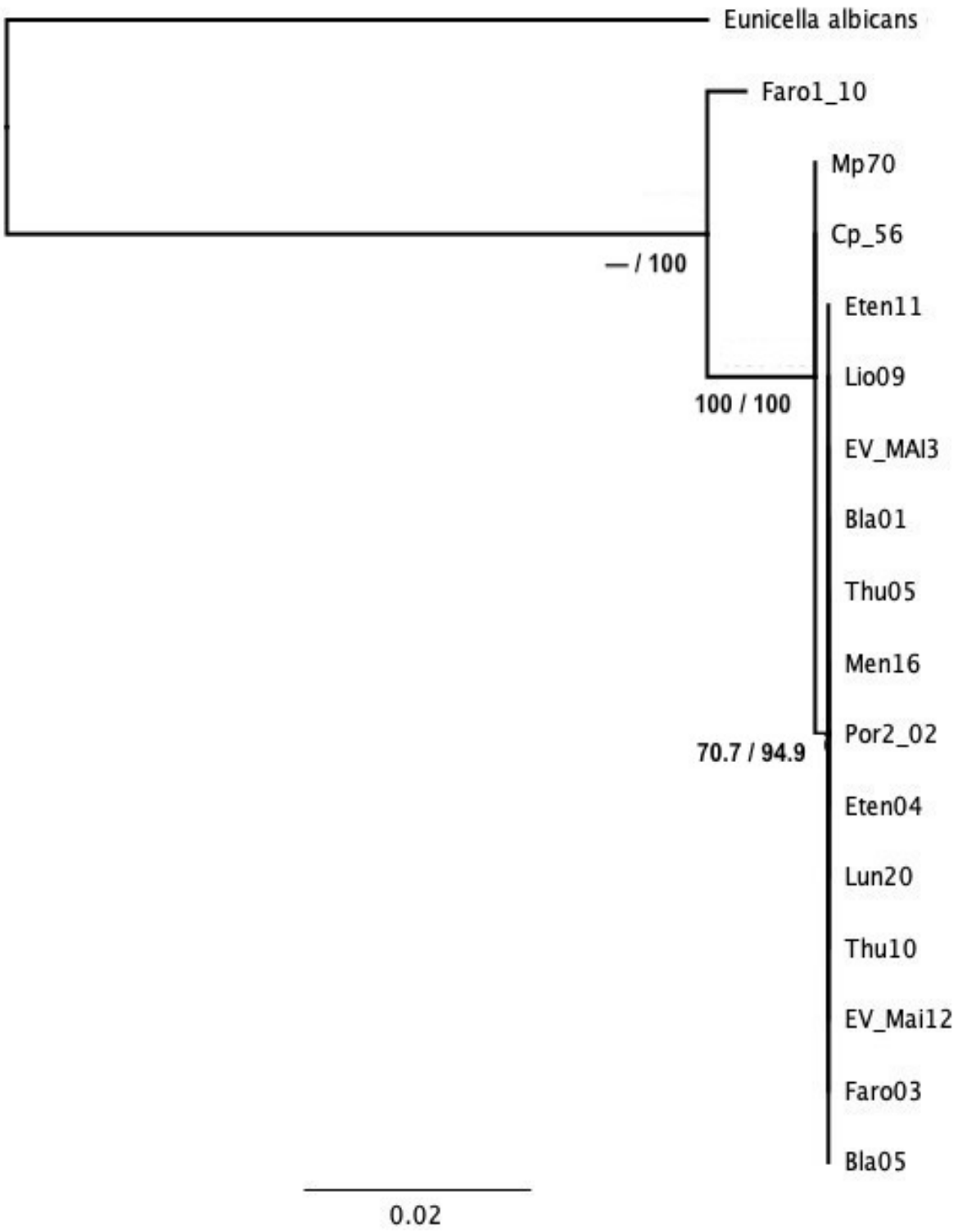

Figure 4 Depicts the maximum likelihood phylogram created in the analysis of the NAD5-IGR-NAD4 contig dataset. Tip labels show individuals ID code, which are outlined in Table 2. The analysis uses 17 NAD5-IGR-NAD4 sequences; including *E. albicans* extracted from complete mitochondrial genome sequence from GenBank and rooted the tree. Values below branches show consensus support values for both the maximum likelihood and Bayesian inference analysis (ML/Bayesian).

Figure 5 Depicts the ML phylogram produced using the partial contigs of mtMutS, a total of 25 sequences were used. The symbol ⋆ indicates branches that had consensus support values of >70% ML and >90% for Bayesian inference analysis. The sea pen *Renilla muelleri* was used to root the tree.

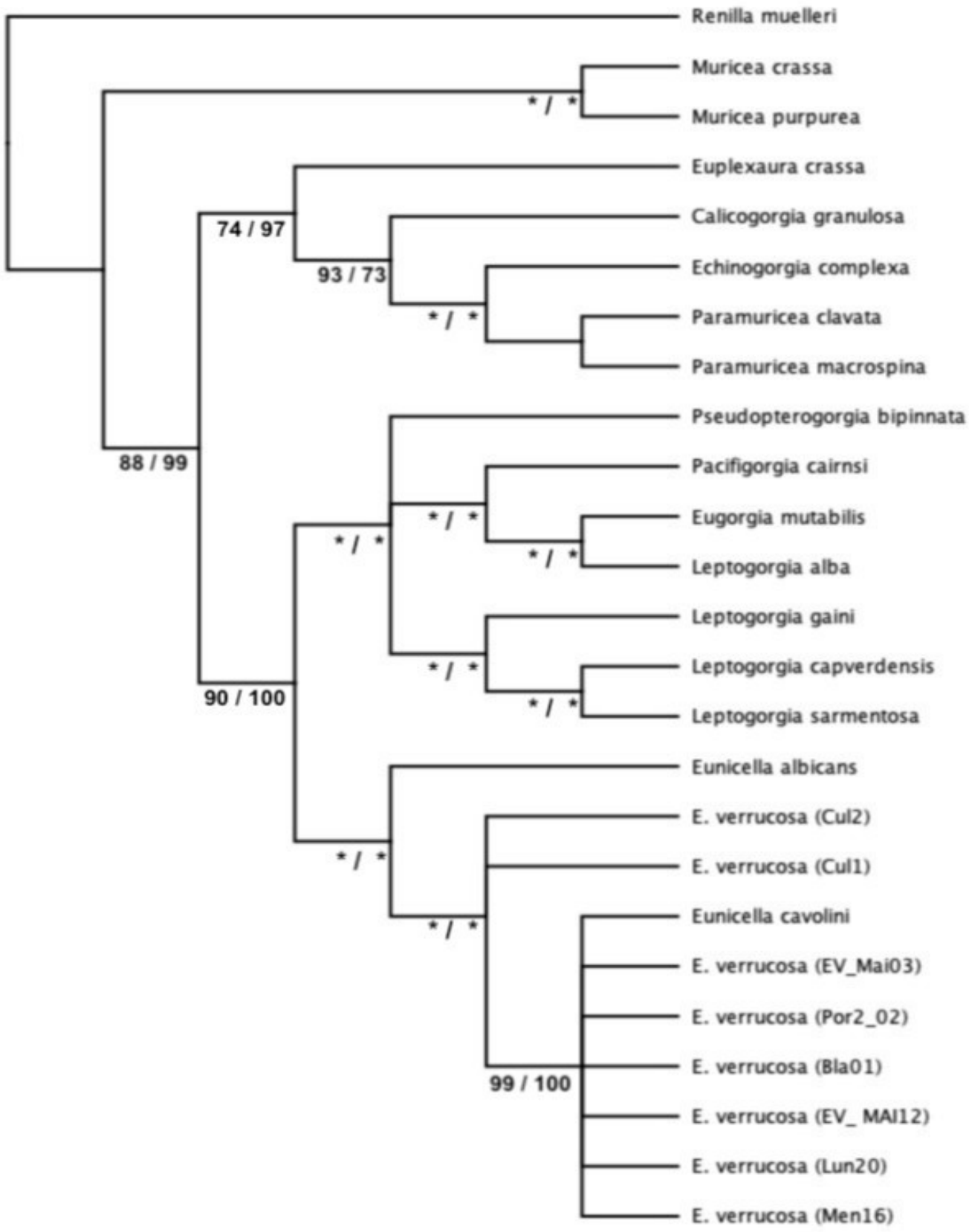

Figure 6 Depicts the ML cladogram produced using the partial contigs of *mtMutS,* a total of 25 sequences were used. Values under the branch show the consensus support value for ML and Bayesian inference (ML / Bayesian inference). Branches with * / * indicates branches that had consensus support values of 100 for both ML and bayesian inference analysis. The sea pen *Renilla muelleri* was used to root the tree.

2.5. Discussion

2.5.1. NAD5-IGR-NAD4

In this study, the newly developed primers, that target NAD5-IGR-NAD4 in the octocoral mitochondrial genome, were unable to distinguish at the conspecific level within *Eunicella.* A total of 13 *E. verrucosa* NAD5-IGR-NAD4 contigs were sequenced in this study and no variation was observed across this 1,156bp contig regardless of their geographic origin. This apparent lack of variation accords with previous findings in Anthozoa (France and Hoover 2002; Shearer et al. 2002; Fukami and Knowlton 2005; Calderón, Garrabou, and Aurelle 2006).

However, this marker was able to distinguish members of *Eunicella* at the congeneric level, with high ML and Bayesian inference consensus support values (>70) for each species' monophyletic clade (Fig. 4). The variation found in this study between the *Eunicella* species provides a real advance for evolutionary studies of taxa within the genus, as previous phylogenetic studies have not found any genetic variation in the *cox1* gene between the European representatives of *Eunicella* studied to date (Calderón, Garrabou, and Aurelle 2006). Calderon et al. (2006) also found that the nuclear marker ITS2 was unable to distinguish between *Eunicella* species. This lack of variation in *cox1* appears to be a widespread phenomenon in Octocorallia with estimations of 18.5% of congeneric species sharing identical *cox1* sequences (Mcfadden et al. 2011). In turn, this has serious implications for genetic barcoding in *Eunicella* and Octocorallia in general, as the *cox1* sequence has been proposed as the universal barcode (Hebert, Ratnasingham, and Jeremy 2003). DNA barcoding

is a method developed to identify all species with the use of a standardised segment of DNA sequence.

