Speciation, Evolution and Phylogeny of some Shallow-water Octocorals (Cnidaria: Anthozoa)

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vorgelegt von
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“Ipse manus hausta victrices abluit unda, anguiferumque caput dura ne laedat harena, mollit humum foliis natasque sub aequore virgas sternit et inponit Phorcynidos ora Medusae. Virga recens bibulaque etiamnum viva medulla vim rapuit monstri tactuque induruit huius percepitque novum ramis et fronde rigorem. At pelagi nymphae factum mirabile temptant pluribus in virgis et idem contingere gaudent seminaque ex illis iterant iactata per undas: nunc quoque curaliis eadem natura remansit, duritiam tacto capiant ut ab aere quodque vimen in aequore erat, fiat super aequora saxum”
(Ovidio, *Metamorphoseon* 4, 740-752)

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Acknowledgements

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Summary

Shallow-water octocorals are among the most abundant macro-benthic organisms inhabiting tropical subtropical and temperate communities. In spite of being worldwide distributed and highly diverse, the systematics of many octocorals remains controversial and the understanding of the processes who led to their diversification is largely unexplored. This study includes five chapters, each dealing with different aspects of the systematics, phylogeny and evolution of six soft coral genera such as *Lobophytum, Sarcophyton, Paramuricea, Leptogorgia, Muricea* and *Pacifigorgia*. One of the main goals of the study was to explore, through the use of molecular methods, the genetic variation within species notoriously difficult to identify. Besides the use of standard molecular methods for phylogenetic reconstruction and species delimitation, the effectiveness of Next Generation Sequencing (NGS) technologies was tested for mitogenomic and genotyping analyses.

In the first chapter the use of single-locus markers (e.g. *COI, mtMutS* and 28S rDNA) was investigated and different automated species delimitation methods (e.g. ABGD, bPTP) were employed to assess species richness among soft coral genera from Western Australia. The methods used appeared suitable for preliminary and rapid diversity assessments especially in the presence of species-rich genera such as *Lobophytum* and *Sarcophyton* where morphological identification is particularly difficult and time consuming.

In the second chapter, along with the sequencing of complete mitogenomes of Mediterranean *Paramuricea* species (*P. clavata* and *P. macrospina*), the biogeography of the genus was investigated. The results revealed nucleotide and genome size polymorphisms, while the biogeographic predictions suggested that the Mediterranean species have resulted from independent speciation events, explaining in part the high phylogenetic divergence detected.

In the third chapter, the sequencing of complete mitogenomes of five *Leptogorgia* species from different geographic areas (eastern Pacific, eastern Atlantic and Mediterranean) was followed by phylogenetic reconstructions based on an extended *mtMutS* dataset. The phylogenetic tree recovered *Leptogorgia* polyphyletic with a clear segregation between the eastern Pacific and eastern Atlantic forms. A time calibrated phylogeny provided insights into the evolution of the genus.

In chapter four, using NGS approaches, the complete mitochondrial genome of two eastern Pacific *Muricea* species (*M. crassa* and *M. purpurea*) has been sequenced. The recovery of complete mitogenomes allowed to evaluate the presence of variable and informative regions and to infer a more robust phylogeny. Overall, the results showed high nucleotide diversity in the intergenic spacers, making these regions new potential molecular markers for species-level identifications.

In the last chapter a genome-wide Single Nucleotide Polymorphisms (SNPs) and a Bayes Factor Delimitation method were used to infer the genetic relationships within species of the genus *Pacifigorgia*. The data obtained showed incongruence between molecular and morphological investigations suggesting the possibility of alternative taxonomic assignments for these species.

This study provides information on the evolution and speciation of ecologically important soft corals, which distribution range from the littoral and sublittoral zones of the Mediterranean to the tropical and subtropical reefs of Western Australia (WA) and eastern Pacific (EP). The use of mitochondrial markers such as *MutS* allowed to shed some light on the biogeography and evolutionary history of widespread gorgonians with special emphasis on the Mediterranean endemics and the Atlantic species. Concerning the Western Australia, the obtained results will support the management and conservation of under-investigated marine biodiversity hotspots and potentially species-rich localities such as the Kimberley. In terms of species delimitation, the application of genome-wide SNPs and the use of NGS technologies showed a higher resolution when compared with the traditional methods based on DNA barcoding and single-locus phylogenies. The data generated have been used to clarify the systematics of the species investigated and will be considered as a baseline for future studies on population genetics with a closer look on the adaptive processes.

Introduction

Octocorallia: general information

The subclass Octocorallia (Cnidaria, Anthozoa) includes more than 3000 species (Daly *et al.* 2007; Cairns, 2007) and is further divided in three orders: Alcyonacea (Lamouroux, 1812), Helioporacea (Bock, 1938) and Pennatulacea (Verrill, 1865). All octocorals typically have polyps with eight pinnulate tentacles and a gastro-vascular cavity (coelenteron) divided by complete mesenteries into eight compartments (Figure 0.1). Within alcyonaceans, the majority of the species are colonial, which means that the polyps of a single individual are linked by protoplasmic connections (gastro-vascular canals) and each polyp is connected to the coelenteron allowing constant water circulation. The gastro-vascular canals are surrounded by an organic matrix, which forms the coenenchyme. This tissue is covered by the ectoderm and is further connected to the polyps. The gastro-vascular canals, coenenchyme and polyps constitute the cenosarc (Figure 0.1).

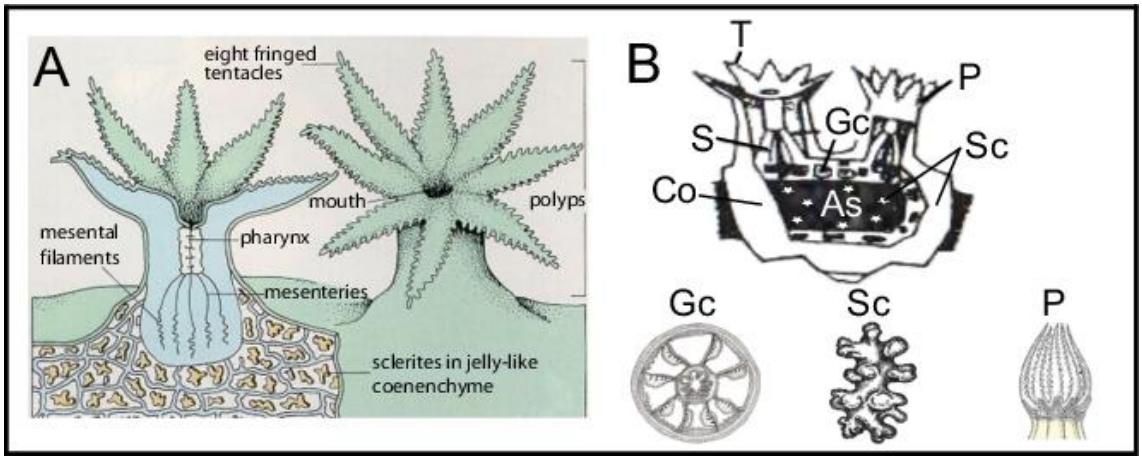

Figure 0.1: Schematic view of octocoral anatomy. A: longitudinal section (from Reader's Digest, 1990). B: Octocoral structure with the principal anatomical components, As: axial skeleton; Co: cortex; Gc: gastrovascular cavity; P: polyp; S: septa; Sc: sclerites; T: tentacle (modified from Luther and Fiedler, 1988).

Alcyonaceans have been divided into six subordinal groups (Alcyoniina, Protoalcyonaria, Stolononifera, Calcaxonia, Holaxonia and Scleraxonia) according to the presence/absence of central axis, the presence of small calcium carbonate ($CaCO_3$) components (sclerites) and the consistence of the coenenchyme. Among the suborders, members of Alcyoniina are commonly known as soft corals due to the lack of rigid

skeletons and the presence of calcareous sclerites in the tissues. Protoalcyonaria includes a restricted number of species mainly distributed in the deep-sea. These organisms are characterised by a cylindrical soft body, which base is enriched in elastic filaments that allow anchoring to the substrata. Stolonifera, on the other hand, comprises more than 150 species characterised by big pinnulate polyps connected by exclusive elastic canals called stolons and in some species, like *Tubipora musica*, sclerites may be fused to form a rigid calcareous skeleton. Members of the suborders Calcaxonia, Holaxonia and Scleraxonia are also known as gorgonians due to the fact that they were formerly assigned to Order Gorgonacea (Lamouroux, 1816). All gorgonians have a colony axis separated into two distinct portions: (1) coenenchyme and (2) sclerax. The sclerax is further divided into an inner portion (medulla) and an outer part (cortex), which is made of gorgonin and provides flexibility to the colony. The cortex can be reinforced by $CaCO_3$ components such as concentric lamellae, solid calcareous material or simply by sclerites (Figure 0.2).

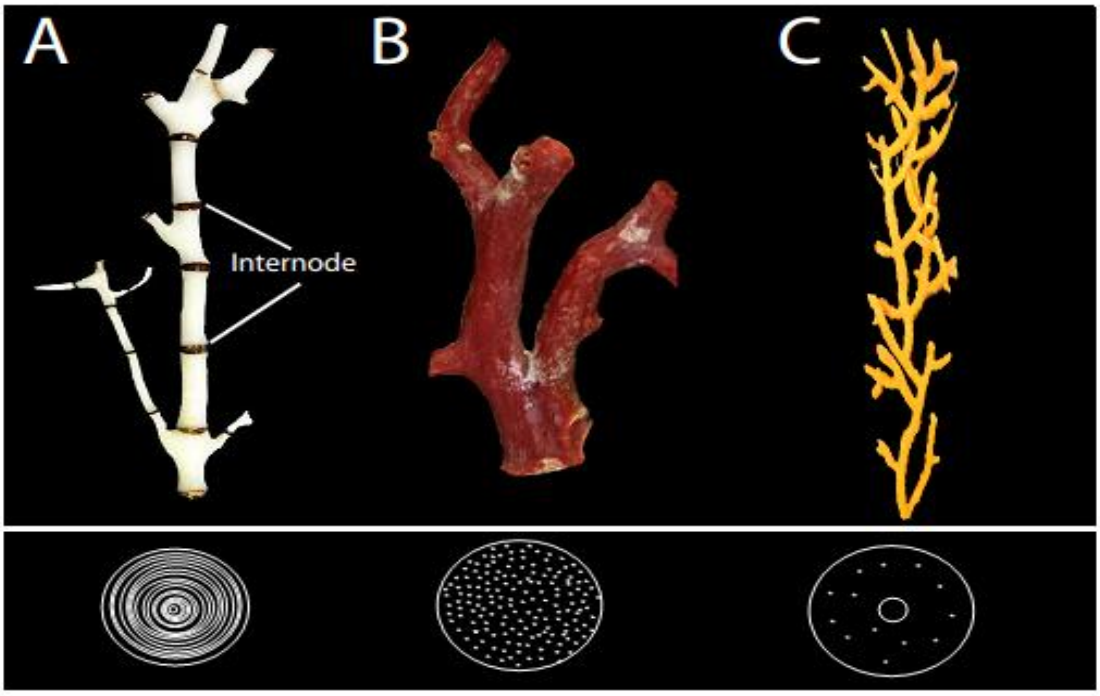

Figure 0.2: Morphological structure of the colony and skeleton in different gorgonian suborders. A: bamboo coral (SO: Calcaxonia) (copyright of the Mountains in the Sea Research Team; IFE; and NOAA), skeleton characterised by $CaCO_3$ concentric lamellae with nodes made by gorgonin; B: *Corallium rubrum* (SO: Scleraxonia), skeleton characterised by the presence of fused sclerites; C: *Leptogorgia capverdensis* (SO: Holaxonia) skeleton composed of gorgonin and characterised by the presence of rare sclerites and a chambered central core without concentric lamellae. On the bottom side of the figure, schematic transversal section of the skeleton.

Unlike alcyonaceans, helioporaceans have a massive skeleton whose composition is similar to that of scleractinians. The order Helioporacea, formerly called Coenothecalia

(Bourne, 1895), includes only few species geographically restricted to the tropics. Among these, *Heliopora coerulea* (Pallas, 1776) and the recently described *Nanipora kamurai* (Miyazaki and Reimer, 2015) have a massive aragonite skeleton and are characterised by the lack of sclerites. The order Pennatulacea includes organisms commonly known as sea pens due to their body shape, which resemble quill pens. Overall, the central stalk of these organisms consists of a lower portion (peduncle) that anchors the colony to the soft bottom and an upper part (rachis) including polyps or branches bearing polyps. These organisms have a broad distribution and can be found down to deep-sea waters up to 7,000 m (Williams, 2011).

Origin of octocorals and fossil records

Recent multidisciplinary studies of putative fossils (Maloof *et al.* 2010; Brain *et al.* 2012), "ancient" biomarkers (see Cryogenian fossil steroids in Love *et al.* 2009) and molecular clocks (Erwin *et al.* 2011) agree on a pre-Ediacaran (1300-600 Mya) origin of metazoans. Within cnidarians, Menon *et al.* (2013) found discoidal fossils resembling modern scyphozoans in the ca. 560 Mya Fermeuse Formation of Newfoundland (Canada) and proposed that their appearance may be dated back to the Ediacaran (635-541 Mya). In the class Anthozoa, soft body organisms such as octocorals have high post-mortem degradation and their presence in the fossil records is infrequent (Schlagintweit and Gawlick, 2009; Whittle *et al.* 2014). Although fossils may potentially show how species have changed across space and time providing evolutionary insights, their identification is difficult and is often restricted to high taxonomic levels (Bayer, 1956; Kokurco and Kokurco, 1992). In this respect, the earliest known octocoral (*Echmatocrinus brachiatus*) was found in the third series of the Cambrian (509-500 Mya) but its taxonomy is to date widely debated (see Sprinkle, 1973; Sprinkle and Collins, 1995, 1998; Ausich and Babcock, 1998, 2000). According to recent paleontological findings (see Cope, 2005), the earliest undisputed octocoral fossil is an alcyonacean of the genus *Petilavenula*. This was found in the Ogof Hên Formation (UK) and was dated back to the Lower Ordovician (480-475 Mya). Other alcyonacean fossils were also described from the upper Landoverian to the lower Wenlockian (435-430 Mya) and from the Campanian-Maastrichtian (83-66 Mya) (see Bengston, 1981; Reich and Kutscher, 2011). In terms of diversity, the fossil assemblage discovered from the Red Bluff Formation in Mississippi (Lower Oligocene, 28.4-33.9

Mya) represents one of the most diverse collections with about 1000 well-preserved sclerites found from a single location (Kocurko and Kocurko, 1992). Among others, fossils of blue corals (Lozouet and Molodtsova, 2008) and sea-pens (Reich and Kutscher, 2011) have been also described in the last decades (Figure 0.3). For instance, fossils of *Epiphaxum* (Lonsdale, 1850) were found from the Aquitane Basin in South-West of France and their dating ranges from 37 to 16 Mya (Lozouet and Molodtsova, 2008). Another helioporacean, *Heliopora coerulea*, was known form the early Cretaceous (145-100 Mya) and its general morphology endured unchanged for more than 100 million years, making this species a perfect keystone for evolutionary studies.

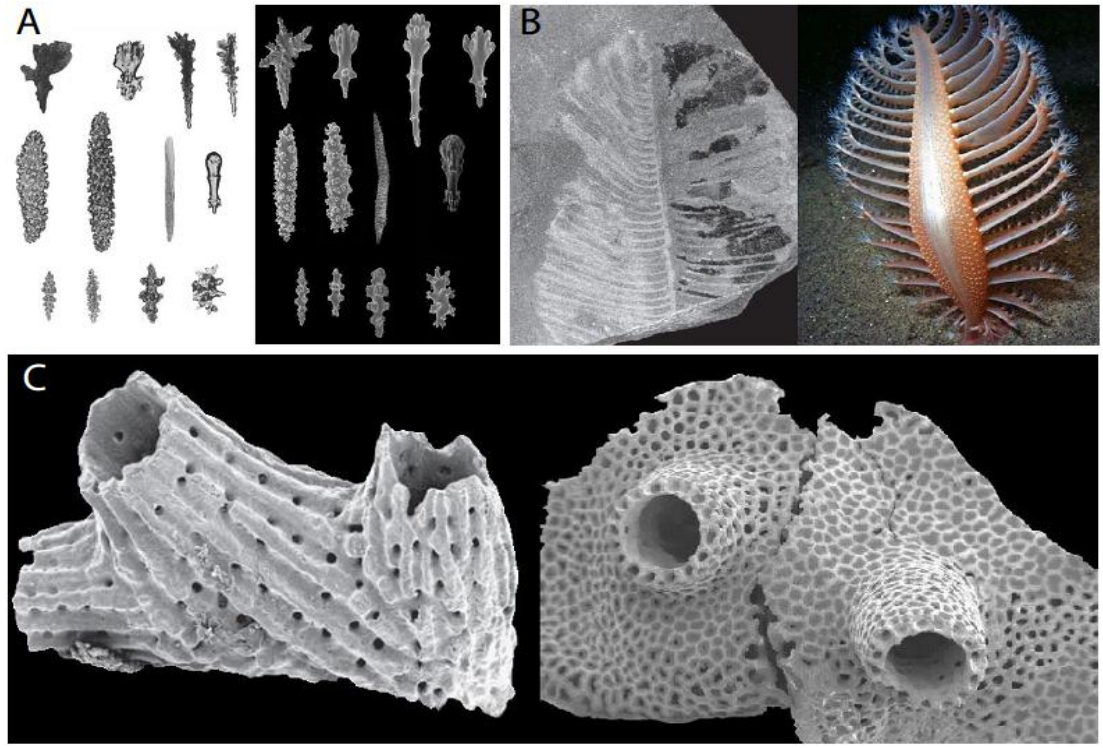

Figure 0.3: Fossil octocorals (left side of each panel) and comparison with the modern forms (right side of each panel). A: sclerites of different plexaurids and gorgoniids (modified from Kokurco and Kokurco, 1992); B: Pennatulacea, left: *Pennalina* sp. (Cope, 2005), right: extant sea pen (photo of Mr. Vittorio Durante); C: aragonite skeleton of helioporaceans, from the left: *Epiphaxum arbuscula* (modify from Lozouet and Molodtsova, 2008) and *Nanipora kamurai* (from Miyazaki and Reimer, 2015).

Pywackia baileyi, dated back to the late Cambrian (497-485 Mya), was supposed to be a precursor of the Pennatulacea lineage (Taylor *et al.* 2013), while Landing *et al.* (2015) proposed for this species a high affinity with the phylum Bryozoa (Ehrenberg, 1831). Due to their body shape, pennatulid-like fossils have been often misinterpreted as belemnites (Reich and Kutscher, 2011), therefore their origin is still debated. Among the few undisputed records, fossils of Pennatulacea were described from the Campanian (72-84 Mya) and several traces of calcareous sclerites were described from the upper Cretaceous (Helm and Schülche, 2003).

Ecology and symbioses

Octocorals have a worldwide distribution and a broad bathymetric occurrence ranging from intertidal to abyssal waters (Bayer, 1961). These organisms are among the most abundant components of littoral benthic communities in tropical and temperate seas (True, 1970; Loya, 1972) and can be ecologically important members of coral reefs (Dinesen, 1983; Fabricius and Alderslade, 2001). Octocoral communities play a key-role in trophic ecology influencing coastal biogeochemical cycles (Cocito *et al.* 2013; Leal *et al.* 2014). Indeed, thanks to the interception of organic and particulate matter and due to the release of metabolic products such as mucus, they may affect the carbon and nitrogen cycle in the water column (Gili and Coma, 1998; Wild *et al.* 2004). Although many octocorals are suspension feeders—organisms feeding on material suspended in the water column—their feeding strategies may be linked to both physiological and environmental aspects (Lasker *et al.* 1983; Ribes *et al.* 1999). For instance, some gorgonians capture zooplanktonic preys (Leversee, 1976; Lasker, 1981; Coma *et al.* 1994), whereas members of the genus *Sarcophyton* are capable of using food sources produced by symbiotic zooxanthellae (e.g. photosynthates) (Muscatine and Hand, 1958). Beside *Sarcophyton* species, several calcaxonian, holaxonian, scleraxonian and stoloniferan species live in symbiosis with unicellular photosynthetic dinoflagellates of the genus *Symbiodinium*, commonly known as zooxanthellae (see van Oppen *et al.* 2005). *Symbiodinium* includes at least nine (A–I) divergent clades each with multiple subclades (Stat *et al.* 2006). Specific *Symbiodinium* clades associated with a particular coral apparently affect its growth rate and its tolerance to thermal stress (Jones *et al.* 2008; Yuyama and Higuchi, 2014). Therefore, the study of the diversity of *Symbiodinium* represents the first step towards understanding the ecological importance of the host-symbiont association and the impact of symbiont lost. In particular, the loss of photosymbionts due to changes in environmental conditions (e.g. heat stress, light stress, CO_2 increment) represents a serious threat to reef communities as it negatively affects the host health and may lead to the loss of critical habitat for coral associated biota (Coffroth *et al.* 2010).

Unlike octocoral-algal symbiosis, which has been widely investigated, the microbial assemblage and their diversity in octocorals is poorly known. Preliminary studies on the bacteria community in the Caribbean gorgonians revealed high diversity and the predominance of Gammaproteobacteria (Brück *et al.* 2007; Santiago-Vázquez *et al.*

2007). Assuming that environmental factors can directly or indirectly affect the microbiota and/or the host physiologies, variation in microbial diversity should be further investigated exploring the effects of climate changes on holobionts.

Reproductive strategies

Both, asexual and sexual reproduction are known in octocorals, however most of the species reproduce only sexually (Kahng *et al.* 2011). Although hermaphroditism has been observed in some soft corals (Benayahu, 1997), most of the species are gonochoristic and they can basically reproduce in two different ways: (1) broadcast spawning and (2) brooding. The second strategy may be further divided into internal fertilization with the planulae brooded in specific compartments in the endoderm and internal fertilization with planulae brooded in the external surface of the polyps. The reproductive strategies used by different octocorals seem to vary among species and according to the environmental conditions (e.g. tropical, temperate). Interestingly, Coll *et al.* (1995) documented that within gorgonians, brooding strategies are preferred to spawning, which is instead quite common among alcyoniids. Independently from the strategy adopted, the spawning of eggs into the water column or the release of planulae allows larvae dispersion. It has been shown that larvae may require up to a week before settling (Babcock and Heyward 1986; Harrison and Wallace, 1990) and that the competency period —span of time before larvae settle onto substrata— may last for several weeks (Wilson and Harrison, 1998). Although brooding species have a shorter competency period (Harrison and Wallace, 1990) and a limited recruitment range (Ayre and Dufty 1994; Benzie *et al.* 1995), variations in larva dispersion may be linked to physiological and reproductive aspects and to the different environmental conditions of each habitat.

Classification and systematics

Classification and identification of octocorals is traditionally based on different morphological aspects of the colony including: (1) the shape and size, (2) pattern of branching, (3) distribution of the polyps, (4) structure of the axis and (5) colour (Bayer, 1961). However, for classification at higher taxonomic ranks (e.g. order, suborder) additional histological features of the coelenteron and the sclerax are also considered.

Except for sea-pens (Pennatulacea) and blue corals (Helioporacea), which were assigned to different orders since the beginning of the last century (Hickson, 1906), the majority of octocorals (soft corals and gorgonians) underwent different taxonomic revisions. For instance, Kükenthal (1925) and Medsen (1944) divided the soft corals and gorgonians into two and six orders respectively, while Hickson (1930) split them into four orders (Alcyonacea, Gorgonacea, Stolonifera and Telestacea). Later Bayer (1981) recognized a three-order system (Alcyonacea, Helioporacea and Pennatulacea) that is currently followed by most octocoral taxonomists (Fabricius and Alderslade, 2001). Based on the skeletal structure and composition, alcyonaceans have been further divided into six sub ordinal groups (Alcyoniina, Calcaxonia, Holaxonia, Protoalcyonaria, Scleraxonia and Stolonifera) but their taxonomic separation has not been corroborated by molecular analyses yet (Berntson *et al.* 2001; McFadden *et al.* 2006).

Species-level identification in octocorals is not a trivial procedure and is often hampered by the lack of diagnostic characters (Bayer, 1961; Sánchez, 2007) and the high morphological homogeneity (Prada *et al.* 2008) (Figure 0.4). The most suitable characters employed to distinguish octocoral genera and species is the study of the form of sclerites (Bayer, 1956, 1961). Among taxonomists, Valenciennes (1855) was the first to investigate sclerite diversity for species identification, later Kölliker (1865) confirmed the value of this character and proposed a new classification scheme based on the presence/absence and the shape of the different sclerites. Surprisingly, after 150 years and despite the use of new integrative approaches, the comparative assessment of sclerite diversity for species-level identification remains a valid strategy among octocoral taxonomists. Nevertheless, the use of DNA barcoding —identification of biological species by comparing different DNA sequences chosen from a standard region of the genome— became an alternative as well as a compulsory method for a better investigation of the taxonomic relationships within morphologically difficult groups (McFadden *et al.* 2006, 2014; Vargas *et al.* 2014).

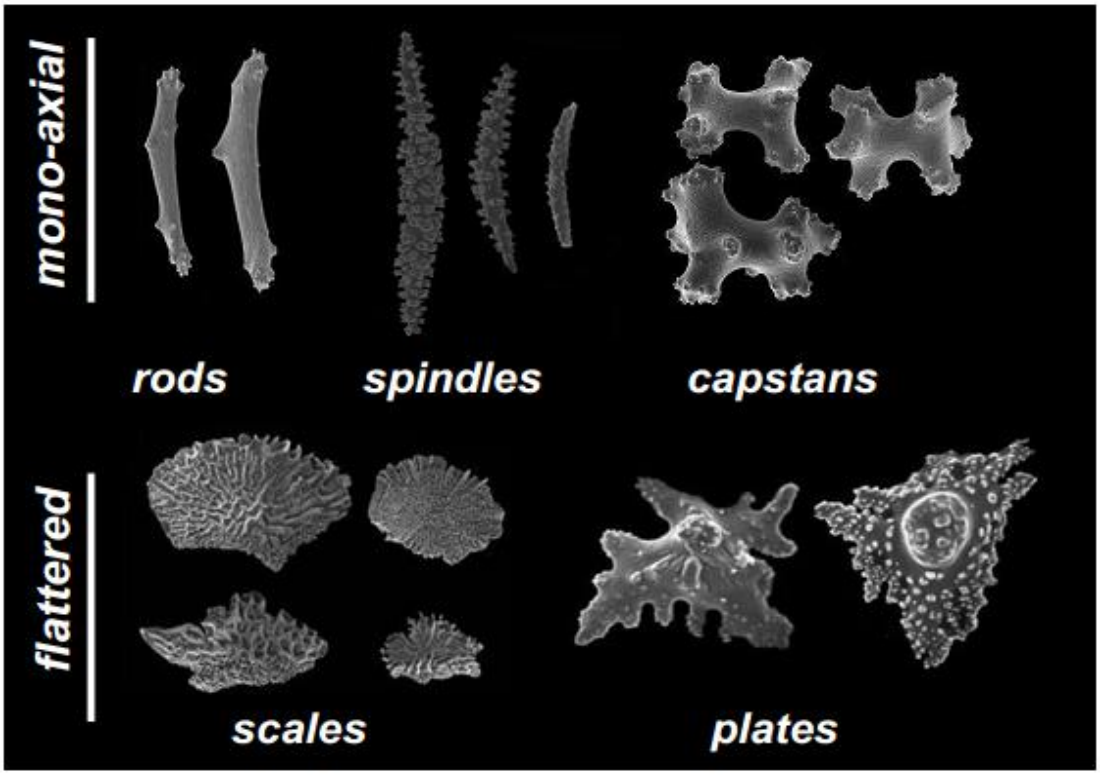

Figure 0.4: Different types of sclerites. Sclerites can be roughly grouped into mono-axial forms (rods, spindles and capstans) and flattered forms (scales, plates). Credits: rods: van Ofwegen and Hermanlimianto, 2014; spindles: DeVictor and Morton, 2007; capstans: van Ofwegen and Hermanlimianto, 2014; scales: courtesy of Dr. Peter Etnoyer (NOAA); plates: DeVictor and Morton, 2007.

Molecular markers and phylogeny

A fragment of the mitochondrial cytochrome c oxidase subunit I (*COI*) was proposed as standard marker for metazoans (Hebert *et al.* 2003). Although this molecular marker has been widely used to discriminate species in many taxa, there are still evident limitations in other groups. In comparison to other animals, anthozoans have slow mitochondrial genome evolution rates (Shearer *et al.* 2002), making the use of *COI*, as a species-specific marker, unsuitable for this group (). In octocorals, the mitochondrial locus *mtMutS*, a *mut*S homolog that is exclusively found in the mitochondrial genome of octocorals (Pont-Kingdom *et al.* 1995, 1998; Culligan *et al.* 2000; France and Hoover, 2001) has been extensively used for molecular and phylogenetic analyses providng significant insights into the taxonomy of the subclass. Interestingly, Chen *et al.* (2009) reported that nuclear genome of some cnidarians evolves up to five times faster than their mitochondrial-genome, suggesting the necessity to consider nuclear markers for a better resolution. Nuclear markers such as the Internal Transcribed Spacers (ITSs), the ATP Synthetase Subunit α (ATPSα) and the 28S ribosomal gene

have been recently used for taxonomy in different octocoral taxa (Haverkort-Yeh *et al.* 2013; McFadden *et al.* 2012; 2012b).

The phylogenetic studies so far published, which are mainly based on a restricted number of mitochondrial and nuclear loci, have shown a certain degree of incongruence. For instance, Berntson *et al.* (2001), using 18S rRNA, recovered Pennatulacea polyphyletic, in contrast McFadden *et al.* (2006) analysing two mitochondrial protein-coding genes (*Nad2* and *mtMutS*) found Pennatulacea monophyletic and deeply nested within alcyonaceans. In the same study, McFadden and colleagues (2006) recovered polyphyly within each of the alcyonacean suborders. Similarly, recent works on deep-sea calcaxonians (Pante *et al.* 2012), South African stoloniferans (McFadden and van Ofwegen, 2012) and shallow-water scleraxonians (Cairns and Wirshing, 2015) corroborated McFadden's results, suggesting that many octocoral groups require extensive taxonomic revisions.

Although molecular analyses are nowadays considered standard procedures in taxonomy, methods such as DNA barcoding and Sanger sequencing will be quickly replaced by new high-throughput sequencing technologies (e.g. Next Generation Sequencing, NGS) which will allow to generate, in short periods, large datasets. Recent applications of NGS in systematics include the recovery of hundreds complete mitochondrial genomes (Maricic *et al.* 2010) and the identification of thousands of informative nuclear loci for shallow-scale phylogeny (Lemmon and Moriarty-Lemmon, 2012). Similar approaches should be considered to investigate the systematics of the whole subclass Octocorallia, aiming to better understand the phylogenetic relationships at different taxonomic levels.

Aims of the study

In this study the species-level diversity of widespread shallow-water alcyonaceans was investigated using molecular approaches. In addition to the traditional methods (DNA barcoding) technologies such as Next Generation Sequencing (NGS) were employed to investigate the genetic variation within taxonomically difficult genera. Besides the use of standard molecular barcodes (e.g. *COI*, *mtMutS* and 28S rDNA), the complete mitogenome of different gorgonian genera has been sequenced and alternative variable traits such as the intergenic regions (IGRs) have been proposed for molecular

species identification. Genome-wide markers like Single Nucleotide Polymorphisms (SNPs) were also tested to delimit morphologically similar species and infer phylogeny.

According to the Australian Department of the Environment (https://www.environment.gov.au/biodiversity/conservation/hotspots) eight out of fifteen national biodiversity hotspots are in Western Australia (WA). Although several biodiversity surveys have been recently performed, the octocoral diversity of the WA reefs remains poorly studied. Using Western Australian taxa as a case in point, was estimated the species richness and phylodiversity of ecologically important soft corals. In particular, the species-level diversity of species-rich genera such as *Lobophytum* and *Sarcophyton* was assessed using different molecular markers and species delimitation methods (Chapter 1).

The Mediterranean Sea is considered a marine biodiversity hotspot. Despite the fact that 30% of the octocoral species are endemics little is known about their evolutionary history. In Chapter 2, the gorgonians *Paramuricea clavata* and *P. macrospina* were analysed to evaluate the mitogenomic variation and the intraspecific diversity in relation to the Atlantic congeners. The mitochondrial sequences obtained were used to reconstruct the biogegraphic history of the genus with particular attention to the Mediterranean endemics.

In Chapter 3, the complete mitochondrial genome of the Mediterranean *Leptogorgia sarmentosa* and that of some of the Atlantic and eastern Pacific congeners were sequenced aiming to investigate the intarspecific diversity and nucleotide variation within the genus. In order to better understand the evolutionary history of the genus, a time-calibrated phylogeny was performed.

Given the high species richness recently shown in the Gulf of Chiriquí (eastern Pacific, EP), the phylognetic relationships of shallow-water genera such as *Muricea* and *Pacifigorgia* have been tested using NGS tecnologies. In particular, the nucleotide diversity and the species-level identificstion within *Muricea* was explored in Chapter 4. Using Single Nucleotide Polymorphisms (SNPs), the delimitation of species boundaries and the phylogenetic relationships between *Pacifigorgia* species were addressed in Chapter 5.

Author Contributions

Chapter 1:

Rapid molecular phylodiversity survey of Western Australian soft-corals: *Lobophytum* and *Sarcophyton* species delimitation and symbiont diversity

Authors: Angelo Poliseno, Benedikt Kuttner, Monika Bryce, Gert Wörheide, Sergio Vargas

Performed the sampling: MB. Conceived and designed the experiments: AP, SV. Performed the experiments: AP, BK. Analysed the data: AP, BK, SV. Contributed reagents/materials/analysis tools: GW, SV. Wrote the paper: AP. Revised the paper: MB, GW, SV.

This chapter will be submitted as a standalone publication to the Journal "Marine Biodiversity"

Abstract

The zooxanthellate octocoral genera, *Lobophytum* and *Sarcophyton* are dominant in many Indo-Pacific and Red Sea benthic communities. They occupy large portions of the primary substrate forming, in some localities, reef-like structures. High intra-specific morphological variability is common among these organisms making many species difficult to identify. As such, the use of molecular methods is attractive as complementary sources of taxonomic information. We used three molecular markers, *mtMutS*, a mismatch repair protein exclusively found in the mitochondrial genome of octocorals, the *COI* and the 28S rDNA. Two different species delimitation methods were utilised, the Automatic Barcode Gap Discovery (ABGD) and Bayesian Poisson Tree Processes (BPTP), to rapidly assess the species richness of the taxonomically challenging genera, *Lobophytum* and *Sarcophyton* along the coast of north Western Australia. Using these markers we also provide a phylogenetic analysis and explore their phylodiversity. Based on a non-coding region of the plastid psbA minicircle ($psbA^{ncr}$) we assessed the molecular diversity of the zooxanthellae (*Symbiodinium*) associated with *Lobophytum* and *Sarcophyton* in this region. Our phylogenetic results were in agreement with previous studies in which *Sarcophyton* and *Lobophytum* species were divided into three main clades. Number of estimated entities varied according to the molecular marker and delimitation method used. Overall, the amount of hypothetical

species inferred with the BPTP method resulted higher than that derived by the ABGD. Finally, among the samples collected we found a high phylogenetic diversity and our analyses on the *Symbiodinium* diversity do not showed any specific association between the symbiont and their hosts.