DNA barcoding sequences can be interpreted via two main methods: distance-based or character-based methods (Hebert, Ratnasingham, and Jeremy 2003; Velzen et al. 2012; Chakraborty, Dhar, and Ghosh 2017). Distance-based methods are the most commonly used barcoding method, which uses a comparative analysis based on distance-based clustering of the species in question and then uses arbitrary distance thresholds to define species boundaries (Hebert, Ratnasingham, and Jeremy 2003; Desalle, Egan, and Siddall 2005; Bergmann et al. 2009). To accurately define a distance threshold value a gap between intraspecific variation and interspecific variation is required (Desalle, Egan, and Siddall 2005; Bergmann et al. 2009) . Character-based methods work upon diagnostic nucleotide traits, they require two data points, the presence or absence of discrete nucleotides and the relative position of this diagnostic nucleotide in the barcoding sequence (Desalle, Egan, and Siddall 2005; Rach et al. 2008).

To overcome the problems of DNA barcoding in Octocorallia, that can occur due to a lack of variation in the *cox1* gene, Mcfadden et al. (2011) proposed to continue to use a distance-based method, but with an extended barcode requiring the sequencing of three separate sections of the mitochondrial genome (*cox1*, *mtMutS* and *igr1*). To be able to delineate congeneric species they also proposed adopting a reduced genetic distance threshold of >0.5% compared to the commonly used 2-3%. Genetic barcodes are meant to be cheap and easy to obtain however, this extended barcode requires the

amplification and sequencing of three separate sequences and thus diminishes the accessibility of this barcode. Moreover, distance-based methods have been criticised as they are seen as a highly subjective approach, with percentage difference thresholds being criticised as arbitrary and not being based on sound biological reasons (Bergmann et al. 2013; Desalle, Egan, and Siddall 2005). Velzen et al. (2012) has shown that, compared to distance-based methods, character-based methods have greater success in identifying recently diverged species, where incomplete lineage sorting can lead to a lack of a barcoding gap. Although Octocorals may not have recently diverged, they do show a lack of a barcoding gap, due to low rates of evolution which cause a lack of interspecific variation (Mcfadden et al. 2011; Shearer and Coffroth 2008); thus they may still be of some value for exploring taxonomic variation within Octocorallia.

The variation observed in this study in the NAD5-IGR-NAD4 mitochondrial genome sequence, while limited, still demonstrates its ability to discriminate between different species of *Eunicella* and its potential as a character-based barcode for this genus. This study has identified potential diagnostic nucleotides within the *nad5-nad4* intergenic region. However, to properly define an unambiguous character based barcode for *Eunicella* further studies are needed; such study would aim to identify any intraspecific variation in NAD5-IGR-NAD4 across the entire species range of *E. singularis* and *E. cavolini,* emulating the research that was carried out for *E. verrucosa* in this current study.

Pursuing such research would be highly valuable, as a character-based barcode would provide many advantages to both the research and conservation

of the genus. Firstly, a character-based approach to delineating species produces a binary result determined from an objective set of criteria, meaning it can easily be combined with classical morphological diagnostics (Chakraborty, Dhar, and Ghosh 2017; Desalle, Egan, and Siddall 2005). Specifically, for *Eunicella*, this means that the few diagnostic morphological characteristics for each species can easily be combined with the few available diagnostic nucleotides, to produce more robust criteria with which to define the species. Moreover, *Eunicella* is a genus whose members' morphological features are highly plastic and which appear to vary due to environmental factors (Gori et al. 2011; Carlos Prada, Schizas, and Yoshioka 2008). A character-based barcode, as suggested here, would allow for a definitive molecular taxonomic identification of the species.

2.5.2. *mtMutS*

Here the partial contig of *mtMutS* has successfully been exploited in order to assess phylogenetic relationships within the octocoral suborder Holaxonia. This marker has shown sufficiently high resolution to infer relationships within Holaxonia and both maximum-likelihood and Bayesian inference analyses produced high consensus support values (>70) for the majority of branches within the *mtMutS*–based phylogeny (Fig. 5 and 6). Within this tree, a well supported monophyletic clade containing all analysed members of the family *Gorgoniidae* and *Plexauridae* is defined; earlier works on gorgonian octocorals, which used morphological and molecular data, also identified distinctions between the highly similar Holaxonian families (Sánchez 2007; Aguilar and Sanchez 2007). The family level relationships observed in these earlier studies are corroborated with the *mtMutS* contig phylogeny produced in this study,

however, neither of these previous studies included the genus *Eunicella* so no comment on the relationships within the *Gorgoniidae* could be made at this time.

Within the Gorgoniidae clade defined in the *mtMutS* molecular phylogeny (Fig. 5 and 6), the genus *Eunicella* formed a monophyletic clade, with ML and Bayesian inference consensus support values of 100. Similarly, in a previous mitogenome-based study of genus Leptogorgia (Poliseno et al. 2017) using complete mitochondrial protein-coding sequences, a high level of congruence was observed between the Holaxonia clade of Poliseno et al. (2017) and that produced in the current study. Within Poliseno et al. (2017) the members of *Eunicella* studied (*E. cavolini* and *E. albicans*) also formed a monophyletic clade within the Gorgonidae clade. Former taxonomic subdivision placed *Eunicella* into the family *Plexauridae* (Bayer 1961); however, the findings of both Poliseno et al. (2017) and the current study confirm the reassignment of *Eunicella* to the family *Gorgonidae*. Both studies identified *Leptogorgia* as being paraphyletic, both placing *Leptogorgia alba* outside the main *Leptogorgia* clade, thereby demonstrating the divergence between Pacific (*L. alba)* and Atlantic Leptogorgia species (= all other *Leptogorgia* studied).

Thus, the molecular studies of *Eunicella*, including the current study, are pivotal, not only for the conservation of this genus, which has recently experienced multiple mass mortalities (Cerrano et al. 2000; Martin, Bonnefont, and Chancerelle 2002; Cupido et al. 2012), but also to understand the wider evolutionary relationships within Ocotocorallia. As aforementioned, there is a general scarcity of fossils records for Octocorallia creatures, as their soft bodies

will decompose rather than fossilise (Juan Armando Sánchez, Lasker, and Taylor 2003; Carlos Prada, Schizas, and Yoshioka 2008). Since very few fossils of Octocorallia are typically found, any that are discovered are pivotal to mapping the evolution of morphological characteristics in octocorals as well as, in calibrating molecular phylogenies. Any octocoral fossils that are found are usually extremely hard to assign with any degree of confidence to a certain clade, due to a lack of diagnostic morphological features in Octocorallia (Kocurko and Kocurko 1992; McFadden, Sánchez, and France 2010). One of the largest and most diverse octocoral fossil assemblages known originated from the Red Bluff Formation in Mississippi and dates back to around 28 –34 million years ago in the Lower Oligocene (Kocurko and Kocurko 1992). The only morphological characteristic that can be positively identified to a particular genus is a distinct balloon club sclerite from a *Eunicella* individual (this balloon club sclerite form does not occur in any other octocoral taxon). An unequivocal identification such as this means that molecular studies into *Eunicella* and its relationship to other octocorals are even more vital, as this taxa of this genus can potentially provide a fossil-based calibration date to any octocoral phylogenetic tree.

Chapter 3 - Complete mitochondrial genome sequencing of *Eunicella verrucosa*

3.1.1. Abstract

Phylogenetic assessment of Octocorallia has been severely hindered by a lack of variation in the molecular markers used to date. However, complete mitochondrial genomes have shown to increase resolution of octocoral phylogenies, when compared to the widely used molecular marker *mtMutS,* and have resolved previously unresolved clades within Octocorallia phylogenies. Despite this only 1% of known octocorals have publicly available mitochondrial genomes. In this study, we apply NGS with rolling circle amplification (RCA), an amplification method novel to Octocorallia, to produce a total of 19 *E. verrucosa* complete mitochondrial genomes. Our results identified only two genotypes across the entire range of *E. verrucosa*. Extremely low genetic distances (0.06%) between the mitochondrial genomes of *E. verrucosa* and *E. cavolini* were observed. This level of variation is low, even when compared with the known low levels of variation observed in other octocoral mitochondrial genomes, suggesting a recent divergence between *E. verrucosa* and *E. cavolini.* A recent divergence would suggest these two Eunicella species diverged after the most recent reopening of the Strait of Gibraltar, calling into question the mechanism of their divergence. The RCA, applied in this study, has shown high efficiency producing octocoral mitochondrial genomes in a cost-effectively with minimal hands-on time; highlighting its potential as an extremely useful tool for phylogeneticists. The increased sequencing breadth complete mitochondrial genomes provide can help resolve relationships within Octocorallia. Understanding relationships within basal metazoans such as octocoral can help the broader understanding of evolution and development of metazoans.

3.2. Introduction

3.2.1. General introduction

Octocoral mitochondrial genomes are unique among Metazoans as they contain a mismatch repair gene, *mtMutS* (Pont-Kingdon et al. 1995; Culligan et al. 2000). The presence of this mismatch repair gene coincides with extremely low levels of genetic variation in the mitochondrial genomes of octocoral (France and Hoover 2002; McFadden, Sánchez, and France 2010). This low level of mitochondrial gene variation has caused a lack of variation in the majority of molecular makers used to date, which has severely hampered insights into the relationships within Octocorallia (McFadden, Sánchez, and France 2010). Recently, Figueroa and Baco (2014) exemplified the advantages that complete mitochondrial genomes can provide to octocoral phylogenetic research. In their study, they were able to resolve previously unresolved clades in Octocorallia (McFadden et al. 2006; Figueroa and Baco 2014); unfortunately, further insights into the relationships within Octocorallia was limited by a paucity of available octocoral mitogenomes. To date, only 1% of the known octocoral species have published mitochondrial genomes (Poliseno et al. 2017).

Common methods that have been used to obtain octocoral mitogenomes are conventional PCR and long-PCR combined with primer walking sequencing; both of these methods are relatively labour intensive and require previous sequence knowledge (Park et al. 2012; Burger et al. 2007; Kayal et al. 2013; Brockman and Mcfadden 2012). Conventional PCR requires the use and optimisation of over 30 primer pairs (Park et al. 2012). Long PCR and primer walking sequencing, this method is more efficient than conventional PCR as the

mitogenome is amplified in 3,000bp fragments, thus reducing the number of amplifications required (Poliseno et al. 2017; Burger et al. 2007; Kayal et al. 2013). Although long-PCR is an efficient process, it still requires the testing of multiple long-PCR primers to acquire complete coverage of the mitochondrial genome (Burger et al. 2007). Moreover, the primer-walking sequencing method that is required can be a slow and costly sequencing method.

With the advantages complete mitogenomes can provide to phylogenetic studies of octocoral the application of a cost-effective and efficient amplification method would show a real advancement for octocoral phylogenetic research. Rolling circle amplification is one such method; details on this method are outlined below.

3.2.2. Rolling Circle Amplification (RCA)

Rolling circle amplification (RCA), also known as multiple displacement amplification, is an alternative amplification method to the commonly used polymerase chain reaction (PCR) (Johne et al. 2009). It is an isothermal reaction that enriches for circular DNA compared to linear DNA (Wang et al. 2014). *In vivo*, the replication of circular DNA molecules often follows a rolling circle amplification mechanism (Kornberg and Baker 1992). *In vitro*, RCA itself has been used since 1989, being applied to multiple different organismal circular DNA templates across a range of different DNA polymerases (Blanco et al. 1989; Shavitt and Livneh 1989; Liu et al. 1996). RCA has several major advantages over conventional PCR, as it does not require the design of specific primers or optimisation of a thermal cycle, as discussed below (Ni et al. 2015).

The general principle of RCA and the way it enriches circular DNA over linear DNA is shown in Figure 7. Initially, specific primers are annealed to the template strand and then the polymerase incorporates nucleotides in the 5'-3' direction, complementary to the template strand. Figure 7a depicts the RCA process for a linear template molecule and shows how RCA would amplify linear DNA until the template ends, yielding a 1:1 ratio of the template sequence in the amplicon and the template DNA.

However, circular DNA generates a much larger amplicon to template ratio as once amplification of the circular DNA reads around to the original primer binding site, displacement of the newly synthesised strand occurs and amplification continues, producing multiple copies of template per single primer binding event (Fig. 7b). The amplicon produced is a concatemer and consists of multiple repeated copies of the original template DNA, hence a greater yield of circular template sequence is obtained compared to amplification from a linear DNA template per single primer binding event**.**

The up-regulation of circular DNA can be further increased by the inclusion of additional primers, the binding sites of which are found on the complementary strand (Fig. 7c). These additional primers can bind to the displaced strand and initiate addition DNA synthesis. Typically, however, the approach often adopted in research is the use of multiple primers (Johne et al. 2009), either specifically designed or random primers, which bind to both strands of the circular DNA template as well as, the newly synthesised DNA sequence. The use of multiple primers produces a hyper-branched RCA amplicon, that contains multiple connected concatemers of the template DNA molecule, as seen in Figure 1c

(Lizardi et al. 1998). Thus, a multi-primed RCA method further increases the efficiency of the amplification of circular template DNA. Multi-primed RCA also allows for enrichment of circular DNA over linear DNA, with practical quantifications of 100- to 10,000-fold enrichment of the mouse mitogenome and, in the worst case, 1:50,000 of nuclear DNA molecules to mtDNA molecules (Marquis et al. 2017).

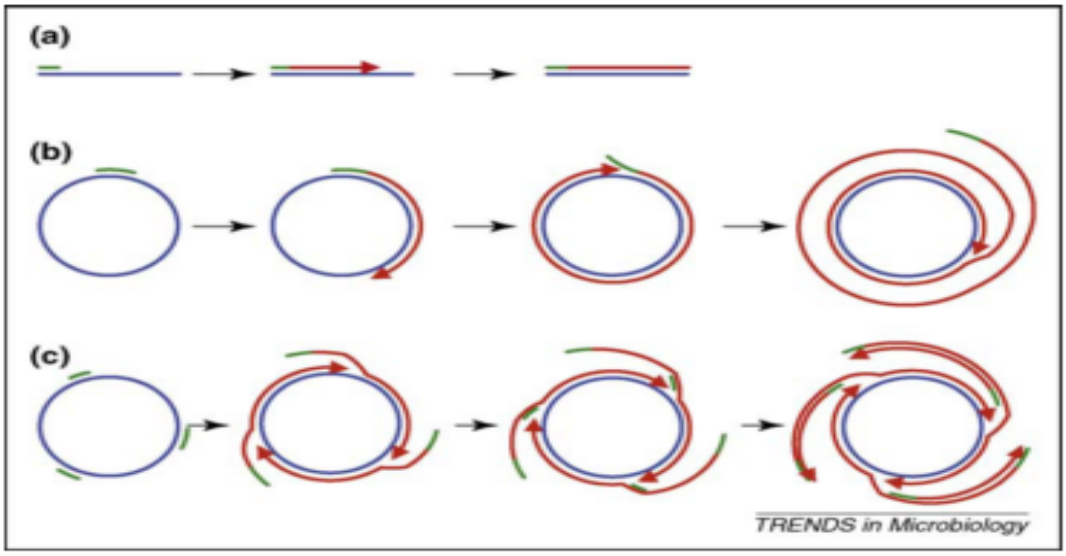

Figure 7 Adapted from Johne et al. 2009 – Rolling-circle amplification of viral DNA genomes using phi29 polymerase. Blue lines depict template DNA sequences, red lines represent newly synthesised DNA sequences and green lines represent primer sequences. (a) Represents the synthesis of linear DNA. (b) Shows the up-regulation of circular DNA by RCA with a polymerase with strand displacement activity and a single primer. (c) Depicts multi-primed RCA and how primers in the reaction mixture can bind to the newly synthesised DNA.

RCA requires the use of a polymerase that possesses specific features and it cannot be performed with Taq polymerase, which is often used in conventional PCR (Johne et al. 2009). The most common polymerases utilised in RCA are phi29, Bst, and Vent exo-DNA polymerase (Ali et al. 2014). phi29 DNA polymerase has been used to induce RCA since 1989 and has been shown to be of great value to exploring questions in viral and microbial biology (Blanco et al. 1989; Johne et al. 2009). phi29 DNA polymerase is part of the B-family of

replicative polymerases and is a single protein with a molecular mass of 68 kDa (Blanco et al. 1989; Berman et al. 2007), which is encoded in the genome of the bacteriophage phi29 that infects *Bacillus subtilis* (Blanco et al. 1989).

phi29 DNA polymerase possesses several features that make it highly suitable for isothermal RCA. Firstly, the strand displacement activity of phi29 DNA polymerase allows the enzyme to displace the complementary strand of DNA, meaning that unlike conventional PCR amplification, it can be carried out isothermally without needing to optimise a thermal cycle (Blanco et al. 1989; Kamtekar et al. 2004; Luthra and Medeiros 2004; Spits et al. 2006). Secondly, phi29 DNA polymerase can produce large products of >70 Kbp; these large products are required in order to up-regulate circular DNA and allow for as many repeats of circular template DNA per concatemer (Blanco et al. 1989; Banér et al. 1998). Moreover, the enzyme has a relatively long half-life, which allows for a prolonged reaction. Studies have estimated that within a 10-hour reaction, phi29-RCA can produce a product with an estimated length of 900 Kbp (Dahl et al. 2004; Banér et al. 1998). phi29 DNA polymerase is also a highly accurate polymerase due to the enzyme's proofreading ability which occurs due to its 3'-5' DNA exonuclease activity (Esteban, Salas, and Blanco 1993). The proofreading activity of phi29 DNA polymerase means it has an estimated error rate of 1 in every $10^6 – 10^7$ bases, two to three orders of magnitude less than Taq polymerase error rates of 1 in every 10^4 bases (Tindall and Kunkel 1988; Esteban, Salas, and Blanco 1993; Paez et al. 2004). This exonuclease activity causes the degradation of primers and can reduce yield, which can be combatted using primers with thiophosphate linkages in the 3' terminal nucleotides (Dean et al. 2001). The ability of phi29 DNA polymerase to

produce large products with low error rates, combined with its strand displacement activity, allows for efficient RCA and thus the up-regulation of circular DNA without the need to optimise a thermal cycle.

The phi29 DNA polymerase induced RCA (phi29-RCA) method was first applied to viral genomes in 2004, when the papillomavirus circular genome was successfully amplified directly from tissue samples (Rector et al. 2004). Multi-primed phi29-RCA has been pivotal in the discovery of unknown and uncultivable viruses, as RCA in combination with random hexamer primers can amplify sequences without the need for previous knowledge of the target sequence (Johne et al. 2009). The application of multi-primed phi29-RCA has led to the discovery and diagnostics of multiple viruses that infect humans, animals and plants (Haible, Kober, and Jeske 2006; Johne et al. 2009). It has also been applied in microbial studies, in which plasmids have been amplified directly from cells or plaques (Dean et al. 2001).

Due to its highly sensitive nature, phi29-RCA has recently been applied in the identification of deleterious heteroplasmic genetic mutations in human mitochondrial genomes (Marquis et al. 2017). The mitochondrial genome is found in multiple copies within a cell, ranging from a few hundred to a few thousand (Ye et al. 2014); heteroplasmy is said to occur when a subset of this mitochondrial gene pool contains a unique mutation and discrete populations of mitochondrial genomes can be found within one cell. phi29-RCA has been used to detect very low-frequency heteroplasmy due to the extremely low error rates observed in the amplification method. Highly sensitive detection of heteroplasmy is vital, as 1 in 200 humans carry one of the top ten most

abundant pathogenic mitochondrial mutations (Elliott et al. 2008). phi29-RCA has shown its ability to be a cheap, easy and highly accurate amplification method, but has mainly been applied to the identification of human diseases and viral genomes. However, a few studies have implemented this method with non-model metazoan mitochondrial genomes (Simison, Lindberg, and Boore 2006; Wolff et al. 2012).