Chapter 2:

Historical biogeography and mitogenomics of two endemic Mediterranean gorgonians (Holaxonia, Plexauridae)

Authors: Angelo Poliseno, Alvaro Altuna, Carlo Cerrano, Gert Wörheide, Sergio Vargas

Performed the sampling: AA, CC. Conceived and designed the experiments: AP, SV. Performed the experiments: AP. Analysed the data: AP. Contributed reagents/materials/analysis tools: GW, SV. Wrote the paper: AP. Revised the paper: AA, GW, SV.

This chapter has been submitted as a standalone publication to the Journal "Organisms Diversity and Evolution"

Abstract

Among the Mediterranean plexaurids, four species are endemics and despite their ecological importance comprehensive studies on the evolution and biogeography of these organisms are lacking. Here we explore the mitogenomic variability of two endemic, ecologically important Mediterranean *Paramuricea* species. We assess their phylogenetic relationships and provide first insights into their evolution and biogeography. Complete mitogenome sequences of *Paramuricea clavata* and *Paramuricea macrospina* were obtained using long range PCR, primer-walking and Sanger sequencing. For an enlarge sample of *Paramuricea* species, maximum likelihood and Bayesian phylogenetic trees of the mitochondrial gene *mtMutS* were obtained and used to study the biogeographic history of *Paramuricea* through a statistical Dispersal-Vicariance method (S-DIVA). Divergence time was estimated under a strict molecular clock model in BEAST using published octocoral mutation rates. Our results revealed high nucleotide diversity (2.6%) among the two Mediterranean endemics; the highest mutation rates were found in the *mtMutS*, *Nad4* and *Nad5*. In addition, we found length polymorphisms in several intergenic regions and diversity in mitochondrial genome size. The red gorgonian *Paramuricea clavata*

was closely related to the eastern Atlantic *P. grayi* rather than its Mediterranean congener, *P. macrospina.* Our biogeographic results provide evidence for the independent speciation of the Mediterranean species and point to a Miocene origin of the two endemics, highlighting the role played by the Messinian Salinity Crisis in the evolutionary history of Mediterranean *Paramuricea*.

Chapter 3:

Comparative mitogenomics, phylogeny and evolutionary history of *Leptogorgia* (Gorgoniidae)

Authors: Angelo Poliseno, Christian Feregrino, Gert Wörheide, Sergio Vargas

Conceived and designed the experiments: AP, SV. Performed the experiments: AP, CF. Analysed the data: AP. Contributed reagents/materials/analysis tools: GW, SV. Wrote the paper: AP. Revised the paper: GW, SV.

This chapter will be submitted as a standalone publication to the Journal "Molecular Phylogenetics and Evolution"

Abstract

Molecular analyses of the ecologically important gorgonian genus *Leptogorgia* are scant and mostly dealing with few species from restricted geographical regions. Here we explore the phylogenetic relationships and the evolutionary history of *Leptogorgia* using the complete mitochondrial genomes of five *Leptogorgia* species from different localities in the Atlantic, Mediterranean and eastern Pacific as well as four other genera of Gorgoniidae and Plexauridae. Our mitogenomic analyses showed high inter-specific diversity, variable nucleotide substitution rates and, for some species, novel genomic features such as ORFs of unknown function. The phylogenetic analyses using complete mitogenomes and an extended *mtMutS* dataset recovered *Leptogorgia* polyphyletic and the species considered in the analyses were split into two defined groups corresponding to different geographic regions, namely the eastern Pacific and the Atlantic-Mediterranean. A time-calibrated phylogeny showed that the separation of eastern Pacific and western Atlantic species started ca. 15 Mya and suggested a recent divergence for eastern Pacific species and for *L. sarmentosa-L. capverdensis*. This last speciation event could be related to the reopening of the Gibraltar Strait after the end of the Messinian crisis (5.96-5.33 Mya). Our results also revealed high inter-specific diversity among eastern Atlantic species highlighting a potential role of the

geographical diversification processes and geological events occurring during the last 30 Ma in the Atlantic on the evolutionary history of these organisms.

Chapter 4:

Complete mitochondrial genome of *Muricea crassa* and *Muricea purpurea* (Anthozoa: Octocorallia) from the eastern tropical Pacific

Authors: Angelo Poliseno, Odalisca Breedy, Michael Eitel, Gert Wörheide, Héctor M. Guzmán, Stefan Krebs, Helmuth Blum, Sergio Vargas

Performed the sampling: OB. Conceived and designed the experiments: AP, SV. Performed the experiments: AP, SK, SV. Analysed the data: AP, ME, SV. Contributed reagents/materials/analysis tools: GW, HMG, SV, HB. Wrote the paper: AP. Revised the paper OB, ME, GW, SK, SV.

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Abstract

We sequenced the complete mitogenomes of two eastern tropical Pacific gorgonians, *Muricea crassa* and *Muricea purpurea*, using NGS technologies. The assembled mitogenomes of *M. crassa* and *M. purpurea* were 19,586 bp and 19,358 bp in length, with a GC-content ranging from 36.0% to 36.1%, respectively. The two mitogenomes had the same gene arrangement consisting of 14 protein-coding genes, two rRNAs and one tRNA. Mitogenome identity was 98.5%. The intergenic regions between *COB* and *NAD6* and between *NAD5* and *NAD4* were polymorphic in length with a high level of nucleotide diversity. Based on a concatenated dataset of 14 mitochondrial protein-coding genes we inferred the phylogeny of 26 octocoral species.

Chapter 5:

Species delimitation and phylogeny of the tropical eastern Pacific gorgonian *Pacifigorgia* using Single Nucleotide Polymorphisms (SNPs)

Authors: Angelo Poliseno, Odalisca Breedy, Héctor M. Guzmán, Gert Wörheide, Sergio Vargas

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reagents/materials/analysis tools: GW, HMG, SV. Wrote the paper: AP. Revised the paper: GW, SV.

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Abstract

Systematics and classification of octocorals is traditionally based on the analysis of morphological characters, which are often conserved and taxonomically ambiguous for species delimitation. As such, the use of short-DNA fragments (molecular barcodes) became a standard procedure for the taxonomy of these organisms. Two-third of the ~12,000 octocoral sequences publicly released in the last twenty years were obtained using Sanger-based methods and the majority concern mitochondrial DNA. In contrast, the genomic data available are still limited and the number of nuclear loci investigated for most of the families is poor. The application of molecular technologies such as Next Generation Sequencing (NGS) has revolutionised the experimental approaches, allowing, even in non-model organisms, to recover large datasets. The vast amount of data generated by NGS is a potential source of sequences for phylogenetic studies. In particular the use of a variety of nuclear loci may be useful to better explore the phylogenetic relatedness among morphological homogeneous organisms such as gorgonians (Octocorallia: Gorgoniidae). Using a Genotyping by Sequencing (GBS) method, we investigate the phylogenetic relationships among nine eastern Pacific gorgonian species of the genus *Pacifigorgia*. Based on genome-wide Single Nucleotide Polymorphisms (SNPs) we delimit species and infer phylogeny. Besides the SNPs recovered, the mitochondrial reads obtained for each of the *Pacifigorgia* specimens were also utilised to infer mitochondrial phylogeny. Similar to what has been recently shown in other octocoral genera (e.g. *Chrysogorgia* and *Paragorgia*), our results showed divergence between the phylogenetic trees inferred from SNPs and mitochondrial data,. The species delimitation carried out on a set of ca. 500 SNPs using Bayes Factor Delimitation (BFD*) methods disagree with the taxonomy of the genus and suggests a possible presence of cryptic species boundaries as the result of adaptive speciation. The low interspecific variation detected among *Pacifigorgia* species may have been driven by rapid radiation events recently occurred in the Gulf of Chiriquí (eastern Pacific).

Chapter 1

Rapid molecular phylodiversity survey of Western Australian soft-corals: *Lobophytum* and *Sarcophyton* species delimitation and symbiont diversity

This chapter will be submitted as a standalone publication to the Journal "Marine Biodiversity"

Chapter 1

Rapid molecular phylodiversity survey of Western Australian soft-corals: *Lobophytum* and *Sarcophyton* species delimitation and symbiont diversity

1.1 **Introduction**

Soft corals are structural components of coral reef communities (Tursch and Tursch, 1982; McFadden *et al.* 2010) and are among the most important contributors to the total biomass of Indo-Pacific coral reef systems where they may cover up to 25% of the total reef substratum (Dinesen 1983; Fabricius and Alderslade, 2001). Some groups, such as the alcyoniid genus *Sinularia*, are active reef-builders, incorporating calcareous sclerites into the rock layers (Jeng *et al.* 2011). Despite their ecological importance, the diversity of octocorals remains poorly known and new species, genera and families are still discovered and described (e.g. Breedy *et al.* 2012; McFadden and van Ofwegen, 2013; Bryce *et al.* 2015).

Among octocorals, species within the genera *Lobophytum*, *Sarcophyton* and *Sinularia* are zooxanthellate and, despite their slow growth, often cover large portions of substrate in shallow reefs (Benayahu and Loya, 1981; Fabricius, 1995, 1997; Fabricius and Dommisse, 2000; Bastidas *et al.* 2004). Taxonomic identification in these genera involves the dissection of the colonies, a difficult and time consuming process and hence morphological identification is often restricted to genus-level determination only. In addition, intra-specific variability and the high number of described species hamper species level identification creating uncertainty in the taxonomy (McFadden *et al.* 2006, 2009, 2011). Despite these difficulties, identification is essential for biodiversity assessment and conservation management (Jamison and Lasker, 2008), especially in under-sampled areas with potentially high numbers of endemic and new species.

DNA barcoding and Sanger sequencing (Hebert *et al.* 2003; Hebert and Gregory, 2005; Stoeckle and Hebert, 2008) have been widely used as rapid methods for first level taxonomic screening due to: (i) their cost effectiveness, (ii) quick implementation and execution, and (iii) the lack of specific taxonomic skills required to increase the rate of specimen identification. Taxonomic determinations and phylogenetic studies in octocorals, in particular, have used multilocus DNA barcodes (i.e. *Cob, COI, mtMutS, Nad2* ITS2 and 28S rDNA) (Sánchez *et al.* 2003; McFadden *et al.* 2006; McFadden and van Ofwegen, 2013; Vargas *et al.* 2014; Wirshing and Baker, 2015). Furthermore, DNA barcoding-based taxon identification and species delimitation have been successfully applied to Red Sea and Palauan octocorals (Haverkort-Yeh *et al.* 2013; McFadden *et al.* 2014). Here we investigate the diversity of two soft coral genera from different locations in north Western Australia (WA) using a molecular approach that includes the sequencing of partial *mtMutS, COI* and 28S rDNA genes. We use the Automatic Barcode Gap Discovery (ABGD) method (Puillandre *et al.* 2011) to estimate primary species hypotheses (PSHs) and the Poisson Tree Processes (PTP) model (Zhang *et al.* 2013) to infer putative species boundaries, otherwise difficult to assess due to the lack of morphological descriptions. Finally, we compare the phylogenetic diversity within Kimberley locations and among different geographical areas (e.g. WA and Palau) and analyse the *Symbiodinium* diversity between genetically different *Lobophytum* and *Sarcophyton* sampled from different Western Australian reefs. Our results provide a first assessment of the molecular phylogenetic diversity of the important shallow reef octocoral genera *Sarcophyton* and *Lobophytum* from WA and attempt to delimit their species using molecular barcodes. In addition, our study provides first insights into the symbiont diversity of these diverse and abundant soft coral genera.

1.2Material and Methods

1.2.1 Sample collection and identification

Lobophytum and *Sarcophyton* specimens were collected as part of a Western Australian Museum, multi-year biodiversity survey of the Pilbara and Kimberley regions of WA. Collections were made intertidally and by SCUBA, at depths ranging from 0–20 meters at the Montebello and Murion Islands (Pilbara) and from the Kimberley at Hibernia, Long and Ashmore Reefs, at Rowley, Heywood, Eugene

McDermott and Vulcan Shoals, and at Cassini Island. Specimens were photographed *in situ* and on deck, and then preserved in 70% ethanol with subsamples for DNA analysis fixed in absolute ethanol and stored at room temperature until further examination. All collected specimens were sorted in the field based on a rapid morphological examination and assigned either to *Lobophytum* or *Sarcophyton*. Further analyses were performed independently using a molecular approach based on DNA barcoding.

1.2.2 Molecular analysis

One hundred and two (102) specimens were available for molecular work. DNA extraction was carried out using either 96-well plates following a modified Ivanova *et al.* (2006) protocol (see Vargas *et al.* 2012), a modified CTAB phenol-chloroform protocol (Porebsky *et al.* 1997) or the Macherey-Nagel NucleoSpin® Tissue kit (M&N, Düren, Germany). The quality of the DNA extracts was assessed visually on 1.0% agarose gels and the DNA concentration (ng/µL) was measured on a Nanodrop 1000. Subsequently, the 5' end of the mitochondrial gene *mtMutS* was amplified using primers ND4-2599F and MUT-3458R (Sánchez *et al.* 2003). Primers COII8068F (McFadden *et al.* 2004) and COIOCTr (France and Hoover, 2001) were used to amplify the *COII*-igr-*COI* region. Additionally, a fragment of about 800 bp of the 28S rDNA was amplified using the conserved forward primer 28S-C2-fwd (Chombard *et al.* 1998) and a specific reverse primer for metazoa (5'-CATCGCCAGTTCTGCTTAC-3') (Voigt *et al.* 2012). In order to assess the zooxanthellae diversity associated with the *Sarcophyton* and *Lobophytum* samples, the non-coding region of the plastid *psbA* minicircle (*psbA*^{ncr}) of the soft coral endosymbionts (*Symbiodinium* sp.) was amplified using the primer pairs 7.4-Forw, 7.8-Rev (LaJeunesse and Thornhill 2011). Amplifications were performed using 3-step PCR in 12.5 µL volumes containing 2.5 µL of 5X Green GoTaq® Flexi Buffer, 1.5 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTP, 0.5 µL of each primers, 0.1 µL of 5 U x μL^{-1} GoTaq® DNA Polymerase (Promega, Madison, WI, USA). PCR products were purified by precipitation with one volume of 20% (w/v) polyethyleneglycol 8000 in 2.5molL^{-1} NaCl and were sequenced directly using the BigDye 3.1 chemistry (Applied Biosystems, Foster City, CA, USA) with the same primers as for PCR. Sequences were assembled into contigs, edited and aligned using the program GENEIOUS 6.0.5 (Kearse *et al.* 2012). Soft corals sequences will be deposited in the European Nucleotide Archive.

1.2.3 Phylogenetic analyses

MtMutS sequences were assembled, aligned and compared against those analysed by McFadden *et al.* (2006, 2009, 2014), Haverkort-Yeh *et al.* (2013) and Benayahu *et al.* (2012) using the program GENEIOUS 6.0.5. Due to the limited number of publicly available *Lobophyton* and *Sarcophyton COI* and 28S rDNA sequences, the sequences obtained were only compared against those analysed by McFadden *et al.* (2014). Reduced datasets, including sequences obtained from Western Australian material, were generated for each molecular marker and exclusively used for species delimitation (see below). A further reduced *mtMutS* dataset, including sequences obtained from WA and Palau (McFadden *et al.* 2014), was generated to assess the phylogenetic diversity between these areas (see below). All the sequences, for each dataset, were aligned in MUSCLE with default options using Seaview 4.5.3 (Gouy *et al.* 2010). The mitochondrial alignments generated (i.e. *COI* and *MutS*) were first analysed independently and then concatenated in a single mitochondrial dataset consisting of about 1,600 nucleotide positions. The concatenated (*COI*+*mtMutS*) and the 28S alignments were finally used to infer mitochondrial and nuclear gene phylogenies, respectively. All the alignments used in this study will be deposited in the Open Data LMU. The best-fit substitution models for the above-mentioned alignments (see Appendix 1.5.1) were selected with the Akaike Information Criterion (AIC) in jModeltest 2.1.3 (Darriba *et al.* 2012). The ML and Bayesian phylogenies were obtained with RAxML 7.2.8 (Stamatakis, 2006) and MrBayes 3.2.5 (Ronquist and Huelsenbeck, 2003), respectively. Maximum Likelihood analyses were performed under the GTRGAMMA substitution model, rate variation was modelled using a discrete gamma distribution with 4 categories (Yang, 1994) and 1000 pseudo-replicates were included for bootstrap analyses. For the Bayesian analyses the Markov Chain Monte Carlo (MCMC) was ran for 10 000 000 generations, sampling trees at 500-generation intervals until convergence between runs was achieved. A standard deviation of the split frequencies <0.009 was assumed to indicate convergence. For each analysis, convergence to the stationary distributions was additionally assessed in Tracer 1.6.0 (Rambaut *et al.* 2014). Upon completion, 25% of the sampled trees were discarded as burn-in.

1.2.4 Species delimitation

Species delimitation was performed using two independent methods, the Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al.* 2011) and the Poisson Tree Processes (PTP) (Zhang *et al.* 2013). The ABGD method relies on genetic distances to sort DNA sequences into primary species hypotheses (PSHs) using a priori defined threshold (i.e. the "barcode gap"). The Bayesian PTP infers putative species boundary starting from a phylogenetic tree and counting the number of substitutions inferred by the length of the branches. Although the ABGD and the Bayesian PTP use different input information (i.e. sequence alignments and phylogenetic trees, respectively) to delimit species, both methods provide information on the number of different candidate/hypothetical species in a collection of sequences. For the ABGD method we ran the analysis online (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) with default options using the Kimura 2-parameters as an evolutionary model. Bayesian PTP analysis was performed in the bPTP web server (http://species.h-its.org/ptp/) using the rooted "best tree" generated by RAxML. The analysis was run for 500,000 generations using a random number seed and a thinning of 100. A quarter of the sampled trees were discarded as burn-in. Species delimitation probability values were calculated with the Bayesian method by considering the frequency of the nodes across the sampling.