3.2.3. Study aims

This study aimed to investigate intraspecific variation in the mitochondrial genome of *Eunicella verrucosa.* This was achieved by applying the aforementioned phi29-induced RCA method in order to sequence multiple *E. verrucosa* complete mitochondrial genomes.

Intraspecific variation is presumed to be low in octocorals, however, this is assumed from a relatively small number of individual genetic barcodes (France and Hoover 2002; Mcfadden et al. 2011; McFadden et al. 2006; McFadden, Sánchez, and France 2010; Calderón, Garrabou, and Aurelle 2006). By sequencing a large number *E. verrucosa* individuals, from their entire range, with a greater breadth of sequencing, by sequencing the whole mitochondrial genome and not just targeting a small segment, we aimed to equivocally define any intraspecific variation observed in their mitochondrial genomes. We anticipated that the identification of any intraspecific variation could be used as the basis of a simple, inexpensive molecular marker.

We also aimed to demonstrate the efficiency of phi29 induced RCA and its ability to sequence a large number of Octocorallia mitochondrial genomes. This

would be a pivotal tool for phylogenetic studies of octocoral species, as, to date, only ~1% of the known 3,000 species of octocorals have publicly available mitochondrial genomes (Poliseno et al. 2017).

3.3. Material and Methods

3.3.1. Sample collection and DNA extraction

Eunicella verrucosa samples were collected from sites ranging from northwest Ireland to Portugal and the northwest of the Mediterranean Sea (Table 4), representing most of their known range. Samples were collected via SCUBA at depths of between 5 and 30 metres via the removal of a 3 cm section of a terminal branch from an individual sea fan. Collection of samples has been on-going from 2007 to the present. To avoid sampling clones, samples were taken from colonies spaced ~1 m apart (as explained in Holland, Jenkins, and Stevens 2017). Once samples were removed from a colony they were placed in a mesh bag, brought to the surface and rapidly immersed in 95 - 100% ethanol. Ethanol was changed 24 - 48 hours after initial immersion to remove any excreted secondary metabolites that might affect downstream analysis. DNA was extracted following the modified salting-out protocol outlined in Chapter 2 (Li et al. 2011). The purity and concentration of the extracted DNA were quantified using a NanoDrop One (Thermo Scientific, Wilmington, DE, USA).

3.3.2. Rolling circle amplification

phi29 induced rolling circle amplification (phi29-RCA) was carried out in 50 µL reactions in a two-stage process. Reactions comprised 2.5 µL of DNA extract, 0.5 µL (200 µg/ml) BSA, 0.5 µL (500 µM) Exo-Resistant Random primers (Thermo Fisher Scientific, Inc.), 2.5 µL Phi29 DNA Polymerase Reaction Buffer

(NEB) and 19 µL of nuclease-free water which was denatured at 95°C for 3 minutes then cooled at 10°C for 15 minutes. To the above reaction 0.5 µL (200 µg/ml) BSA, 0.5 µL (500 µM) Exo-Resistant Random primers (Thermo Fisher Scientific, Inc.), 2.5 µL Phi29 DNA Polymerase Reaction Buffer (NEB), 19.25 µL of nuclease-free water, 1.25 µL (10mM) dNTP solution mix (NEB) and 1 µL (10 units) Phi29 DNA Polymerase (NEB) were added. The reaction was heated at 34°C for 16 hours, after which the temperature was raised to 65°C for 15 minutes to heat inactivate the polymerase.

A sodium acetate-ethanol precipitation was carried out to purify the amplified DNA. For this, 5 µL of 3M sodium acetate and 125 µL 100% ethanol was added to the final 50 µL RCA reaction and cooled in the freezer (-20°C) for two hours. Subsequently, reactions were centrifuged at 13.3 rpm for 20 minutes and the supernatant was then removed, leaving just a DNA pellet in the bottom of the reaction tube. The DNA pellet was washed twice, by adding 200 µL of 70% ethanol, centrifuging at 13.3 rpm for 5 minutes and removing the supernatant. The DNA pellet was then left to dry, prior to being re-suspended in 12 µL of EB buffer (Qiagen).

3.3.3. Sequencing and Bioinformatics

The sequencing was outsourced and carried out by Exeter Sequencing Service and Computational Core Facilities at the University of Exeter. Library preparations were carried out using a Mosquito LV (ttpLabTech) using Nextera XT. Minimal PCR cycles were used in library preparation, with the maximum number of cycles being 12. Individuals were barcoded and pooled onto a single

lane of paired-end 300bp Illumina MiSeq v2, which has a capacity of 24-30 million reads.

Assembly of all mitochondrial genomes was carried out in Linux. In summary, the bioinformatics process proceeded as follows: low-quality reads and adaptors were removed via the program Fastq-mcf v.1.04.636; a *de novo* SPAdes (Nurk et al. 2013) assembly was then carried out. Due to the large amount of contamination that was observed the *de novo* contigs produced by SPAdes were blast searched against the *Eunicella cavolini* complete mitochondrial genome (NCBI accession number: KY559408.1). The contigs with significant hits were then extracted; this was often in the form of one large 19,267 bp contig representing the complete mitochondrial genome of *E. verrucosa.* The extracted contig was then used as a reference genome to assemble the raw reads back on to.

As well as the *de novo* SPAdes assembly, raw reads were also mapped against a reference genome. Due to the known similarity between *E. cavolini and E. verrucosa* mitochondrial genomes, as evidenced in chapter 2, the *Eunicella cavolini* mitochondrial genome (NCBI accession number: KY559408.1) was used as a reference genome against which to assemble individuals. The Burrows-Wheeler Aligner (BWA) (Li and Durbin 2009) program was used to align the trimmed reads against the reference genome. The Samtools package (Li et al. 2009) was then used to sort, index and remove duplicates. The freebayes (Garrison and Marth 2012) and Real Time Genomics packages (Real Time Genomics Limited©, 2015) were then used to filter and call variant sites; only variant sites with a Q score of >30 were kept. These variant data were put

into BCFtools to create a consensus sequence. BAM files were loaded into the Integrative Genomics Viewer (IGV) (Ribison et al. 2011) to visualise variant sites and to assess the overall assembly.

3.3.4. Phylogenetic analysis

The program Geneious 6.1.8 was used for phylogenetic analysis of the mitochondrial genome sequences of *Eunicella verrucosa.* Sequences were aligned using the ClustalW alignment software, with default settings. The Akaike information criterion corrected value (AICc) obtained from MEGA 7.0.26 (Kumar, Stecher and Tamura 2015) was used to select the most appropriate nucleotide substitution model for each alignment. Maximum-likelihood trees were created with the Geneious 6.1.8 plugin PhyML (Guindon and Gascuel 2003) with 100 bootstrap replicates. The Geneious 6.1.8 plugin MrBayes (Ronquist et al. 2012) was used to produce Bayesian inference trees; four Monte-Carlo chains were selected at a chain length of 1,100,000 with sampling frequency set at 200, burn-in length set at 100,000, and a random number seed setting. The Consensus Tree Builder in Geneious was used to build consensus trees for both maximum-likelihood and Bayesian reconstructions after removing the initial 10% burn-in, and the support threshold set at 50%. For the phylogenetic analysis of *Eunicella* complete mitochondrial genomes, a GTR+G+I nucleotide substitution model was selected, with 5 gamma categories and a transition/transversion ratio of 3.42.

For the analysis of the Holaxonia clade, only two *Eunicella verrucosa* sequences (Lio13 and Bla04) were used to represent the two genotypes observed in this study. Additional Holaxonia mitochondrial genome sequences were obtained from GenBank. Holaxonian mtDNA genomes included in the

analysis are the same as in Chapter 2, except for the removal of *Euplexaura crassa,* due to unsuccessful alignment. As in chapter 2, *Renilla muelleri* (O. Pennatulacea) was used as the outgroup. Due to the extreme length variation of intergenic regions, protein-coding genes were extracted from each mitochondrial genome. The 14 protein-coding gene sequences were concatenated for each individual and aligned using ClustalW in Geneious 6.1.8**.** Maximum-likelihood and Bayesian inference trees were produced as outlined above. A GTR+G+I nucleotide substitution model was implemented with 5 gamma distribution categories and a transition/transversion ratio of 2.59.

3.3.5. PCR amplification and Sanger sequencing

A unique genotype was observed in two samples from Lion Rock, Isles of Scilly, southwest England (Lio03 and Lio13). Two single nucleotide polymorphisms (SNPs) seen in the genotype unique to Lion Rock were found within the *nad5-nad4* intergenic region. To confirm the unique polymorphisms were not observed because of an error in the RCA amplification method or NGS sequencing, PCR amplification and Sanger sequencing of this intergenic region was carried out using the primer pair NAD5-IGR-NAD4, developed in Chapter 2.

All PCR reactions were performed in 25 µL reactions with: 0.375 µL of each of the forward and reverse primer, 0.25 µL BSA (NEB), 12.5 µL HotStart Taq Master Mix (Qiagen), 9.0 µL RNase free water and 2.5 µL extracted DNA. A 60˚C to 55˚C touchdown protocol was used for the NAD5-IGR-NAD4 primers. The PCR thermal cycle for the 60˚C to 55˚C touch down was as follows: an initial denaturing stage at 94˚C for 5 minutes; followed by 35 cycles of 30 seconds denaturing at 94˚C, 30 seconds annealing at 60˚C (decreasing by

0.5˚C every cycle for 10 cycles then remaining at 55˚C for the remaining 25 cycles), and finally a 30 second extension at 72˚C; after these 35 cycles, a final extension for 5 minutes at 72˚C was undertaken.

A 5 µL aliquot of each amplified PCR product was viewed using gel electrophoresis on a 1% agarose gel in order to check successful amplification and to assess the rough size of each amplicon. A 20 µL aliquot was then purified using a Qiagen Minielute PCR purification kit (Qiagen GmbH, Germany) following the manufacturer's instructions. The DNA concentration of the purified amplicon was measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, USA). Sanger sequencing was completed externally by a commercial sequencing facility, EUROFINS® (Eurofins Genomics, United Kingdom).

Table 4. Specimens used in the analysis of Holaxonia complete mitochondrial genome sequences.

Species	Family	GenBank accession no	Sequence from
***Eunicella verrucosa* (Bla04)**	*Gorgoniidae*	n/a	This study
***Eunicella verrucosa* (Lio13)**	*Gorgoniidae*	n/a	This study
Leptogorgia sarmentosa	*Gorgoniidae*	NC_035670.1	Genbank
Leptogorgia alba	*Gorgoniidae*	NC_035669.1	Genbank
Pacifigorgia cairnsi	*Gorgoniidae*	NC_035668.1	Genbank
Eunicella cavolini	*Gorgoniidae*	NC_035667.1	Genbank
Eunicella albicans	*Gorgoniidae*	NC_035666.1	Genbank
Eugorgia mutabilis	*Gorgoniidae*	NC_035665.1	Genbank
Leptogorgia gaini	*Gorgoniidae*	NC_035664.1	Genbank
Leptogorgia capverdensis	*Gorgoniidae*	NC_035663.1	Genbank
Paramuricea macrospina	*Paramuriceidae*	NC_034750.1	Genbank
Paramuricea clavata	*Paramuriceidae*	NC_034749.1	Genbank
Muricea purpurea	*Plexauridae*	NC_029698.1	Genbank
Muricea crassa	*Plexauridae*	NC_029697.1	Genbank
Calicogorgia granulosa	*Acanthogorgiidae*	NC_023345.1	Genbank
Echinogorgia complexa	*Plexauridae*	NC_020457.1	Genbank
Pseudopterogorgia bipinnata	*Gorgoniidae*	NC_008157.1	Genbank
***Renilla muelleri* (O. Pennatulacea)**	*Renillidae*	NC_018378.1	Genbank

Where applicable GenBank accession numbers are shown. For the two *Eunicella* specimens sequenced in this study, individual's ID code is shown in brackets adjacent to species name. *Renilla muelleri*, a member of the sea pen suborder *Pennatulacea*, was used as the outgroup in all analyses.

3.4. Results

3.4.1. Mitogenomes

A total of 19 *E. verrucosa* mitochondrial genomes were sequenced in this study and two distinct genotypes were observed. Two *E. verrucosa* members from Lion Rock, Isles of Scilly, southwest England (Lio03 and Lio13) shared a distinct genotype; the second genotype was shared by all other *E. verrucosa* sequenced analysed. The complete mitogenomes were 19,267 bp and 19,248 bp in length for the widespread genotype and the Lion Rock genotype, respectively. Both of the mitogenomes showed the ancestral gene arrangement (Type A), which includes 14 protein-coding genes, two ribosomal RNA subunits (12S and 16S) and one methionine tRNA gene (tRNAMet) (Fig. 8). GC content was 37.3% for both genotypes. All protein-coding genes had the start codon ATG. The stop codons were either TAG or TAA, except for the *Cox1* gene, which terminates with CTTT.

In comparison to *Eunicella cavolini* (GenBank KY559408.1), the widespread genotype showed a total of six single nucleotide polymorphisms (SNPs) and a single 49 bp deletion in the *nad5-nad4* intergenic region. Compared to *Eunicella cavolini* (GenBank KY559408.1), Lion Rock individuals have 12 SNPs, a 21 bp deletion and a separate 49 bp deletion both in the *nad5-nad4* intergenic region, as well as, a 2 bp insertion in the *atp6-atp8* intergenic region. A third individual (Lio06) from Lion Rock was sequenced that did not present the unique Lion Rock genotype, but shared that of the other 'standard' *E. verrucosa* sequenced. To test the validity of these unique polymorphisms, Sanger sequencing was carried out. Two SNPs, unique to the Lion Rock genotype, occurred in the *nad5-nad4* intergenic region and thus PCR amplification was carried out using

the primers developed in Chapter 2 of this thesis, NAD5-IGR-NAD4. Sanger sequencing *nad5-nad4* intergenic region confirmed these unique Lion Rock SNPs were not just error of the NGS library preparation or sequencing.

Table 5. Genetic distances between *Eunicella* mitogenomes

	E. verrucosa **(Bla04)**	***E. verrucosa*** **(Lio13)**	***E. cavolini***
E. verrucosa **(Bla04)**			
E. verrucosa **(Lio13)**	0.0727 (14)		
E. cavolini	0.0312 (6)	0.0624 (12)	

Genetic distances are presented as a percentage and the number of substitutions is given in brackets.

3.4.2. Phylogenetic analysis

A total of 21 complete mitogenome sequences were analysed to produce the *Eunicella* phylogenetic tree, of which 19 were derived from *E. verrucosa* newly sequenced in this study. Both maximum-likelihood and Bayesian inference consensus support values were greater than 95 for all nodes. The two Lion Rock individuals showing the unique genotype were resolved as being more distinct than *E. cavolini* to the other *E. verrucosa* individuals analysed (Fig. 9).

The holaxonian phylogenetic analysis used 18 concatenated sequences, containing all 14 mitochondrial protein-coding genes. In this analysis, mitogenome sequences derived from only two *E. verrucosa* individuals (Lio13 and Bla04) were used, representing the two distinct mtDNA genotypes observed in this study. Holaxonian mitochondrial genome sequences were

extracted from GenBank, details are outlined in Table 4. Maximum-likelihood and Bayesian inference trees both had the same topology and showed high support for the majority of nodes (Fig. 10 and 11). For both maximum-likelihood and Bayesian inference, consensus support values were >83 for all nodes, except a single node that connects *Muricea sp.* to the other *Plexuaridae* species studied.

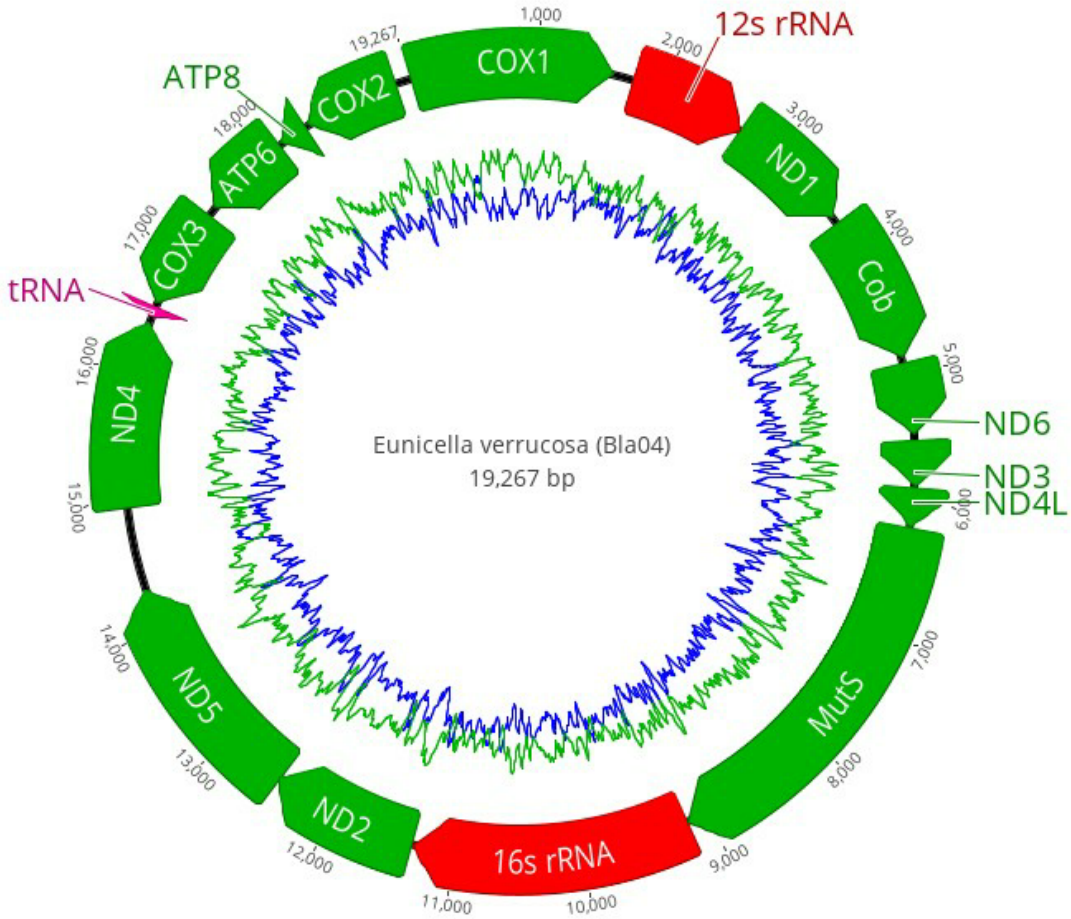

Figure 8. Depicts the fully annotated mitochondrial genome of *Eunicella verrucosa.* Inner circles shows GC-content and AT-content in blue and green, respectively.