1.2.5 Phylodiversity

The *mtMutS* Maximum Likelihood topology was used to estimate and compare the inclusive phylogenetic diversity (PD_I) (Lewis and Lewis, 2005) of Kimberley (Ashmore Reef; Rowley Shoals), Pilbara (Montebello Islands) and Palau. The inclusive phylogenetic diversity corresponds to the length of the path starting at the root of the tree and leading to all branches connecting the members of a specific group (Lewis and Lewis, 2005; Vargas *et al.* 2014, 2015). Samples included in the dataset used for PD_I analyses were collected across a broad geographic range, however the PD_I was only estimated for Kimberley, Pilbara and Palau, as the number of samples from other regions was typically low. In order to account for different sampling efforts across areas, PD_I values obtained for each location were compared using rarefaction (Sanders, 1968). A python script for tree-based rarefaction analysis was provided by Vargas *et al.* (2014).

1.3 Results

1.3.1 DNA Sequences and alignments

Among the 102 specimens analysed we obtained 82 sequences for *mtMutS*, 78 for *COI* and 72 for 28S rDNA (see Appendix 1.5.2 for detailed list of the samples). *MtMutS* sequences were used for phylogenetic analyses, species delimitation and phylodiversity investigations, while *COI* and 28S sequences were considered only for phylogenetic and species delimitation analyses. We obtained both *COI* and *mtMutS* for 57 specimens and these were aligned with those available from Palau (McFadden *et al.* 2014). A concatenated alignment (*COI+MutS*) including 126 taxa and 1,642 nucleotide positions was further used for phylogenetic analyses and for comparison against the nuclear (28S) phylogeny. A clear length polymorphism was observed in the 28S sequences with some of the Palauan specimens having a shorter sequence (~140 bp) compared to other specimens. The 28S dataset consisted of 142 taxa and 624 nucleotide positions.

Within the 102 specimens analysed we obtained 41 *Symbiodinium* sequences, 19 derived from *Lobophytum* specimens, 21 from *Sarcophyton* and one from *Sinularia* originating from six Kimberley and two Pilbara reefs. Amplification of the non-coding region of $psbA^{ncr}$ was not possible for the majority of the samples collected from Cassini Island and Long Reef. However, the blast sequence analyses performed on the obtained non-ambiguous and unique sequences revealed that the majority of WA *Lobophytum* and *Sarcophyton* assemblages investigated are dominated by *Symbiodinium* clade C3, while only one specimen (GW8669) harboured a different *Symbiodinium* lineage (*S. goreaui*).

1.3.2 *MtMutS* phylogeny

The phylogenetic trees obtained by Maximum Likelihood and Bayesian methods have the same topology, differing only in the support values reported for some branches. The topology was consistent with previous studies (McFadden *et al.* 2006) and included three well supported clades, namely *Sarcophyton* (S), *Lobophytum* (L) and Mixed (M) (see Figure 1.1). Except for three specimens (GW8639, GW8651 and GW8652) grouping in a small, well-supported clade (Clade 1) and also including *Lobophytum crassocaule* and *Lobophytum* sp. (DQ280951), the remaining specimens were split into the S, L and M clades of McFadden *et al.* (2006). Unexpectedly, one

sample analysed (GW8680) was sister to *Sinularia digitata* and *Sinularia finitima* and this clade was used to re-root the tree. Among the 82 specimens analysed, 34 were placed in the *Sarcophyton* clade and were split into two main clades further divided in subclades with slight differences in the support values of some internal nodes in both analyses. The phylogenetic relationships within these clades were well defined and each of the sub-clades included several specimens assigned to a restricted number of species (Figure 1.1). The Bayesian tree reported higher support for the shallower nodes, yet the phylogenetic placement of some samples remained unresolved (e.g. GW8637 and GW8689).

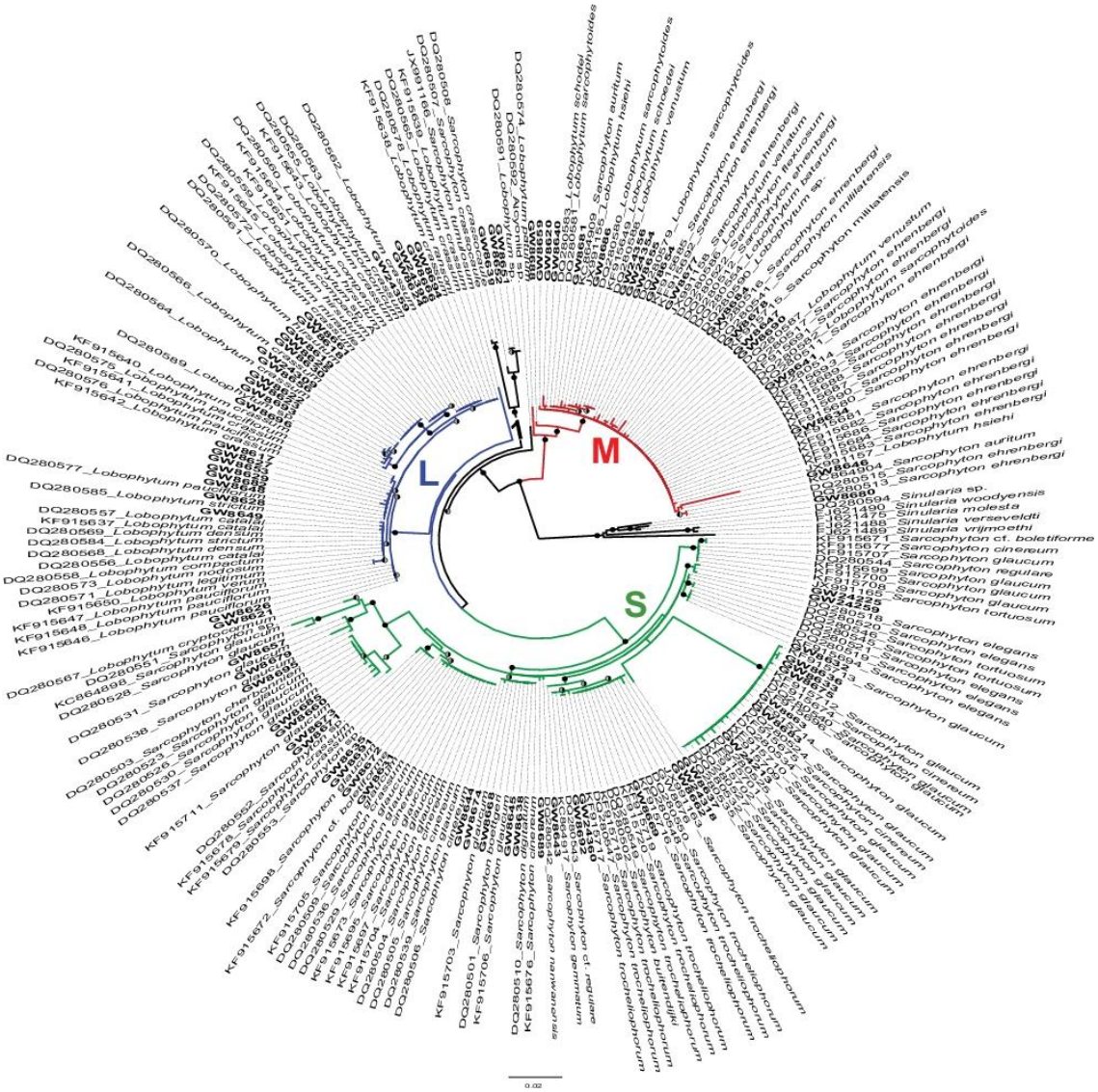


Figure 1.1 Phylogenetic tree of *mtMutS*. Western Australia specimens are in bold. Colours highlight the three main clades of the tree (green, S=*Sarcophyton*; blue, L=*Lobophytum* and red, M=Mixed) and the clade including *S. crassocaule* (clade 1). Circles at the nodes indicate bootstrap (BP) and posterior probability (PP) values; filled black circles indicate high support for both ML (BP ≥70) and Bayesian (PP ≥0.95) analyses. Split circles indicate high support for one analysis only (black: high support; grey: low support); the left and right half of the circles refer to ML and Bayesian analyses, respectively.

Notably, *Sarcophyton glaucum* grouped in five distinct genetic sub-groups, four of which also included Western Australian specimens. The *Lobophytum* clade was divided into three sub-clades, supported only in the Bayesian analysis. The internal structure of these was well defined. Twenty-six specimens from WA were included in this clade and were distributed in each of the *Lobophytum* subclades. *L. cryptocormum*, *L. pauciflorum* (KF915646-48), GW8621 and GW8626 were considerably different from all other samples in this clade. We found high genetic variability among specimens assigned to *Lobophytum crassum*, which formed four genetically distinct sub-groups. The third main clade, the mixed clade (M), was sister to *Sarcophyton* and *Lobophytum* clades with high support values in both analyses and included 18 WA specimens. *L. patulum* and GW8688 were remarkably different from the other species grouped in this clade. Specimens GW24354, GW24356 and GW8655 grouped in a clade, supported only by the Bayesian analysis, including also a specimen of *L. sarcophytoides* (DQ280579). Two *S. ehrenbergi* specimens (DQ280513, DQ280515) were sister to *S. auritum* and GW8646, whereas the remaining specimens assigned to *S. ehrenbergi* had identical sequences.

1.3.3 Mitochondrial and nuclear phylogenies

Some discrepancies can be observed between the phylogenies derived from the analyses of mitochondrial (*COI+MutS*) and nuclear (28S) genes (Appendix 1.5.3 and Figure S2). In the mitochondrial phylogeny, the relationship between S and L clades was not resolved. Nodes with bootstrap values <70 and posterior probabilities <0.95 were collapsed into polytomies and, as a result, those specimens formerly included into S and/or L clades were placed in a single main clade (S+L) (see Appendix 1.5.3). Within S+L clade, 55 *Sarcophyton* and 34 *Lobophytum* specimens grouped into five and three clades, respectively. These specimens were sister to a well-supported clade (M) including 27 specimens. *Sarcophyton* and *Lobophytum* specimens were divided into two major clades in the nuclear phylogeny. One clade was further split into three main clades (S, L and L1) and two smaller groups (clade 1 and clade 2), which included three specimens respectively (Appendix 1.5.4). A second well-supported clade (M) consisted of 23 *Sarcophyton* and nine *Lobophytum* specimens. As in the mitochondrial phylogeny (*MutS+COI*), sample GW8688 resulted remarkably different from the other species grouped in the Mixed clade.

Notably, specimens included in clade 1, which were sister to the *Sarcophyton* and *Lobophytum* clades in the mitochondrial trees, were not closely related in the 28S phylogeny. The position of sample GW8660 was also dissimilar; this sample nested within the S+L clade in the mitochondrial phylogeny, but in the 28S tree was included in a well-supported clade (clade 2) together with GW24352 and GW8667. Interestingly, the nuclear phylogeny showed a clear separation between the WA and Palauan specimens included in the *Sarcophyton* clade.

1.3.4 Species delimitation

The Automatic Barcode Gap Discovery and the Bayesian Poisson Tree Processes analyses yielded different numbers of putative species. For *mtMutS*, the ABGD method recovered a total of 30 PSHs (Figure 1.2 and Appendix 1.5.5) with an intra-specific divergence threshold of 0.001. For the Bayesian PTP analysis, the most supported partition identified using a simple heuristic search resulted in 35 putative species. Except for GW8680 and clade 1 formed by GW8639, GW8651 and GW8652, the remaining specimens were divided into different groups and/or candidate species within the three main clades (see Figure 1.2 and Appendix 1.5.5). Estimation of hypothetical species was different according to the methods used, varying in both composition and the number of specimens included in each PSH cluster. Overall, the BPTP method recovered a higher number of putative species. In the Mixed clade, for 18 specimens the ABGD and BPTP analyses recognised 12 and 11 different PSHs, respectively. Within *Sarcophyton*, 34 specimens were split into four (ABGD) and ten candidate species (BPTP). Twenty-six samples included into the *Lobophytum* clade were assigned to 12 and 14 putative species, respectively. Except for four putative species, which reported posterior delimitation probability values ≥ 0.95, the remaining were poorly supported. Regardless of the method used to delimit species, only nine specimens out of 85 (10%) were assigned to the same hypothetical species clusters (Appendix 1.5.5). Species delimitation using *COI* and 28S recovered 37 and 31 PSHs with ABGD and 62 and 46 putative species with BPTP. Among the markers used for species delimitation, the number of hypothetical species recovered using *COI* was the highest.

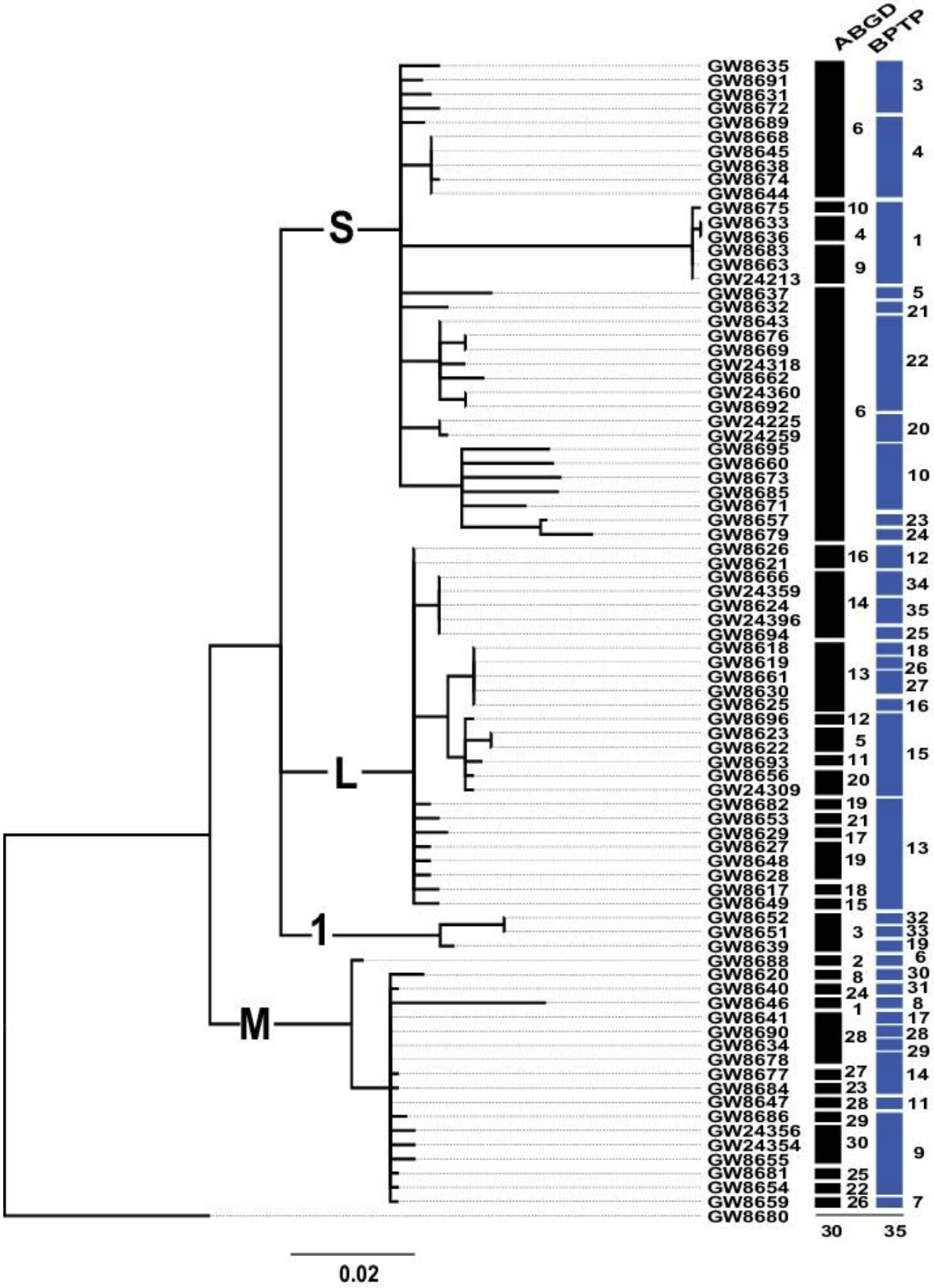


Figure 1.2 Phylogenetic tree of *mtMutS* based on Maximum Likelihood analysis and species delimitation inferred with the Automatic Barcode Gap Discovery (black columns) and Bayesian Poisson Tree Processes (blue columns). Black numbers on the right side of the tree correspond to the Primary Species Hypothesis clusters (ABGD) and putative species (BPTP), respectively. Nodes with low support values (BP <70) have been collapsed into polytomies.

1.3.5 Phylodiversity

The PD_I analyses revealed high variation within the Western Australian locations (Figure 1.3). Ashmore Reef was the most diverse, while Rowley Shoals had the lowest PD_I values. Overall, the Montebello Islands and Ashmore Reef had higher phylodiversity than Palau at the comparable sampling efforts. Interestingly, the phylogenetic diversity in the two ocean regions (WA and Palau) kept constantly growing in relation to the accumulated number of samples.