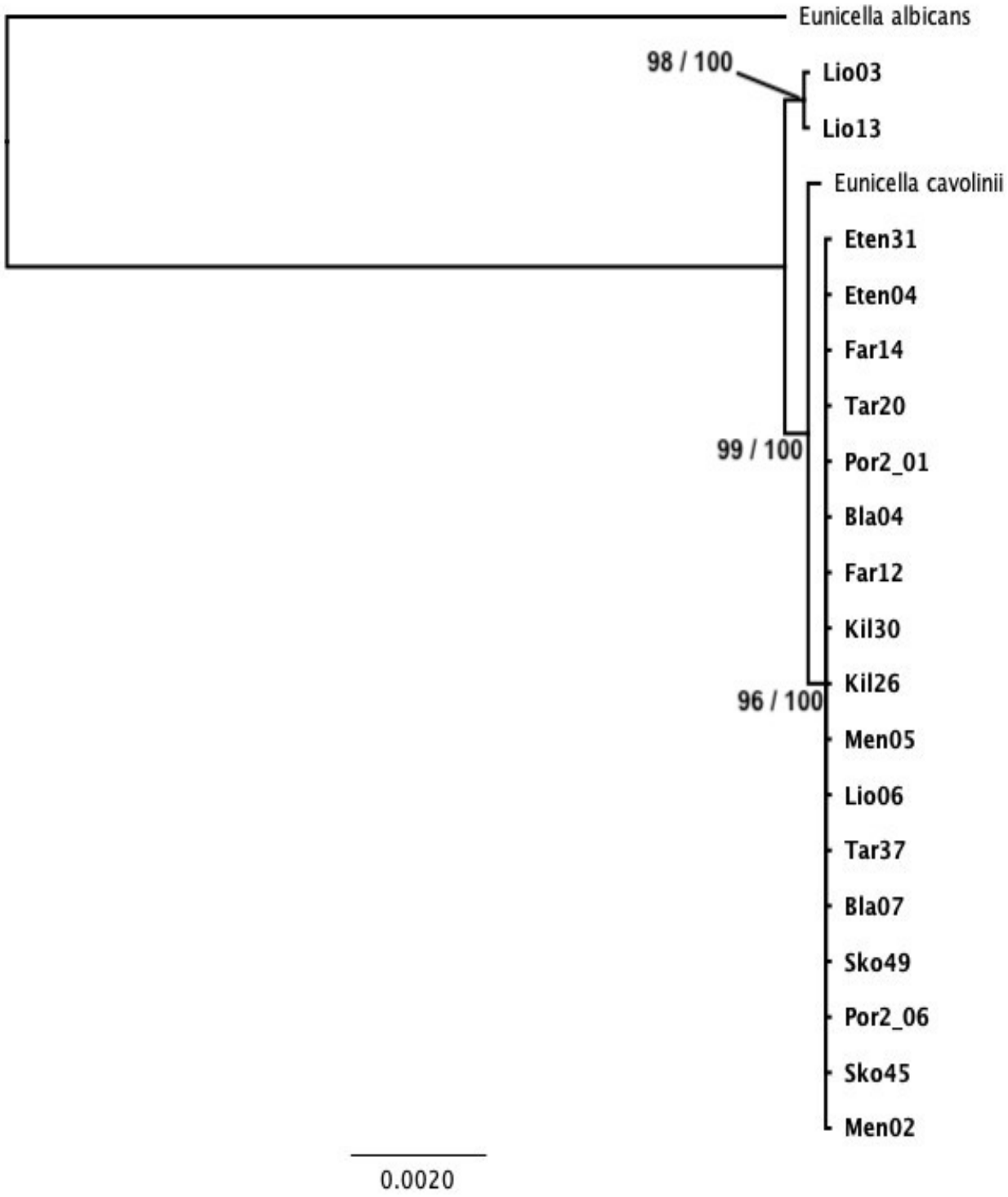

Figure 9. Phylogram constructed by maximum likelihood analysis of 21 complete *Eunicella* mitochondrial genomes. Both maximum likelihood (ML) and Bayesian inference consensus support values are shown on each major node (ML/Bayesian). Sequences derived from individuals sequenced in the current study are labelled in bold.

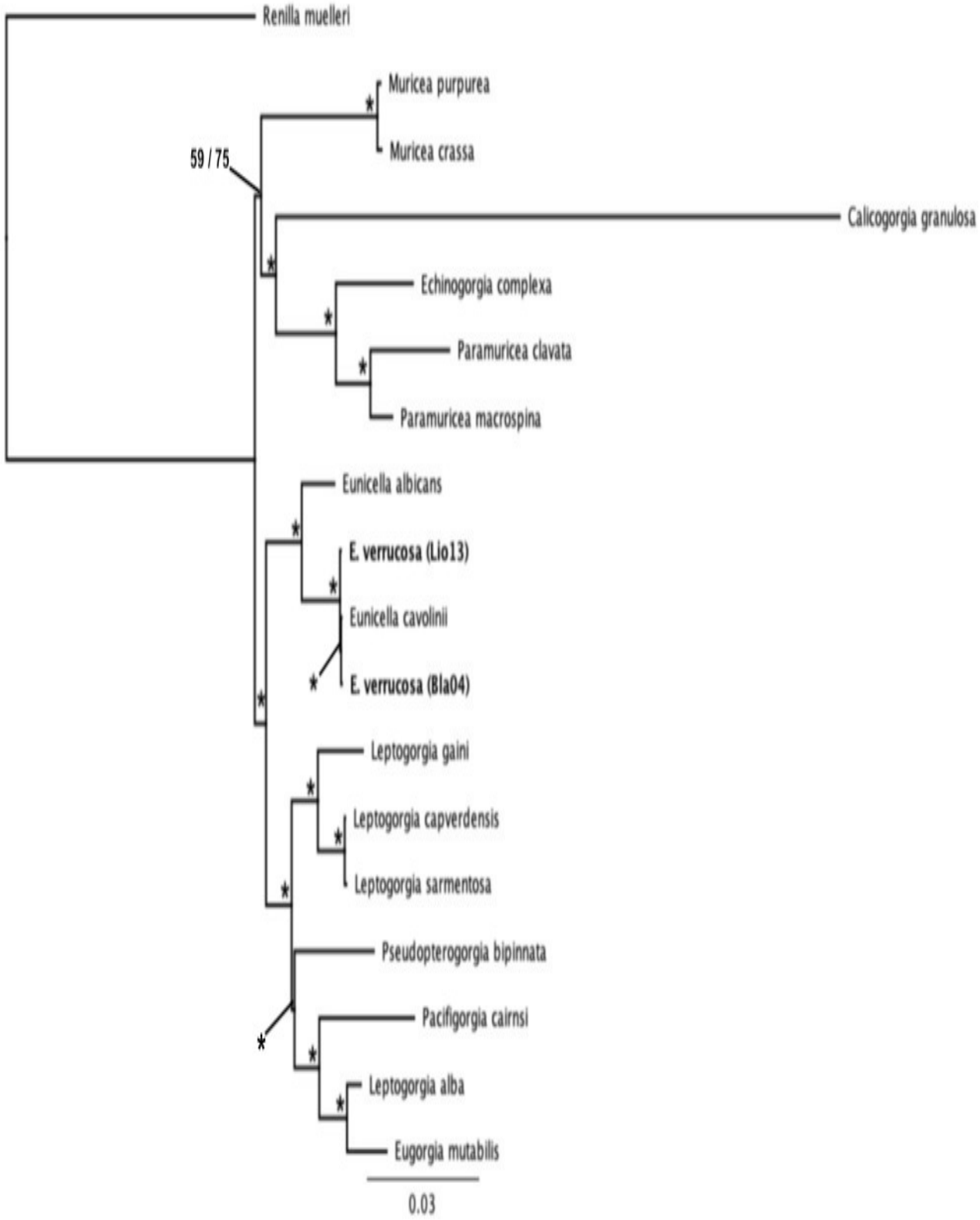

Figure 10. Phylogram constructed by maximum likelihood analysis of 18 Holaxonia mitochondrial genomes. All 14 mitochondrial protein-coding genes were extracted from complete mitochondrial genomes. The star symbol * indicates nodes with consensus support values of >70 for maximum likelihood and >90 for Bayesian inference if values are below ML and Bayesian inference values are stated (ML/ Bayesian). Sequences derived from individuals sequenced in the current study are labelled in bold.

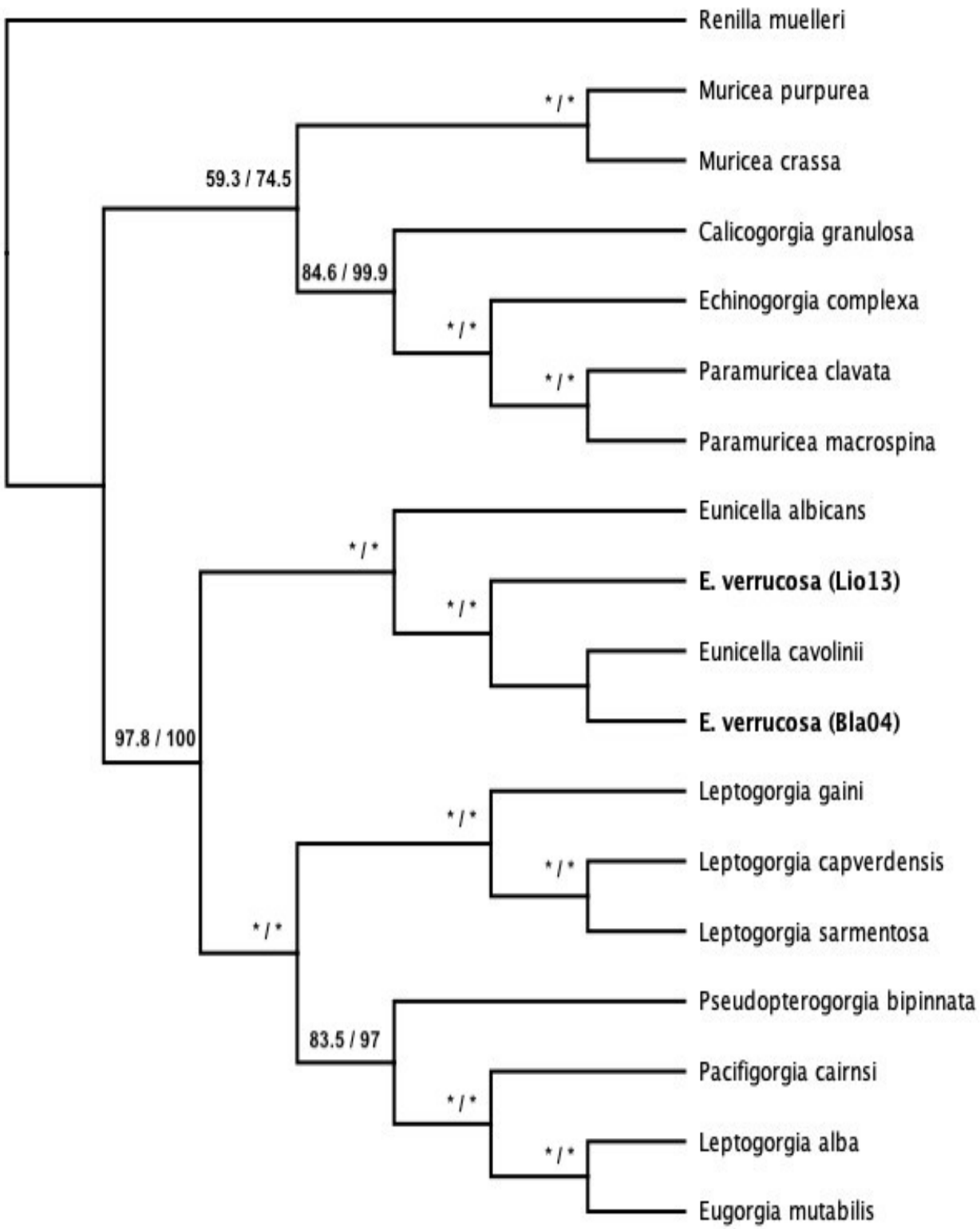

Figure 11. Cladogram constructed by maximum likelihood analysis of 18 Holaxonia complete mitochondrial protein-coding sequence. Both maximum likelihood (ML) and Bayesian inference consensus support values are shown above each node (ML/Bayesian). * / * indicates nodes with conesus support values of 100 for both ML and Bayesian inference analysis. Sequences derived from individuals sequenced in the current study are labelled in bold.