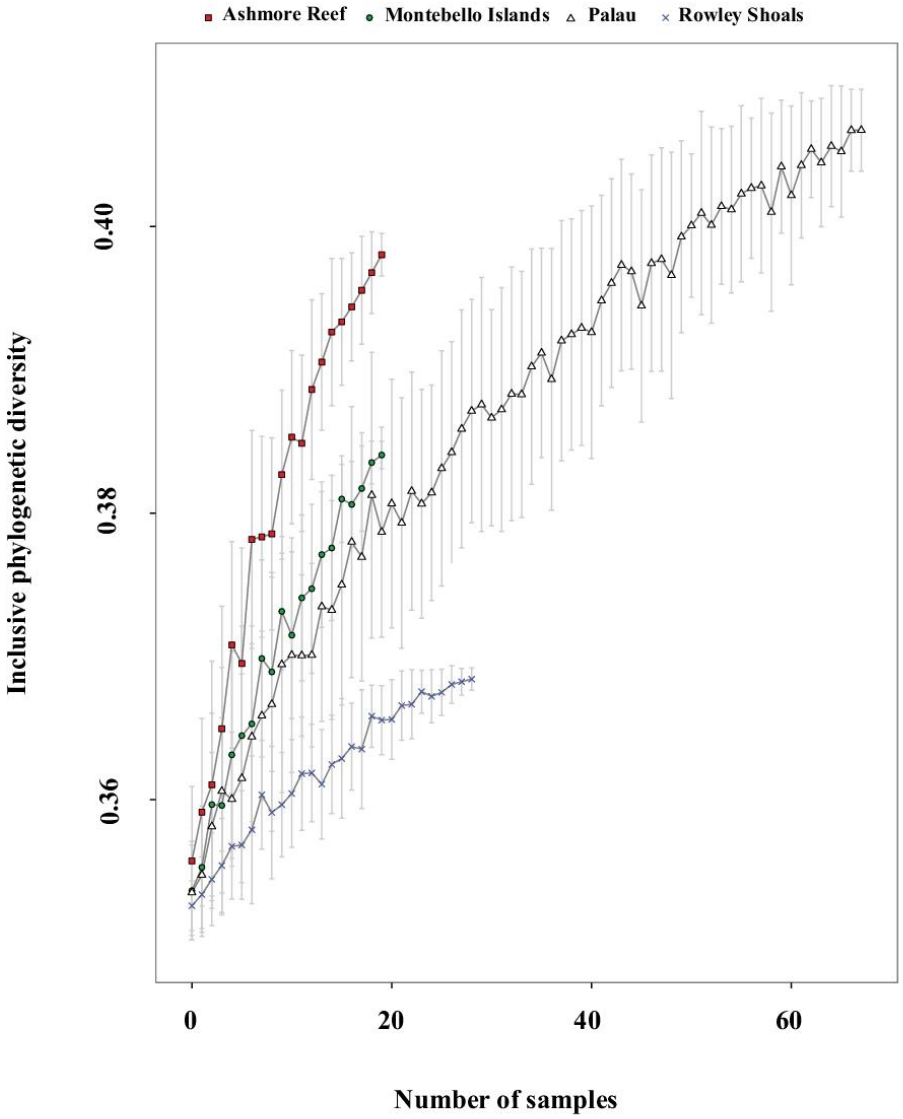


Figure 1.3 Inclusive phylogenetic diversity of *Lobophytum* and *Sarcophyton* specimens from Palau and from three WA locations (Ashmore Reef, Rowley Shoals and Montebello Islands).

1.3.6 *Symbiodinium* phylogeny

The phylogenetic tree of the non-coding region *psbA*^{ncr} was largely unresolved and the majority of the branches were not supported (Figure 1.4). We did not find any specific association between the *Symbiodinium* type and their hosts, but genetic variation (i.e. nucleotide substitutions and indels) was found among the sequenced symbionts. This variation was not explained by geographic segregation, as no phylogeographic pattern could be observed in the analyses.

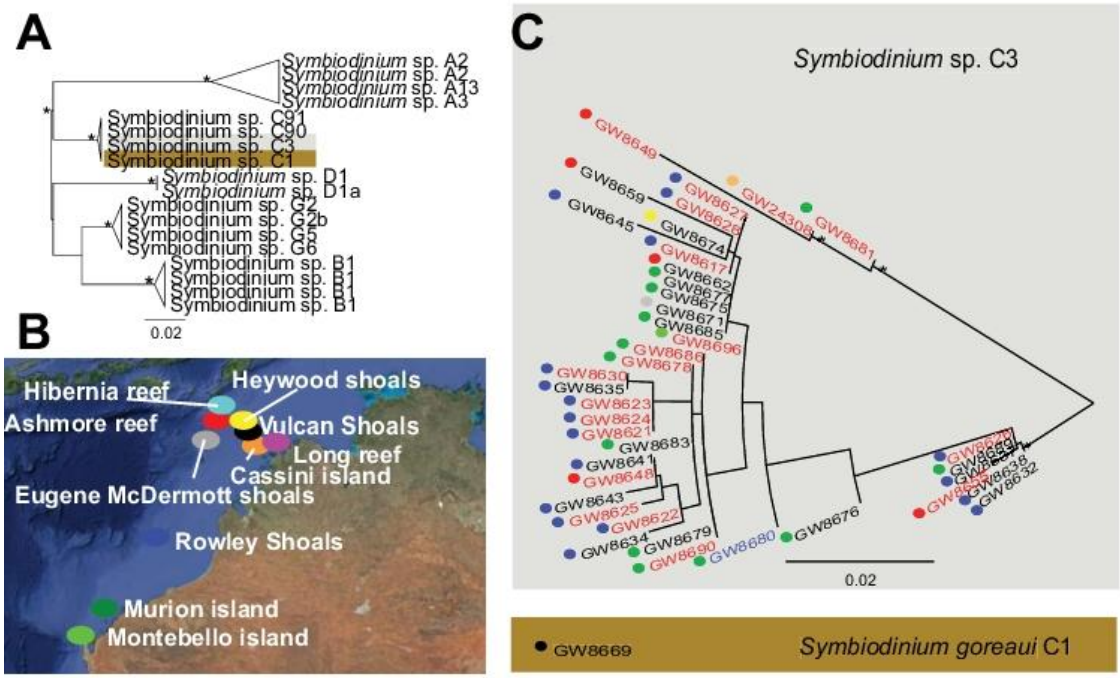


Figure 1.4 Phylogenetic reconstruction of *Symbiodinium* spp. A: phylogenetic tree of *psbA*^{ncr} including different *Symbiodinium* clades (A, B, C, D, G); *Symbiodinium* types C3 and C1 are enclosed in grey and brown boxes respectively. B: sampling sites along Western Australia. C: phylogenetic relationships within the *Symbiodinium* sp. C3 harbored by WA *Lobophythum* and *Sarcophyton.*

1.4 Discussion

1.4.1 Phylogeny, phylodiversity and species delimitation

Mitochondrial tree topologies (*MutS* and *COI+MutS*) were consistent with those obtained in previous studies (McFadden *et al.* 2006; Aratake *et al.* 2012) and show the separation of *Sarcophyton* and *Lobophytum* in three main clades with a fourth well-supported group (clade 1), including *S. crassocaule* and one unidentified *Lobophytum* species from Okinawa (DQ280591). The phylogenetic placement of GW8639, GW8651 and GW8652 outside the three main clades has significant implications for the taxonomy of these genera as already proposed for *Sarcophyton crassocaule*. In this respect, based on the colony shape, *S. crassocaule* has been described as a typical

Sarcophyton-clade species, while its interior basal sclerites, to some degree, resembled those of *Lobophytum* (McFadden *et al.* 2006). Similarly, GW8639 appears morphologically to be a typical *Sarcophyton*-clade species with a smooth disc distinct from a stalk, while in the interior thick spindles with girdles of warts, typical for *Lobophytum,* are present. Based on the colony shape, GW8651 and GW8652 conform to the Mixed clade. They have massive, flat colonies with discs not distinct from the stalk and with low, rounded ridges. The sclerites have *Lobophytum*-clade characters with surface clubs between 0.10-0.27 mm long and the interior sclerites being ovals, 0.20-0.32 mm long, and with two to three girdles of warts. Why these morphologically different species are united within the same clade cannot be explained at this stage. In order to better understand the phylogenetic relationships among *S. crassocaule* and related samples, further molecular and morphological analyses will be required to clarify the taxonomy of those specimens that seem to neither belong to the *Lobophytum* or *Sarcophyton* clades.

Concerning the *mtMutS* phylogeny, the Western Australian specimens were spread across the tree and some (e.g. GW8637 in *Sarcophyton* clade, GW8688 in the Mixed clade) were, in terms of genetic distance, markedly distinct from the other samples included in the tree. The colony of GW8637 consists of a smooth disc with an extremely convoluted margin and a distinct stalk and sclerites typical for the *Sarcophyton*-clade. No obvious morphological cues suggest why it is distinctly different from the other *Sarcophyton* specimens as genetic distance indicates. GW8688 clearly fits morphologically into the Mixed clade. The colony has a smooth disc, which is not distinct from the stalk, and superficially resembles a *Lobophytum* with a flat surface. The sclerites of the surface of the polypary are small, well-formed clubs and the colony interior contains long sticks and distinct spindles. These specimens deserve further taxonomic investigations as they may represent undescribed, endemic species. Detailed morphological determinations, involving sclerite analyses from five different parts of the colony will be necessary for detailed species descriptions and to complement the genetic findings. The use of *mtMutS* has been criticised because of the lack of resolution for species-level identification (McFadden *et al.* 2011). However, by using this marker we were capable of inferring a restricted number of putative species, which can be considered as a baseline for detailed morphological species determinations. *MtMutS*, despite its limitations, appears to be a powerful tool for the rapid sorting of specimens collected from potentially species-rich localities.

We would also like to comment on the use of nuclear markers (e.g. 28S rDNA) as a complementary source for molecular studies and DNA barcoding. A ~800 bp fragment of the 28S nuclear ribosomal gene has been recently used to reconstruct the phylogenetic relationships of different octocoral groups (McFadden and van Ofwegen, 2012; Bryce *et al.* 2015; Cairns and Wirshing, 2015). Nevertheless, its application for phylogenetic studies and species delimitation in specious genera in need of taxonomic revisions, such as *Lobophytum* and *Sarcophyton*, has so far been ignored. Overall, the genetic distances of 28S gene sequences obtained among and within the three main clades (data not shown) were higher than those obtained with the mitochondrial loci. This, in addition to the high genetic divergence between WA and Palauan *Sarcophyton* species, suggests that 28S rDNA can be more extensively applied to resolve species-level questions. However, the current limited taxon sampling of 28S sequences from octocorals, unlike the *mtMutS*, at present do not allow comprehensive phylogenetic reconstructions. Our results demonstrate the importance of a multi-gene approach, including both mitochondrial and nuclear genes that typically have different mutation rates, to enhance our understanding of the diverse octocorals.

Regarding the WA *Lobophytum* and *Sarcophyton* species richness, our estimates lead to a number of primary species hypotheses ranging from 30 (*mtMutS*) to 37 (*COI*) with the ABGD method, and from 35 (*mtMutS*) to 62 (*COI*) with the Bayesian PTP analyses. Cross-comparisons between PSHs obtained by using different molecular markers were not possible due to the different number of specimens considered among the samples used for species delimitation. However, the number of hypothetical species derived by BPTP was generally higher than obtained with ABGD. Based on material deposited at the Western Australian Museum, Bryce and Sampey (2014) identified 20 *Sarcophyton* and *Lobophytum* species collected from the Kimberley area. Thus, the number of species inferred by the Bayesian PTP method could be inflated and the ABGD prediction might be more realistic and most likely reflect the real diversity of the area. Species delimitation methods exclusively based on DNA sequences can provide accurate approximations to the species richness estimated using classical methodologies and can be used to canalize efforts to investigate the morphology and ecology of octocorals.

Finally, we observed that the phylogenetic diversity of Western Australian areas sampled was higher than that of Palau. In particular, among the Western Australian locations sampled, Ashmore Reef reported the highest PD_I values. This result is in

agreement with Bryce and Sampey (2014) finding that WA octocoral diversity appears to be higher at offshore sites, like Ashmore Reef, than at inshore and coastline locations. The high biodiversity of Ashmore Reef in the North West Shelf bioregion can be attributed to the high habitat diversity and the close proximity and biogeographic affinity with the species-rich Central Indo-West Pacific region (Wilson, 2013).

1.4.2 *Symbiodinium* diversity and ecological evidences

The multi-copy internal transcribed spacer 2 (ITS2) has been formerly used to compare the *Symbiodinium* diversity among coral populations inhabiting different reefs (Stat *et al.* 2011; Arif *et al.* 2014). Both *psbA*^{ncr} and ITS2 show high intragenomic variation. However, *psbA*^{ncr}, considered the fastest evolving genomic marker so far known for *Symbiodinium* (LaJunesse and Thornhill 2011), has less overlap between intragenomic, inter-individual and inter-species diversity than ITS2, resulting in a suitable marker to infer phylogenetic relationships on close-related species. In addition, the rapidly evolving plastid marker has a higher diversity on broad geographic distances (LaJunesse and Thornhill 2011). PCR amplification, using specific primers for *Symbiodinium* clade *C psbA*^{ncr} haplotypes, was successful for the majority of the samples suggesting a high level of symbiont specificity for WA *Lobophytum* and *Sarcophyton* species. However, ~23% of the sequences obtained show several ambiguous nucleotides and double peaks, most likely due to the presence of mixed community symbionts as already proposed for the scleractinian coral, *Montipora capitata* (Stat *et al.* 2011) or intragenomic variation. On the other hand, the paucity of positive amplification for those specimens collected from Cassini Island and Long Reef suggests a possible presence of multiple *Symbiodinium* clades. However, this needs to be further validated by testing different primer pairs and screening additional samples. Beyond the lack of phylogeographic structure and host specificity, we reported on noteworthy ecological data. For instance, the sample collected from Vulcan Shoals (GW8669) was the only one to host a distinct *Symbiodinium* lineage (C1). *Symbiodinium* types C1 and D appear to have a higher tolerance to environmental stresses than type C3 (see Abrego *et al.* 2008; Jones *et al.* 2008). Although our data requires further corroboration with analysis of a larger sample size from a broader area, the discovered symbiont diversity may be due to intensive pollution events such as the 72 days of gas and oil spillage from the Montara wellhead disaster combined with changes in sea-water temperature that recently occurred off Vulcan Shoals.

In conclusion, with the present study we highlighted the importance of biodiversity surveys and rapid, DNA-based species delimitation analyses to explore the diversity of ecologically important soft coral communities distributed along WA. We estimated species richness and assessed the phylogenetic diversity of different Kimberley locations and compared these locations with Palau Archipelago, a known biodiversity hot-spot. We also investigated the genetic diversity of the symbionts hosted by different *Sarcophyton* and *Lobophytum* species. Our study provides an example of the exclusive use of DNA-based classification tools to analyse the diversity of abundant soft coral communities across a geographic range. We hope our contribution helps supporting management and conservation plans of under-investigated marine Western Australian regions.

1.5 Appendix

Appendix 1.5.1 List of best-fit substitution models for Bayesian analyses

Appendix 1.5.2 List of Western Australian specimens used in our analyses

Appendix 1.5.3 Mitochondrial phylogenetic tree (*COI*+*mtMutS*)

Appendix 1.5.4 Nuclear phylogenetic tree (28S rDNA)

Appendix 1.5.5 Species delimitation and number of estimated entities obtained for the mitochondrial (*mtMutS* and *COI*) and nuclear (28S) markers

Appendix 1.5.1 List of best-fit substitution models for Bayesian analyses

Dataset	Model Selected	fA	fC	fG	fT	R [AC]	R [AG]	R [AT]	R [CG]	R [CT]	R [GT]	gamma shape (Γ)	p-inv (I)
mtMutS	TPM1uf+G	0.31	0.17	0.18	0.34	1.00	8.80	0.74	0.74	8.80	1.00	0.47	
COI+mtMutS	TPM3uf+I+G	0.29	0.17	0.18	0.36	1.51	6.99	1.00	1.51	6.99	1.00	0.65	0.53
28S rDNA	TIM3+G	0.22	0.29	0.32	0.17	0.41	2.00	1.00	0.41	4.75	1.00	0.35	

Best-fit substitution models for the Bayesian analyses selected with the Akaike Information Criterion in jModeltest.