3.5. Discussion

3.5.1. *Eunicella verrucosa* mitochondrial genomes

The phi29 induced rolling circle amplification utilised in this study enabled the generally extremely low sequence variation occurring throughout the complete *Eunicella* mitochondrial genomes to be observed across a relatively large number of *E. verrucosa* individuals – 19 in total. Two distinct genotypes were observed, a widespread genotype and a distinct Lion Rock genotype that was only observed in two individuals. Throughout the 19,267 bp mitogenome, of the widespread *E. verrucosa* genotype, only 6 variant sites were found when comparing the widespread *E. verrucosa* mitogenome genotype to *Eunicella cavolini,* equating to a genetic distance of 0.03%. Anthozoans are acknowledged to have extremely low rates of mitochondrial evolution, with France et al. (1996) showing Hexacorallia to have a congeneric genetic distance of 16.1-26.3%, with that of Ocotocorallia being even lower at 2.7-6.3%, for the mitochondrial gene encoding 16S rDNA. However, France et al. (1996) only studied one segment of the mitochondrial genome. McFadden et al. (2011) provided a more comprehensive analysis, which included 75 individuals and three different segments of the octocoral mitochondrial genome, totalling 1844 bp. McFadden et al. (2011) showed an average within-genus pairwise genetic distance of 2.36% and an average intraspecific genetic distance of 0.16%. This intraspecific variation equates to five times the variation found between *E. verrucosa* and *E. cavolini,* in the current study. The members of *Eunicella* analysed in this study exhibit extremely low genetic variation, even when compared to the typically low variation observed in other octocoral mitogenomes (France et al. 1996; France and Hoover 2002; McFadden, Sánchez, and France 2010; Mcfadden et al. 2011).

Similarly, low genetic distances have been observed in *Leptogorgia,* Poliseno et al. (2017) found a genetic distance of <0.05% between *L. sarmentosa* and *L. capverdensis.* The study included a fossil-calibrated molecular dating analysis, where the authors suggested a relatively recent divergence of *L. sarmentosa* and *L. capverdensis,* occurring approximately 3 Mya. Both *Eunicella* and *Leptogorgia* are Atlantic-Mediterranean members of the octocoral family *Gorgonidae*. Logic suggests that the extremely low divergence observed in *Eunicella,* in this current study, is most likely also due to a recent diversification event between *E. verrucosa* and *E. cavolini*. As no molecular dating analysis was carried out in the current study, due to time constraints, no definitive estimation can be made on the divergence time between *E. verrucosa* and *E. cavolini*. However, a divergence time of 3 Mys or lower, would be inline with the divergence times estimated by Poliseno et al. (2017), and would place the divergence of *E. verrucosa* and *E. cavolini* after the most recent re-opening of the Strait of Gibraltar (5.96 - 5.33 Mya) (Krijgsman et al. 1999; Patarnello, Volckaert, and Castilho 2007; Garcia-Castellanos and Villaseñor 2011). A divergence after the re-opening of the Strait of Gibraltar would call into question the whether these species diverged via peripatric, parapatric or sympatric speciation.

Interestingly, two *E. verrucosa* individuals from Lion Rock, Isles of Scilly, southwest England (Lio03 and Lio13), showed a unique genotype, which differed by 14 substitutions from the widespread *E. verrucosa* genotype. This Lion Rock genotype showed a pairwise genetic distance with the widespread *E. verrucosa* of twice that observed when comparing of *E. cavolini* against the widespread *E. verrucosa* genotype (Table 5). Figure 9 shows the phylogenetic

tree produced from the alignment of *Eunicella* mitochondrial genomes, both maximum-likelihood and Bayesian inference show the same topology and resolve the Lion rock genotype as more distinct than *E. cavolini* to *E. verrucosa*, with strong consensus support (>95) for both the maximum-likelihood and Bayesian inference analyses. A third individual (Lio06) from Lion Rock was sequenced that did not present the unique Lion Rock genotype, but shared that of the other 'standard' *E. verrucosa* sequenced. This calls into question the validity of these sequences and whether sequencing error has occurred in the library preparation or sequencing process (Pfeiffer et al. 2018). As mentioned previously, Sanger sequencing of the *nad5-nad4* intergenic was carried out and confirmed these unique polymorphisms were not just error of the NGS library preparation or sequencing. Therefore, the evidence presented in this study suggests the two members of Lion Rock have a unique genotype, which is not seen in any other *E. verrucosa* studied to date.

To date, the study by Holland, Jenkins, and Stevens (2017) is the only research to assess the population structure of *E. verrucosa* in the Atlantic. These authors were able to identify regional population structure in *E. verrucosa* by using 13 microsatellite loci. In their study, a total of 22 individuals from the Lion Rock sample site were included, as well as 23 individuals from a separate sample site in the Isles of Scilly. With this comprehensive coverage, their study showed no distinction of Lion Rock samples compared to other samples from southwest Britain; this can be seen in both a principal component analysis (PCA) and a Bayesian iterative analysis of genetic variants among populations – a STRUCTURE analysis using the program STRUCTURE v2.3.4 (Pritchard et al., 2000).

Preliminary restriction site associated DNA sequencing (RAD-seq) analysis has been performed on *E. verrucosa* across their natural range (T. Jenkins, unpublished data). RAD-seq is a relatively new genome-wide method of analysis and has shown great potential in the field of population genetics. RAD-seq combines next generation sequencing (NGS) with enzymatic fragmentation of the genomic DNA to provide sequence data from sequences adjacent to the target cut sites (Baird et al. 2008). Ultimately, these sequences provide thousands of loci from across the genome, including both coding and non-coding DNA, where SNPs are identified and can be used to infer relationships within the study species (Cariou, Duret, and Charlat 2013). Preliminary restriction site associated DNA sequencing (RAD-seq) analysis of *E. verrucosa* suggests no distinction of the Lion Rock individuals (T. Jenkins, unpublished data). This RAD-seq analysis included 13 *E. verrucosa* samples from Lion Rock, including the individuals that showed the unique mitogenome in the current study (Lio03 and Lio13). In the preliminary RAD-seq analysis, individuals from the Lion Rock sample site showed no major distinction when compared to other British *E. verrucosa* samples; genetically, the two individuals, Lio03 and Lio13, showed no obvious distinction to the other Lion Rock samples analysed (T. Jenkins, unpublished data).

The Lion Rock specimens showing pairwise mtDNA-based genetic distances twice that of *E. cavolini,* when *E. cavolini* is compared to the widespread *E. verrucosa* mitogenome, could lead to it being inferred that the Lion Rock individuals are a distinct taxonomic entity, perhaps a separate species or subspecies. However, the nuclear, genome-wide, multiple loci data, produced

by both microsatellite analysis and the RAD-seq method, clearly show that the two Lion Rock individuals are in fact *E. verrucosa* (Holland, Jenkins, and Stevens 2017; T. Jenkins, unpublished data). This study shows the importance of comprehensive sampling and the need for multiple independent sources of evidence –the use of multiple genome-wide genetic markers– when inferring phylogenetic relationships. This is especially important in Octocorallia, a clade of species with extremely low levels of mitogenomic variation, where even minor levels of genetic variation can cause relationships to be inferred.

3.5.2. Holaxonia mitogenome phylogeny

Published octocoral mitochondrial genomes were successfully used to analyse the relationships within Holaxonia. The analysis used all 14 protein-coding genes from the mitochondrial genomes of 18 octocoral taxa; two mitochondrial genomes sequenced in this study were used to represent the two distinct *E. verrucosa* genotypes identified during this research. High levels of congruence of topology are apparent when comparing the Holaxonia mitogenome phylogeny (Fig. 10 and 11) to the *mtMutS* Holaxonia phylogeny (Fig. 5) presented in Chapter 2 of this study. However, the analysis of complete mitochondrial genomes (Chapter 3) showed a slight increase in consensus support for the majority of major nodes. The relative placement of all *Gorgonidae* members was the same, with distinct, highly supported *Eunicella* and *Leptogorgia* clades, while some previously ambiguous classifications were resolved by the use of whole mtDNA genomes. For example, the position of *Pseudopterogorgia bipinnata* within the *Leptogorgia* clade (which was unresolved in the *mtMutS* phylogeny) has been resolved in the mitogenome

phylogeny, with high consensus support of 83.5 and 97, respectively, in the maximum-likelihood and Bayesian inference analyses.

Until recently, the majority of octocoral phylogenies have been based on limited numbers of mitochondrial protein-coding genes (Sánchez et al. 2003; Herrera, Baco, and Sánchez 2010; McFadden, Sánchez, and France 2010). More than 10 years on, the phylogeny presented by McFadden et al. (2006), which utilised partial contigs of the mitochondrial genes *mtMutS* and *nad2,* is generally still considered the most comprehensive octocoral phylogeny available. Unfortunately, many of the deeper nodes of the McFadden et al. phylogeny were not resolved and a large amount of sequence data would be required to resolve these nodes (McFadden et al. 2006). Nonetheless, the power of complete mitochondrial genome sequences to resolve relationships in Octocorallia is evident (Uda et al. 2011; Poliseno et al. 2017), most notably in the study by Figueroa and Baco (2014). The latter authors used complete mitochondrial genomes to produce a tree with a high degree of congruence to that of McFadden et al. (2006), but with better resolution of previously unresolved deeper nodes. Often, however, octocoral mitogenome studies, such as these, are hampered by the lack of availability of published octocoral mitochondrial genomes; tellingly, Poliseno et al. (2017) suggest that currently only 1% of the 3,000 known octocoral species have publicly available mitochondrial genomes.

3.5.3 Implications

The extremely low variation found in members of the *Eunicella* genus has hampered any inference on phylogeographic relationships in *Eunicella*

verrucosa, in the current study. However, even when compared to the lack of mitochondrial sequence variation seen in other Octocorallia species, the level of variation observed in this study is extremely low, providing an interesting subject for further evolutionary research. For example, if estimations of the divergence time for *Eunicella* taxa are comparable to the *Leptogorgia* species studied in Poliseno et al. (2017), this raises an interesting question regarding the mechanism of divergence between *Eunicella verrucosa* and *Eunicella cavolini*. Further research is needed in this area to produce an accurate estimate of their divergence times. However, as observed in the current study, the extremely low genetic distances observed between the complete mitochondrial genomes of *E. verrucosa* and *E. cavolini,* would suggest that no area of the mitochondrial genome has sufficient variation to accurately and unambiguously define the evolutionary relationship between these two *Eunicella* species.

Moreover, the genetic differences observed between the Lion Rock genotype and the widespread *E. verrucosa* genotype exemplifies why researchers cannot rely on a single locus to infer taxonomic and evolutionary relationships. The mitochondrial genome replicates as a single unit and a single-locus tree can present a biased inference of the evolutionary history of the species being studied due to the effect of selection or the stochastic nature of coalescence processes (Ballard and Whitlock 2004; Galtier et al. 2009; Drovetski et al. 2015; Hung, Drovetski, and Zink 2016). In a species group such as Octocorallia, that has low levels of mitochondrial gene variation, the bias of using only a single locus might be amplified, as even a relatively small stochastic variation in the

mitochondrial sequence may lead to invalid inferences regarding relationships within the group.

Nonetheless, the breadth of sequence data that complete mitochondrial genomes can provide makes them valuable in providing insights into the deeper nodes of the Octocorallia and in resolving previously unresolved nodes (Figueroa and Baco 2014). This was exemplified in the current study, where mitochondrial genomes increased consensus support for the majority of nodes, as well as resolving several previously unresolved nodes – unfortunately, as discussed above, only 1% of the known members of Octocorallia have publicly available mitochondrial genomes (Poliseno et al. 2017).

Finally, although this study has focused primarily on sequencing samples of *E. verrucosa,* it has also highlighted the potential of the RCA method to sequence complete mitochondrial genomes in a cost effective and highly efficient manner. The RCA method has a minimal hands-on laboratory time of just 20 minutes, with no need to optimise thermal cycles and it is also a highly scalable process (Johne et al. 2009). RCA in combination with NGS tools mean that it is now possible to produce a relatively large number of complete mitochondrial genomes quickly and cheaply, with an average cost of less than £200 per mtDNA genome in the current study.

Chapter 4 - General Conclusion

Gorgonian octocorals, like *Eunicella,* are pivotal members of the marine ecosystem, as their three-dimensional structures provide vital habitat for a range of other organisms and thus, are considered ecosystem engineers (Buhl-Mortensen et al. 2010). Unfortunately, they have also been affected by multiple mass mortality events, which have been linked to thermal anomalies (Cerrano et al. 2000; Martin, Bonnefont, and Chancerelle 2002; Cupido et al. 2012). Members of this genus are also vulnerable to anthropogenic impacts, for example, the IUCN redlist species *Eunicella verrucosa* being subject to damage from bottom trawling fishing gear (Hall-Spencer et al. 2007). Phylogenetic studies into the relationships between threatened gorgonian octocorals are vital to inform conservation effort, as the conservation of ecosystem engineers, like *Eunicella,* can have a broader positive impact on other species (Boogert, Paterson, and Laland 2006). However, phylogenetic studies of the Anthozoan sub-class Octocorallia have been severely hampered by the lack of variation shown in their mitochondrial genome (McFadden, Sánchez, and France 2010). This lack of variation is highly evident in *Eunicella,* such that, previous studies have shown no variation in the mitochondrial protein-coding gene *cox1* gene between these European members of *Eunicella* (Calderón, Garrabou, and Aurelle 2006).

This study aimed to explore the mitochondrial genomes of *E. verrucosa, E. cavolini* and *E. singularis* to identify any genetic variation, allowing us to assess better and understand the phylogenetic relationships within *Eunicella,* and their relationship to other members of the octocoral sub-order Holaxonia.

As previous molecular markers have been unsuccessful in differentiating *Eunicella spp.* novel primers were designed in chapter 2 to target areas of the mitochondrial genome suspected to have the highest levels of variation. Mitochondrial DNA was targeted as the ease of amplification means the molecular markers developed in this study could be widely applied by researchers to inform conservation actions.

In this study, the variation observed in *the nad5-nad4* intergenic region enabled us to differentiate between the studied *Eunicella species*; *E. verrucosa, E. cavolini* and *E. singularis*. Although the variation was limited, this is one of the first studies to identify molecular variation between the mitochondrial genomes of these three *Eunicella* morphospecies. The variation found in this intergenic region demonstrates its potential as a genus-specific character-based genetic barcode. Character-based barcoding methods have been shown to have greater success, than the more common, distance-based methods when identifying recently diverged species, as they don't rely on an arbitrary threshold gaps between intraspecific and interspecific variation (Bergmann et al 2009; Velzen et al 2012). Due to the low levels of variation observed between the studied *Eunicella* species a binary barcoding method, like a character-based barcode, would allow for a more robust criterion on which to define the studied *Eunicella* species on (Desalle, Egan, and Siddall 2005; Chakraborty, Dhar, and Ghosh 2017). Moreover, it will allow for the combination of the scarce molecular and morphological data, and in turn, allow us to better conserve these species.

The variation found in this intergenic region demonstrates its potential as a genus-specific character-based genetic barcode. A binary barcoding method, like a character-based barcode, would allow for a more robust criterion on which to define these *Eunicella* species on (Desalle, Egan, and Siddall 2005;

Chakraborty, Dhar, and Ghosh 2017); allowing us to combine the scarce molecular and morphological data, and in turn, allow us to better conserve these species.

Interestingly, two *E. verrucosa* genotypes were observed. The widespread genotype was found in 17 *E. verrucosa* individuals and was present in individuals from all sampling sites. The unique Lion Rock genotype was only observed in two individuals collected from Lion Rock, Isle of Scilly, Britain. Interestingly, when comparing these findings to previous microsatellite (Holland, Jenkins, and Stevens 2017) and preliminary RAD-seq data (T. Jenkins, unpublished data), which included these Lion Rock individuals, there was no suggestion that these Lion Rock individuals differentiated from the other British *E. verrucosa* surveyed. Inline with their studies, the current study highlights the importance of using multi-loci data, when inferring relationships within Octocorallia and not just relying on the single-locus data mitochondrial sequences produce (Hung, Drovetski, and Zink 2016).

The analysis undertaken in both Chapters 2 and 3 produced highly congruent Holaxonia phylogenies. Partial contigs of *mtMutS* were used to produce the Holaxonia phylogeny in Chapter 2; this gene was targeted as it has shown to have twice the levels of variation when compared to other octocoral mitochondrial protein-coding genes (Mcfadden et al. 2011). The Holaxonian phylogeny produced in Chapter 3 utilised the sequence of all 14 protein-coding genes, extracted from complete mitogenomes. Former taxonomic assignment placed *Eunicella* into the family *Plexauridae* before they were reassigned to *Gorgonidae* (Bayer 1961); both Holaxonia phylogenies, produced in this study,

support the reassignment of *Eunicella* to the *Gorgonidae* family, in line with findings by Poliseno et al. (2017). Both Holaxonia trees produced, in this study, show highly congruent topologies, but the increased sequencing breadth in the complete mitogenome phylogeny of Chapter 3 resolved the position of *Pseudopterogorgia bipinnata*. The analysis in chapter 3 also showed increased consensus support for the majority of nodes. This study has shown the advantage complete mitogenomes can have over single protein-coding genes in the analysis of relationships within Octocorallia, as has been shown in other studies (Uda et al. 2011; Figueroa and Baco 2014; Poliseno et al. 2017).

Complete mitogenomes have been shown to increase the resolution of octocoral phylogenies (Uda et al 2011; Figureoa and Baco 2014). However, only 1% of known octocorals have currently published mitochondrial genomes (Poliseno et al 2017). The phi29-RCA amplification method, implemented in Chapter 3, has shown its ability to produce mitochondrial genomes cost-effectively with minimal hands-on laboratory time. Thus, showing its potential as a useful tool for octocoral phylogeneticst, as it allows for the production of large quantity of octocoral mitochondrial genomes, in an cost effective, efficient and highly scalable manor.

In this study, genetic variation in the mitochondrial genomes of *E. verrucosa, E. cavolini* and *E. singularis* was successfully explored. The *nad5-nad4* intergenic region was highlighted as a potential candidate for a genus-specific character-based barcode for *Eunicella*. The ability of phi29-RCA to produce a large number of mitochondrial genomes was shown. The complete mitochondrial genomes of *E. verrucosa* showed extremely low divergence from *E. cavolini,*

suggesting a recent divergence of the two species and raising the question of their diversification. Complete mitochondrial genomes were shown to increase the resolution of phylogenies compared to partial contig of the protein-coding gene, *mtMutS*.

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