Appendix 1.5.2 List of Western Australian specimens used in our analyses

Museum Voucher	Genus	Species	GW number	WA region	Location	*mtMutS*	*COI*	28S	*psbA*
WAM Z59656	*Sarcophyton*	sp.	GW24212	Kimberley	Cassini island				
WAM Z59657	*Sarcophyton*	sp.	GW24213	Kimberley	Cassini island				
WAM Z59663	*Sarcophyton*	sp.	GW24219	Kimberley	Cassini island				
WAM Z59669	*Sarcophyton*	sp.	GW24225	Kimberley	Cassini island				
WAM Z59697	*Sarcophyton*	sp.	GW24259	Kimberley	Cassini island				
WAM Z59742	*Sarcophyton*	sp.	GW24296	Kimberley	Cassini island				
WAM Z59738	*Lobophytum*	sp.	GW24308	Kimberley	Cassini island				
WAM Z59749	*Lobophytum*	sp.	GW24309	Kimberley	Cassini island				
WAM Z59756	*Sarcophyton*	sp.	GW24310	Kimberley	Cassini island				
WAM Z59762	*Sarcophyton*	sp.	GW24318	Kimberley	Cassini island				
WAM Z59767	*Lobophytum*	sp.	GW24323	Kimberley	Cassini island				
WAM Z59792	*Lobophytum*	sp.	GW24348	Kimberley	Long reef				
WAM Z59795	*Sarcophyton*	sp.	GW24351	Kimberley	Long reef				
WAM Z69796	*Lobophytum*	sp.	GW24352	Kimberley	Long reef				
WAM Z59798	*Lobophytum*	sp.	GW24354	Kimberley	Long reef				

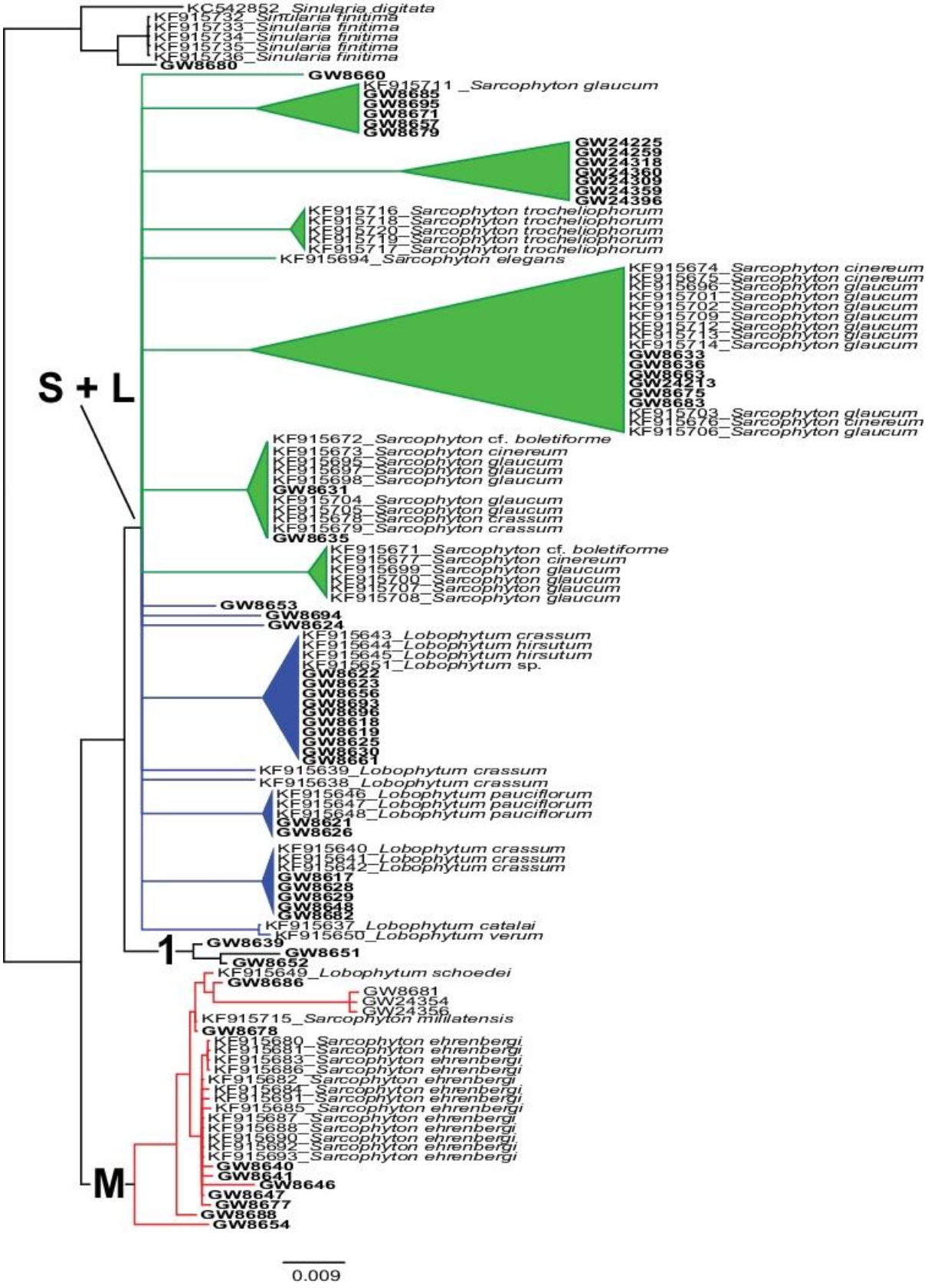
WAM Z50800	*Lobophytum*	sp.	GW24356	Kimberley	Long reef
WAM Z59803	*Lobophytum*	sp.	GW24359	Kimberley	Long reef
WAM Z59804	*Sarcophyton*	sp.	GW24360	Kimberley	Long reef
WAM Z59817	*Sarcophyton*	sp.	GW24373	Kimberley	Long reef
WAM Z59831	*Sarcophyton*	sp.	GW24378	Kimberley	Long reef
WAM Z59840	*Lobophytum*	sp.	GW24395	Kimberley	Cassini island
WAM Z59839	*Lobophytum*	sp.	GW24396	Kimberley	Cassini island
WAM Z67215	*Lobophytum*	sp.	GW8617	Kimberley	Rowley shoals
WAM Z67236	*Lobophytum*	sp.	GW8618	Kimberley	Rowley shoals
WAM Z67237	*Lobophytum*	sp.	GW8619	Kimberley	Rowley shoals
WAM Z67270	*Lobophytum*	sp.	GW8620	Kimberley	Rowley shoals
WAM Z67310	*Lobophytum*	sp.	GW8621	Kimberley	Rowley shoals
WAM Z67312	*Lobophytum*	sp.	GW8622	Kimberley	Rowley shoals
WAM Z67314	*Lobophytum*	sp.	GW8623	Kimberley	Rowley shoals
WAM Z67315	*Lobophytum*	sp.	GW8624	Kimberley	Rowley shoals
WAM Z67321	*Lobophytum*	sp.	GW8625	Kimberley	Rowley shoals
WAM Z67332	*Lobophytum*	sp.	GW8626	Kimberley	Rowley shoals
WAM Z67392	*Lobophytum*	sp.	GW8627	Kimberley	Rowley shoals
WAM Z67394	*Lobophytum*	sp.	GW8628	Kimberley	Rowley shoals
WAM Z67416	*Lobophytum*	sp.	GW8629	Kimberley	Rowley shoals
WAM Z67420	*Lobophytum*	sp.	GW8630	Kimberley	Rowley shoals
WAM Z67238	*Sarcophyton*	sp.	GW8631	Kimberley	Rowley shoals
WAM Z67264	*Sarcophyton*	sp.	GW8632	Kimberley	Rowley shoals
WAM Z67210	*Sarcophyton*	sp.	GW8633	Kimberley	Rowley shoals
WAM Z67218	*Sarcophyton*	sp.	GW8634	Kimberley	Rowley shoals
WAM Z67246	*Sarcophyton*	sp.	GW8635	Kimberley	Rowley shoals

WAM Z67248	*Sarcophyton*	sp.	GW8636	Kimberley	Rowley shoals
WAM Z67263	*Sarcophyton*	sp.	GW8637	Kimberley	Rowley shoals
WAM Z67265	*Sarcophyton*	sp.	GW8638	Kimberley	Rowley shoals
WAM Z67266	*Sarcophyton*	sp.	GW8639	Kimberley	Rowley shoals
WAM Z67306	*Sarcophyton*	sp.	GW8640	Kimberley	Rowley shoals
WAM Z67307	*Sarcophyton*	sp.	GW8641	Kimberley	Rowley shoals
WAM Z67308	*Sarcophyton*	sp.	GW8642	Kimberley	Rowley shoals
WAM Z67311	*Sarcophyton*	sp.	GW8643	Kimberley	Rowley shoals
WAM Z67320	*Sarcophyton*	sp.	GW8644	Kimberley	Rowley shoals
WAM Z67386	*Sarcophyton*	sp.	GW8645	Kimberley	Rowley shoals
WAM Z67390	*Sarcophyton*	sp.	GW8646	Kimberley	Rowley shoals
WAM Z67414	*Sarcophyton*	sp.	GW8647	Kimberley	Rowley shoals
WAM Z67052	*Lobophytum*	sp.	GW8648	Kimberley	Ashmore reef
WAM Z67083	*Lobophytum*	sp.	GW8649	Kimberley	Ashmore reef
WAM Z66921	*Lobophytum*	sp.	GW8650	Kimberley	Ashmore reef
WAM Z67004	*Lobophytum*	sp.	GW8651	Kimberley	Ashmore reef
WAM Z66922	*Lobophytum*	sp.	GW8652	Kimberley	Ashmore reef
WAM Z67051	*Lobophytum*	sp.	GW8653	Kimberley	Ashmore reef
WAM Z67132	*Lobophytum*	sp.	GW8654	Kimberley	Eugene McDermott shoals
WAM Z66995	*Lobophytum*	sp.	GW8655	Kimberley	Ashmore reef
WAM Z67105	*Lobophytum*	sp.	GW8656	Kimberley	Hibernia reef
WAM Z66911	*Sarcophyton*	sp.	GW8657	Kimberley	Ashmore reef
WAM Z66912	*Sarcophyton*	sp.	GW8658	Kimberley	Ashmore reef
WAM Z66914	*Sarcophyton*	sp.	GW8659	Kimberley	Ashmore reef
WAM Z66916	*Sinularia*	sp.	GW8660	Kimberley	Ashmore reef
WAM Z66972	*Sarcophyton*	sp.	GW8661	Kimberley	Ashmore reef

WAM Z66984	*Sarcophyton*	sp.	GW8662	Kimberley	Ashmore reef
WAM Z66988	*Sarcophyton*	sp.	GW8663	Kimberley	Ashmore reef
WAM Z66989	*Sarcophyton*	sp.	GW8664	Kimberley	Ashmore reef
WAM Z67021	*Sarcophyton*	sp.	GW8665	Kimberley	Ashmore reef
WAM Z67025	*Sarcophyton*	sp.	GW8666	Kimberley	Ashmore reef
WAM Z67061	*Sarcophyton*	sp.	GW8667	Kimberley	Ashmore reef
WAM Z67081	*Sarcophyton*	sp.	GW8668	Kimberley	Ashmore reef
WAM Z67121	*Sarcophyton*	sp.	GW8669	Kimberley	Vulcan shoals
WAM Z67133	*Sarcophyton*	sp.	GW8670	Kimberley	Eugene McDermott shoals
WAM Z67139	*Sarcophyton*	sp.	GW8671	Kimberley	Eugene McDermott shoals
WAM Z67147	*Sarcophyton*	sp.	GW8672	Kimberley	Heywood shoals
WAM Z67148	*Sarcophyton*	sp.	GW8673	Kimberley	Heywood shoals
WAM Z67151	*Sarcophyton*	sp.	GW8674	Kimberley	Heywood shoals
WAM Z67517	*Sarcophyton*	sp.	GW8675	Pilbara	Montebello islands
WAM Z67522	*Sarcophyton*	sp.	GW8676	Pilbara	Montebello islands
WAM Z67523	*Sarcophyton*	sp.	GW8677	Pilbara	Montebello islands
WAM Z67531	*Lobophytum*	sp.	GW8678	Pilbara	Montebello islands
WAM Z67554	*Sarcophyton*	sp.	GW8679	Pilbara	Montebello islands
WAM Z67555	*Lobophytum*	sp.	GW8680	Pilbara	Montebello islands
WAM Z67557	*Lobophytum*	sp.	GW8681	Pilbara	Montebello islands
WAM Z67560	*Lobophytum*	sp.	GW8682	Pilbara	Montebello islands
WAM Z67561	*Sarcophyton*	sp.	GW8683	Pilbara	Montebello islands
WAM Z67566	*Lobophytum*	sp.	GW8684	Pilbara	Montebello islands
WAM Z67569	*Sarcophyton*	sp.	GW8685	Pilbara	Montebello islands
WAM Z67573	*Lobophytum*	sp.	GW8686	Pilbara	Montebello islands
WAM Z67585	*Sarcophyton*	sp.	GW8687	Pilbara	Montebello islands

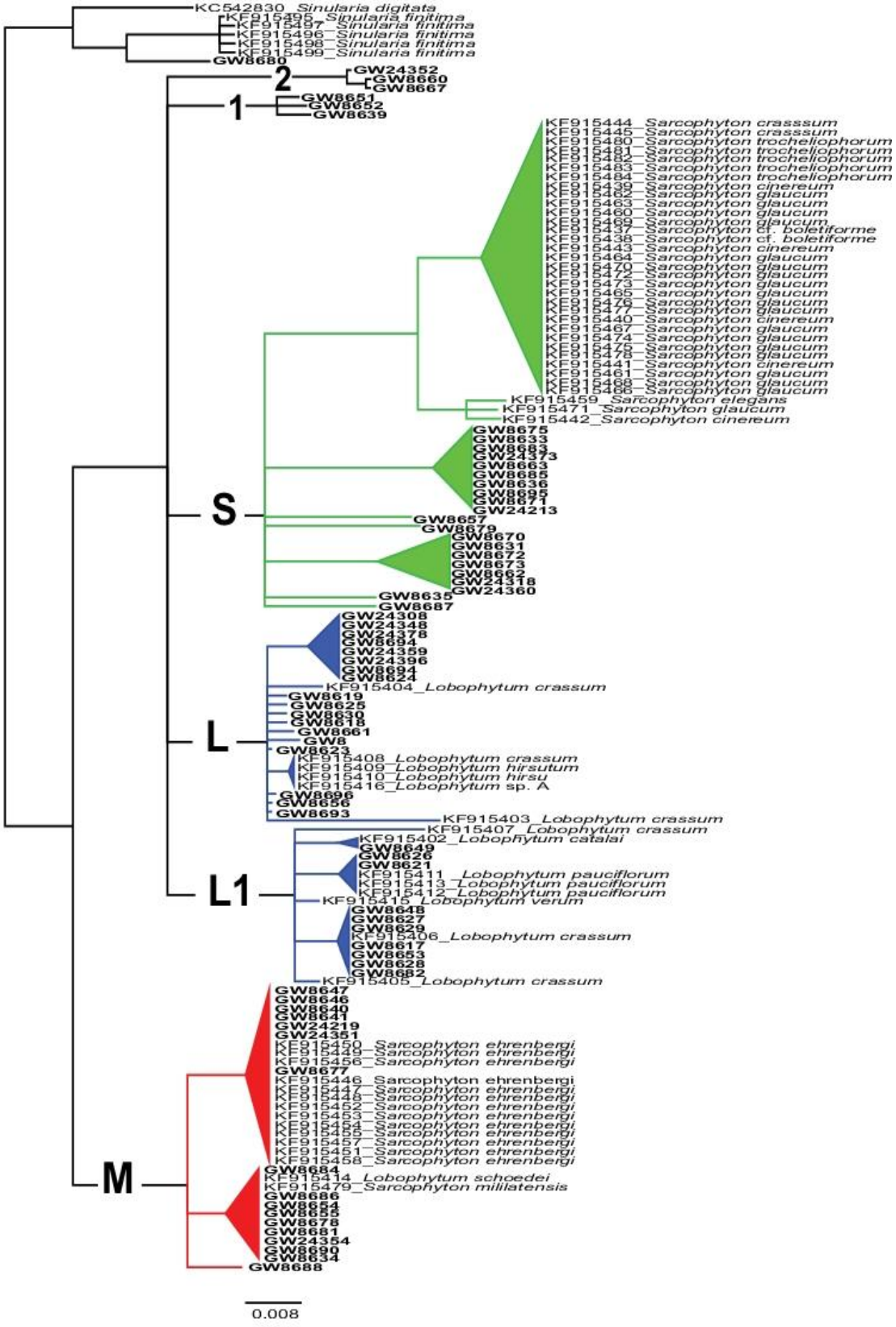
WAM Z67590	*Sarcophyton*	sp.	GW8688	Pilbara	Montebello islands
WAM Z67596	*Sarcophyton*	sp.	GW8689	Pilbara	Montebello islands
WAM Z67615	*Lobophytum*	sp.	GW8690	Pilbara	Montebello islands
WAM Z67624	*Sarcophyton*	sp.	GW8691	Pilbara	Montebello islands
WAM Z67626	*Sarcophyton*	sp.	GW8692	Pilbara	Montebello islands
WAM Z67627	*Lobophytum*	sp.	GW8693	Pilbara	Montebello islands
WAM Z67628	*Lobophytum*	sp.	GW8694	Pilbara	Montebello islands
WAM Z67630	*Sarcophyton*	sp.	GW8695	Pilbara	Murion island
WAM Z67639	*Lobophytum*	sp.	GW8696	Pilbara	Murion island

Appendix 1.5.3 Mitochondrial phylogenetic tree (*COI+mtMutS*)



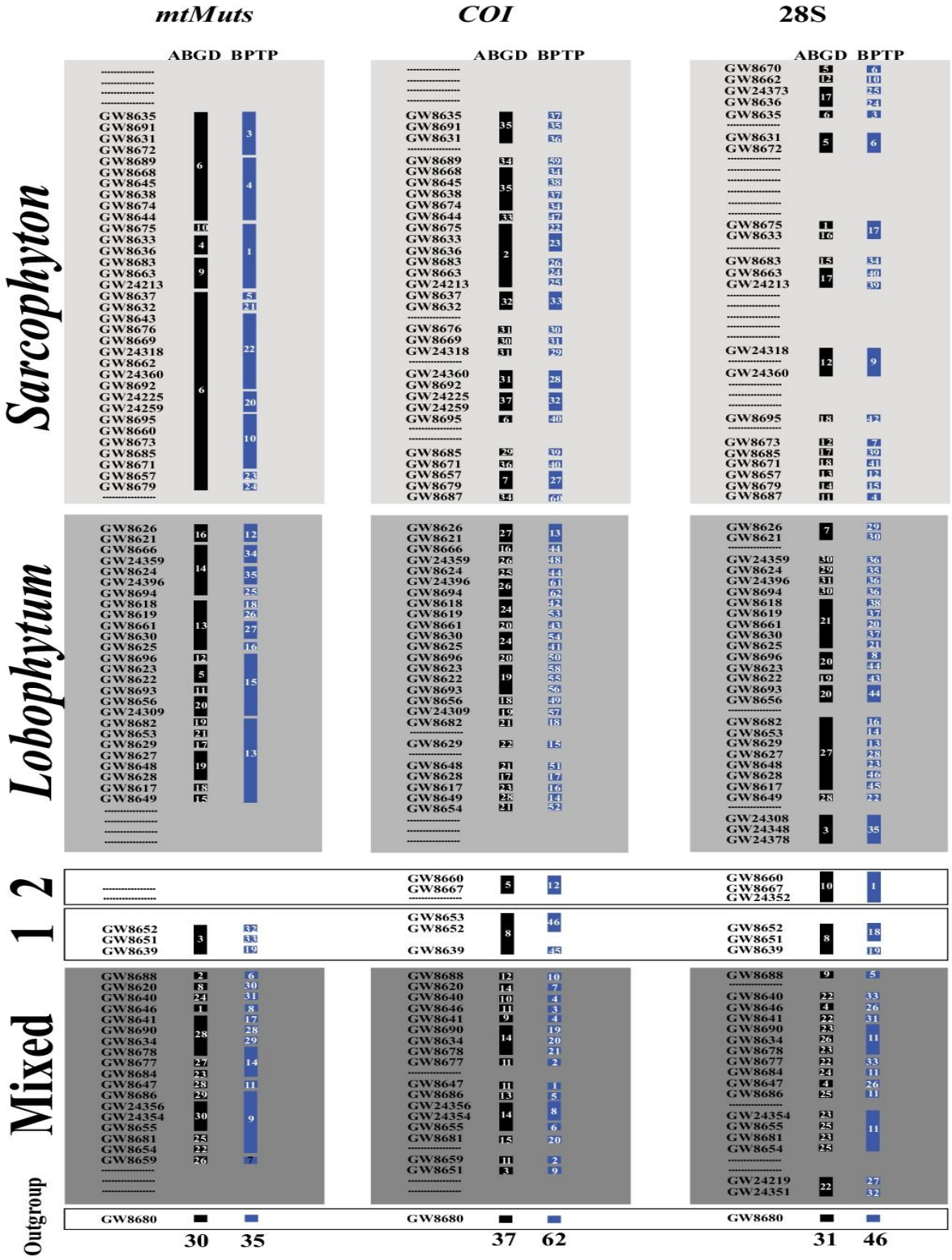
Maximum Likelihood tree obtained from a concatenated mitochondrial dataset (*COI+mtMutS*). Nodes with low support (bootstrap <70) have been collapsed into polytomies. Letters and numbers at the nodes indicate the main clades (S= *Sarcophyton*; L= *Lobophytum*; M= Mixed).

Appendix 1.5.4 Nuclear phylogenetic tree (28S rDNA)



Maximum Likelihood tree obtained from 28S rDNA. Nodes with low support (bootstrap <70) have been collapsed into polytomies. Letters and numbers at the nodes indicate the main clades (S= *Sarcophyton*; L and L1= *Lobophytum*; M= Mixed).

Appendix 1.5.5 Species delimitation and number of estimated entities obtained for the mitochondrial (*MutS* and *COI*) and nuclear (28S) markers



Species delimitation and number of estimated entities obtained for the mitochondrial (*MutS* and *COI*) and nuclear (28S) markers. The black columns indicate the Primary Species Hypothesis inferred with the ABGD, while the blue columns indicate the putative species derived from the BPTP. White numbers correspond to the PSH clusters (ABGD) and putative species (BPTP), respectively. Numbers at the bottom indicate the amount of candidate species for each marker and delimitation method used.

Chapter 2

Historical biogeography and mitogenomics of two endemic Mediterranean gorgonians (Holaxonia, Plexauridae)

This chapter has been submitted as a standalone publication to the Journal “Organisms Diversity and evolution”

Chapter 2

Historical biogeography and mitogenomics of two endemic Mediterranean gorgonians (Holaxonia, Plexauridae)

2.1 Introduction

The Mediterranean Sea, the largest semi-enclosed sea on Earth, is characterized by unique oceanographic conditions (e.g. limited water exchange, distinctive subsurface-water circulation and deep overturning circulation; see Pinardi et al. 2006; Tanhua et al. 2013) and strong environmental gradients (Danovaro et al. 1999). This wide range of climatic and hydrological conditions within the Mediterranean sea have been used to define up to 10 biogeographical regions based on the combination of different geological, physical and biological parameters (Bianchi and Morri 2000) and results in the coexistence of temperate and sub-tropical organisms in this basin (Coll et al. 2010).

The Mediterranean is considered a hot spot of marine biodiversity with a high percentage of endemic species (Bianchi and Morri 2000; Boudouresque 2004). The Mediterranean marine biota is closely related to that of the Atlantic Ocean (Sarà 1985; Bianchi and Morri 2000) and its evolutionary history has been shaped by episodes of isolation and reconnection with the Atlantic. In this regard, evidence for a radiation after the Messinian Salinity Crisis (5.2 Ma) — the isolation and restriction of the Mediterranean due to the closure of marine gateways between the Atlantic ocean and the Mediterranean Sea— have been reported for invertebrates (Sanna et al. 2013) and vertebrates (e.g. Carreras-Carbonell et al. 2005). A second event, the Gelasian Crisis occurred 2.6 Ma (Dov Por 2009) likely affecting the Mediterranean biota and changing its general composition and diversity patterns. Although a wealth of oceanographic and ecological data exist for the Mediterranean Sea (e.g. Sarà 1985; Pinardi et al. 2006; Bas 2009) little is known about the evolutionary history of different Mediterranean taxonomic groups. Among octocorals in particular, the few studies so far published have been mostly focused on the population genetics of economically and/or ecologically relevant species in the

region such as the precious coral *Corallium rubrum* (Costantini et al. 2013; Pratlong et al. 2015) and the red gorgonian, *Paramuricea clavata* (see Angell et al. 2009; Moktar-Jamaï et al. 2011).

The genus *Paramuricea* (Koelliker 1865) has been reported from different latitudes including the Gulf of Mexico (Doughty *et al.* 2014), North-Western Atlantic (Thoma et al. 2009), Mediterranean (Bo et al. 2012; Angeletti et al. 2014) and South Eastern Australia (Alderslade et al. 2014). The genus currently includes more than 20 species, two of which, *P. clavata* and *P. macrospina* are endemic to the Mediterranean. *P. clavata* is one of the most common gorgonians dwelling the coralligenous assemblages of the Mediterranean. *P. macrospina* has a more restricted distribution occurring mainly in detritic or sandy deep-sea bottoms up to 200 m deep (Carpine and Grasshoff 1975) and is also common in the oligotrophic Sea of Marmara (Topçu and Öztürk 2015). Despite their ecological importance and their widespread distribution across the Mediterranean basin, the phylogenetic affinities of the Mediterranean *Paramuricea* and their historical biogeography remain largely unstudied. In order to shed light on the molecular (phylo)diversity of the Mediterranean *Paramuricea* and on the evolutionary processes leading to the diversification of these species, here we (I) sequence the complete mitochondrial genome of the two Mediterranean endemic species, (II) assess their phylogenetic relationships with other members of this widespread genus and (III) infer the biogeographic history of *Paramuricea* to clarify the processes involved in the diversification of the Mediterranean species and shed light on the possible historical events that shaped the evolutionary history of this genus in the Mediterranean.

2.2 Materials and methods

2.2.1 Study area, sampling and taxonomic identification

Paramuricea macrospina (LT576168, LT576169) and *P. clavata* (LT576167) were collected in the Mediterranean, whereas *P. grayi* was sampled in Galicia (LT576170) and the Bay of Biscay (LT576171) (North-Eastern Atlantic). All the samples were collected by SCUBA diving at depths ranging from 10 to 90 m and were preserved in absolute ethanol for molecular analyses. Species assignments done in the field were further corroborated by the morphological analysis of the colony and sclerome.

2.2.2 Molecular analyses

Genomic DNA was extracted following three different protocols. The Macherey-Nagel NucleoSpin® Tissue kit (M&N, Düren, Germany), a modified CTAB phenol-chloroform (Doyle and Doyle 1987) and a salting out extraction methods (Aljanabi and Martinez 1997) were used to obtain high molecular weight DNA. The quality of the DNA was assessed on a 1.5% agarose gel. Mitochondrial DNA fragments of different sizes (500-4000 bp) were amplified using published octocoral specific primers (i.e. France and Hoover 2002; Sánchez et al. 2003; McFadden et al. 2004) as well as additional primers designed to bind regions conserved in the octocoral mitochondrial genomes available to date (see Supplementary Table 1). For *P. grayi* and *P. macrospina* (LT576169) the 5' end of *mtMutS* was amplified using the primers ND42599F (France and Hoover 2002) and Mut-3458R (Sánchez *et al.* 2003) following previously published protocols. PCR products were purified using a polyethylene glycol (PEG)-NaCl precipitation and were sequenced with different internal primers (see Supplementary Table 1) using the BigDye® Terminator v3.1 chemistry in an ABI PRISM® 3700 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The mitochondrial genomes were assembled, edited and annotated using Geneious 6.0.5 (Kearse et al. 2012). This program was also used to estimate GC-content and to annotate and create a graphical map of the genomes. Nucleotide diversity (π) between genomes was calculated in DnaSP 5.10.1 using a sliding window of 500bp (Librado and Rozas 2009). The complete mitochondrial DNA sequences were deposited in the European Nucleotide Archive (ENA accession numbers: LT576167-LT576171).

2.2.3 Extended Phylogenetic analyses

A fragment of about 750 bp of the octocoral mitochondrial gene *mtMutS* was extracted from the mitochondrial genome of the two Mediterranean species and was aligned with the sequences of *P. grayi* here generated and with sequences of other plexaurids deposited in public sequence repositories. A dataset consisting of 166 taxa of which 150 belong to the genus *Paramuricea*, was used to assess the phylogeny of the genus *Paramuricea.* This dataset contained many identical genotypes. Thus a second, reduced dataset (see Supplementary Table 2 for the list of species used) was generated including only 26 *Paramuricea* representative species. This dataset was used for phylogenetic inference and Dispersal-Vicariance analyses (S-DIVA, see below). All sequences were aligned in MUSCLE (Edgar 2004) with the default options available through Seaview 4.5.3 (Gouy *et al.* 2010). Alignments

used in this study are available at OpenDataLMU (DOI: http://dx.doi.org/10.5282/ubm/data.89).

A maximum likelihood tree was inferred in RAxML 7.2.8 (Stamatakis 2006) under the GTR + Γ model (General Time Reversible + Gamma). Branch support was assessed through a rapid bootstrap analysis (Stamatakis et al. 2008) with a random seed number and 1000 pseudo-replicates. For the Bayesian analyses we used BEAST 2.3.1 (Bouckaert et al. 2014) under the best-fit substitution model selected using the AIC (i.e. GTR + Γ + I) in the program jModeltest 2.1.3 (Darriba et al. 2012). The Markov chain Monte Carlo was run for 10,000,000 generations sampling every 1000 generations. Convergence of the Markov chains was assessed in Tracer 1.6 (Rambaut and Drummond 2009). One tenth of the sampled trees (i.e. 1000 trees) were discarded as burn-in and the species: *Eunicea fusca*, *Muricea elongata*, *Plexaura homomalla* and *Pseudoplexaura porosa* were used as outgroup.

2.2.4 Biogeographic analyses

The geographic distribution of the species was based on collection localities gathered from the literature (Sánchez et al. 2003; Wirshing et al. 2005; McFadden et al. 2006, 2011; Thoma et al. 2009; Doughty et al. 2014; Vargas et al. 2014). Seven biogeographical areas were considered: (A) Eastern Pacific (Panama and Galapagos); (B) Caribbean; (C) North-West Atlantic; (D) Eastern Atlantic (Gulf of Biscay); (E) Mediterranean; (F) Central Indo-Pacific (Indonesia, Papua New Guinea, Palau, Western Australia and Philippines) and (G) South-West Pacific (Supplementary Table 2). For simplicity the Gulf of Mexico and the Caribbean were included in a single geographic area (B). Ancestral distributions were reconstructed with the Statistical DIVA (S-DIVA; implemented in RASP 3.2, Yu et al. 2014) method. S-DIVA is an extension of classical DIVA (see Ronquist 1997, 2001) that reconstructs ancestral areas using sets of trees (e.g. those sample by the Markov Chain in Bayesian analyses) instead of a fixed topology (Yan et al. 2010). Hence, S-DIVA treats tree topology as a nuisance parameter and allows to account for the effect of uncertainty in tree topology on ancestral area optimization. The method is of special interest in groups, such as octocorals (e.g. Wirshing and Baker 2015; see also Supplementary Fig. 1 and Supplementary Fig. 2), where phylogenetic signal is weak at shallow scales and topological uncertainty is high. In these groups, integrating over a set of possible topologies allows to extract the biogeographic signal that is present in the set of trees independently of whether the summary of the trees (e.g. the consensus) is highly supported or not. For the reconstruction of the biogeographic history of *Paramuricea* we used a set of 10,000 trees generated for the Bayesian analysis and sampled

1000 random trees from the posterior tree space. 10% of the total sampled trees were discarded as burn-in and the Maximum Clade Credibility tree (MCC) from the BEAST analysis was used as the summary tree to display ancestral area reconstruction. The maximum number of areas allowed per node was four.

2.2.5 Molecular dating

For many soft-bodied organisms such as octocorals, with little or no fossil remains, the estimation of divergence times among lineages rely on the use of geological events or mutation rates to calibrate the molecular clock and provide taxon-ages. We used the Bayesian MCMC analysis implemented in BEAST 2.3.1 (Bouckaert et al. 2014) under a GTR + Γ + I substitution model and a strict molecular clock model to date the *mtMutS* tree here inferred (see above). We assumed that the general mutation rate of *mtMutS* ranges between 0.14% and 0.25% Myr $^{-1}$ (see Lepard 2003 in Thoma et al. 2009 and Pante et al. 2015) and tested both rates to derive a conservative (assuming 0.14% mutation rate) and a speculative (assuming 0.25% mutation rate) molecular dating for *Paramuricea*. For each molecular clock analysis the Markov chain was run for 10 million generations, sampling every 1000 generations. The Effective Sample Size (ESS) of each parameter was assessed using Tracer 1.6 (Rambaut and Drummond 2009). Ten percent of the trees were discarded as burn-in and the remaining set of trees was visualised using DensiTree 2.0 (Bouckaert 2010) (Supplementary Fig. 1 and Supplementary Fig. 2). The maximum clade credibility (MCC) tree with mean divergence times and 95% highest posterior density (HPD) interval was summarised in TreeAnnotator.

2.3 Results

2.3.1 Mitochondrial genomes

The mitogenomes of *P. clavata* and *P. macrospina* were 18,669 base pairs (bp) and 18,921 bp respectively (Fig. 1a). The architecture of the two genomes, in terms of genome content and gene order was consistent with that of other octocorals with genome arrangement A (see Brockman & McFadden, 2012). Both genomes contained all 14 mitochondrial protein-coding genes reported in octocorals, the 12S rDNA (SSU) and 16S rDNA (LSU) and one tRNA gene ($tRNA^{Met}$). GC-content was 37% in *P. macrospina* and 37.3% in *P. clavata*. Except for *Nad2* and *Nad4*, which overlapped by 13 bp, the remaining genes were separated by intergenic regions (IGRs) of variable length. In both species the shortest IGRs were 12S rDNA-*Nad1* and

16S rDNA-*Nad2*, which were 4 bp long. The longest IGR was located between *Cox1* and 12S rDNA (150 bp) in *P. clavata* and between *Nad5* and *Nad4* (208 bp) in *P. macrospina*. In total, non-coding regions spanned about 4% of the genome in *P. clavata* and 5.4% in *P. macrospina*. Among the intergenic regions, the highest variability in terms of sequence length was observed between *Cob* and *Nad6* (13 bp and 178 bp in *P. clavata* and *P. macrospina* respectively). Between *Nad5* and *Nad4* we found a variable IGR with single base substitutions and insertion/deletion (Fig. 1b). Sequence variability across the two genomes was 2.6%, excluding gaps. The highest level of nucleotide diversity (π) was found within *Nad4* (3.59%), *mtMutS* (3.46%) and *Nad5* (2.75%). The least variable gene was *Atp8* (0.46%) and other conserved regions were identified within *Nad1*, *Cox3* and *Atp6* (Fig. 1b).

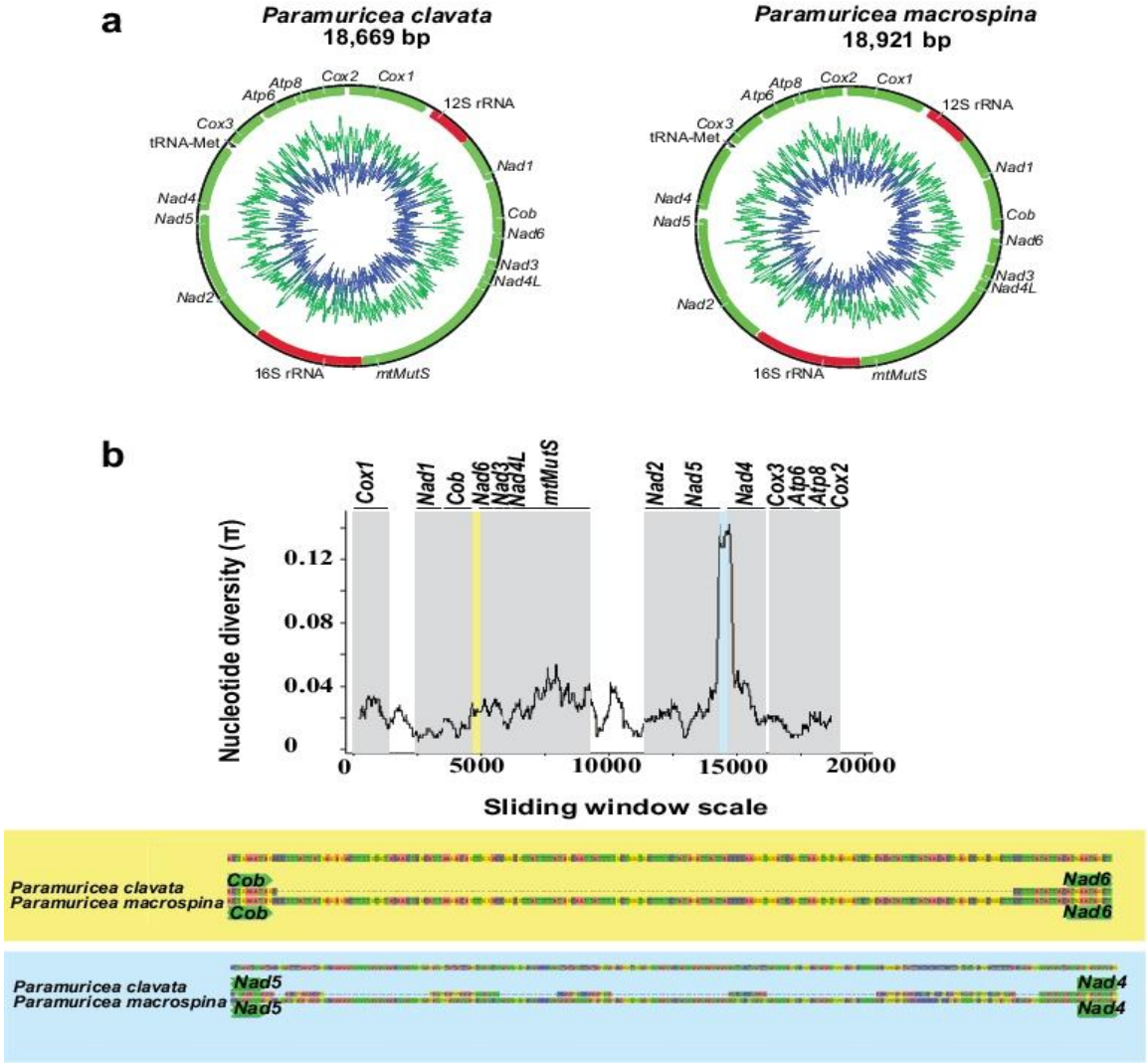


Figure 2.1 Mitochondrial genome structure and genes variability. (a) Mitogenomes of *Paramuricea clavata* and *Paramuricea macrospina* with genome size and genes annotation. GC-content and AT-content are shown in blue and green on the inner and outer surface of the ring, respectively. (b) Sliding window analysis of the complete mitochondrial genomes of *P. clavata* and *P. macrospina*. The black line indicates nucleotide diversity across the miogenome in a window of 500 bp (10 bp steps). Coding protein genes are in grey, white boundaries represent rDNA genes, tRNA gene and 14 intergenic regions (IGRs). The IGRs between *Cob*-*Nad6* and *Nad5*-*Nad4* are highlighted in yellow and blue, respectively, alignments are showed in the two panels below.

2.3.2 Molecular phylogeny

The phylogenetic tree of the partial *mtMutS* gene (~750 nt) showed that the 17 *Paramuricea* species sampled grouped in a clade supported by high bootstrap (BP=89) and posterior probability (PP=0.95) values and that the closest related genera were *Echinomuricea*, *Menella* and *Placogorgia* (Fig. 2). Among *Paramuricea*, most of the shallow nodes are poorly supported (BP <70; PP <0.95) and the phylogenetic relationships within NW Atlantic specimens were not resolved. Twenty-six *Paramuricea* specimens were divided in three clades: the first (I) includes *P. clavata* and *P. grayi*, the second (II) groups *Paramuricea* sp. (DQ297420), *P.* nr. *biscaya* (KF856184) and three different *Paramuricea* sp. types (C, G, H) and the third (III) consists of *P. macrospina*, *P. multispina*, *P. biscaya*, *P.* nr. *biscaya* (KF856209), *P.* nr. *grandis*, and five different *Paramuricea* sp. types (e.g. A, B, D, E, F).

2.3.3 Historical biogeography of Mediterranean *Paramuricea* species

According to our S-DIVA analysis, a total of six dispersal and eight vicariance events occurred during the evolutionary history of the genus *Paramuricea* (Fig. 2). Node 1 included all 26 *Paramuricea* specimens from four main geographic areas (e.g. Caribbean, North-West Atlantic, East Atlantic and Mediterranean). S-DIVA assigns this node, the ancestor of *Paramuricea*, to four different ancestral area reconstructions with similar frequency: NW Atlantic + Mediterranean (30%), NW Atlantic + E Atlantic (25%), NW Atlantic + E Atlantic + Mediterranean (25%) and Mediterranean (19%). The most probable ancestral range at node 2 (i.e. *P. clavata* + *P. grayi* ancestor) is E Atlantic + Mediterranean with an occurrence frequency of 100%. This reconstruction suggests that *P. clavata* and *P. grayi* originated through vicariance of this Atlanto-Mediterranean ancestor. S-DIVA reconstructs a Caribbean + NW Atlantic as the ancestral area of node 3 with an occurrence frequency of 100%. This node is further split in two lineages with distributions in the North Atlantic (Node 4) and the Caribbean (*Paramuricea* sp. H). For node 5 (i.e. the ancestor of the clade including *P. macrospina*) S-DIVA postulates a NW Atlantic + Mediterranean ancestral range with an occurrence frequency of 93%. This ancestral distributions implies a vicariance led to the separation of *P. macrospina* in the Mediterranean and the ancestor of clades 6 and 7 in the NW Atlantic. These nodes (i.e. 6 and 7) are predicted to have two possible ancestral ranges: Caribbean (61%) and Caribbean + NW Atlantic (39%), and NW Atlantic (72%) and Caribbean + NW Atlantic (28%) respectively.

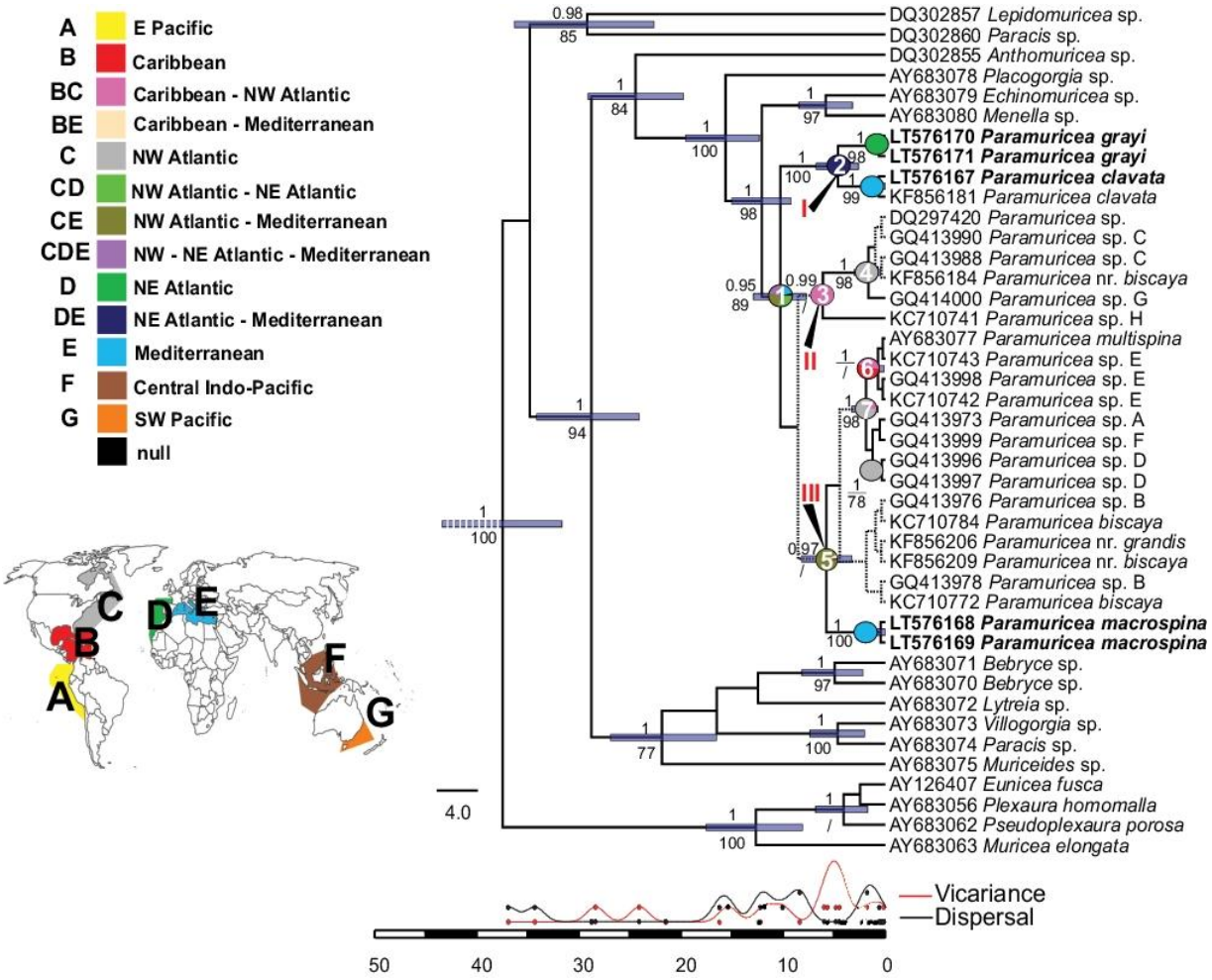


Figure 2.2 Phylogenetic relationships, divergence times and ancestral area reconstructions of the genus *Paramuricea*. The tree is a chronogram inferred using a strict molecular clock and conservative mutation rate (0.14% per million years) based on a BEAST Markov chain Monte Carlo (MCMC) analysis. Numbers above and below the nodes represent posterior probability (PP) and bootstrap (BP) values, respectively. Dashed lines indicate branches which lead to nodes with PP <0.95 and BP <70. Roman numbers indicate the three main groups within the genus *Paramuricea* and the Eastern Atlantic and Mediterranean species analysed in this study are shown in bold. The pie charts indicate the ancestral area reconstruction of a given node based on seven pre-assigned geographic distribution (A-G) for the S-DIVA analysis. The biogeographic regions considered for the analysis are the following: (A) E Pacific (Panama and Galapagos); (B) Caribbean; (C) NW Atlantic; (D) NE Atlantic; (E) Mediterranean Sea; (F) Central Indo-Pacific (Indonesia, Papua New Guinea, Palau, Western Australia and Philippines) and (G) South-West Pacific. Node bares indicate 95% HPD age ranges. The time-event curves for dispersal (red) and vicariance (red) events obtained with RASP are shown on the bottom side of the chronogram. The scale bar in million years.

2.3.4 Molecular dating

Using either a conservative or a speculative molecular rate (see Fig. 2 and Supplementary Fig. 3), molecular dating resulted in a divergence time of 4.6 Ma (95% HPD: 3-7 Ma) or 2.6 Ma (95% HPD: 1-4 Ma), respectively, for *Paramuricea clavata*. The speciation event leading to the diversification of *Paramuricea macrospina* from a NW Atlantic + Mediterranean ancestor was dated at 5.8 Ma (95% HPD: 3-8 Ma) or 3.2 Ma (95% HPD: 2-5 Ma).

2.4 DISCUSSION

2.4.1 Mitogenomics, phylogeny and evolution of Paramuricea

The genus *Paramuricea* is restricted in the Mediterranean to two endemic species that according to our phylogenetic analyses are not closely related. The level of mitochondrial sequence divergence between these two species was high (~2.6%), well above the 1% threshold proposed for *mtMutS*-based species delimitation in octocorals (McFadden et al. 2011). Intrageneric nucleotide diversity in octocorals changes according to the taxonomic group considered and the molecular marker used. In the Mediterranean *Paramuricea* the *Nad5* and *Nad4* showed the highest mutation rate among mitochondrial protein coding genes, and the intergenic region between these two genes was remarkably different in terms of size (100-500bp). For members of *Paramuricea*, this particular mitogenomic region (*Nad5*-igr-*Nad4*) may be of interest as a complementary barcode to the *mtMutS* and *Cox1* regions and should be further investigated. Differences in the length of IGRs in non-bilaterian metazoans (i.e. Cnidaria, Ctenophora, Placozoa and Porifera) contribute wide range of size variation observed in the mitochondrial genomes of these animals and is mainly caused by the presence of repetitive elements such as hairpin-forming elements, palindromes and duplicated segmental DNA (Erpenbeck et al. 2009; Lavrov 2010; Park et al. 2011). Octocorals display a wide range of mitochondrial genome sizes —between 18,668 bp and 20,246 bp among sequenced members of Holaxonia reported in GenBank and the two genomes obtained in this study. Interestingly, neither direct variant repeats (DVRs) or duplicated segmental DNA, as it has been reported in *Calicogorgia granulosa* (for details see Park et al. 2011), were found in any of the other published octocoral mitochondrial genomes or those sequenced here leaving open questions about the molecular mechanisms responsible for mitochondrial genome expansion/contraction in octocorals. A broader sampling effort across the subclass may help to better understand mitochondrial genome evolution among octocorals.

Concerning our phylogenetic results, they showed the shallow-water *P. clavata* sister to E Atlantic *P. grayi*, and *P. macrospina* closely related to deep-sea congeners from the Gulf of Mexico and NW Atlantic. Our analyses also revealed a close genetic relation between the genera *Paramuricea, Echinomuricea* and *Placogorgia,* in agreement with previous studies (see Wirshing et al. 2005; McFadden et al. 2011). The genus *Echinomuricea* includes approximately 30 species with a broad geographic distribution, but generally is regarded as limited to the Indo-West Pacific (Williams and Chen 2014). Grasshoff (1977) reassigned the

Mediterranean endemic *Echinomuricea klavereni* to *Spinimuricea* and the status of the *Echinomuricea* species from the West Indies and the Gulf of Mexico is not clear and needs revision. Similarly, *Placogorgia* represents another poorly known group in need of taxonomic and systematic treatment. It is interesting to note that these three genera as well as those with thorn-scale sclerites (i.e. *Muriceides*, *Thesea*, *Villogorgia*) were grouped into the same family (Paramuriceidae) by Bayer (1956). The phylogenetic affinity of paramuriceids (sensu Bayer 1956) with similar sclerites highlights the importance of morphological characters in evolutionary studies. For instance the thorn-scale sclerites, reported in the family Paramuriceidae, represent a synapomorphic character that was likely present in the most recent common ancestor of this family.

2.4.2 Historical biogeography, vicariance and speciation

Paramuricea and most members of the family Paramuriceidae occur in different biogeographic provinces such as Atlantic, Pacific and Mediterranean. Due to their broad geographic distribution, a Tethyan common ancestor has been proposed for this family (Bayer 1961; Vargas et al. 2010). The Mediterranean is considered to be the evolutionary centre of Tethys fauna remnants (Dov Por 2009) and putative Tethyan relicts have been found in different groups of organisms in this region (Pérès and Picard 1964; Boury-Esnault et al. 1992; Dov Por 2009). These relicts are thought to have survived the Messinian and Gelasian crises (5.2 and 2.6 Ma), during which a dramatic decrease in the number of marine species occurred due to the extreme geological and climatic changes driven by the closing of the strait of Gibraltar and the following desiccation of the Mediterranean Sea (Hsü et al. 1973).

Among octocorals, the existence of early Miocene (~20 Ma) fossils belonging the family Corallidae (Vertino et al. 2014) suggests that a Mediterranean ancestor of this group survived the Messinian crisis 5.2 Ma. In the case of gorgonians, where fossils are lacking, it is hard to establish whether Mediterranean species are derived from an Atlantic ancestor (i.e. they speciated after an ancestral range expansion into the Mediterranean Pérès 1985; Sarà 1985) or speciated *in situ* from a wide spread Atlanto-Mediterranean ancestor. In historical biogeography, however, the distribution of extant species can be used to reconstruct ancestral distributions and clarify the speciation process of taxa when other information sources are lacking. Our biogeographic analysis suggests that the two Mediterranean endemic species originated from two independent vicariance events and we pose that these events are related to the Messinian and Gelasian crises. Interestingly, our molecular clock estimates are in line with this hypothesis. Based on conservative or speculative mutation rates, the divergence estimates

of 4.6 Ma and 2.6 Ma for *P. clavata* and 5.8 and 3.2 Ma for *P. macrospina* match the geological age of these crises. Hence, the origin of the Mediterranean *Paramuricea* seems to be in agreement with the canonical hypothesis about the role played by the Mesinian (and Gelasian) crisis in shaping the evolutionary history of the marine fauna of the Mediterranean. Indeed, after the Messinian crisis the Mediterranean Sea was "refilled" with Atlantic water (Garcia-Castellanos et al. 2009). According to our results, this event could have provided the conditions for the ancestral vicariance event we detected leading to the diversification of the "new" Mediterranean biota due to the new environmental conditions offered by this basin. Our results also demonstrate the importance of vicariance as a driving-force for speciation in the ocean. In this respect, recent studies on the evolution and speciation of Mediterranean organisms (e.g. Domingues et al. 2005; Tougard et al. 2014) reveal that the endemic species are either the result of inter-basin speciation after the Messinian Salinity Crisis or derived from Atlantic ancestor populations which became extinct after the "colonization" of the Mediterranean.

Despite our phylogenetic trees included some branches with uncertainty, the majority of the deep nodes are well supported and the ancestral reconstruction analysis seems to be robust to topological uncertainty. In this regard, the high occurrence frequency values obtained by S-DIVA indicate that the underlying biogeographic signal is strong enough to overcome the topological uncertainty present in the phylogeny of *Paramuricea* at shallow levels. Finally we would like to highlight the somewhat unexpected high phylodiversity of the Mediterranean *Paramuricea*, which represent two highly divergent lineages resulting from independent speciation events. Interestingly, this diversity is also reflected in the ecology of the endemic *Paramuricea* species with the red gorgonian occurring along the littoral and *P. macrospina* restricted to the deeper habitats.

We hope our study provides further information on the genus *Paramuricea* in the Mediterranean that can be used to guide conservation planning and decision making in the area by taking into account the evolutionary and biogeographic history of these organisms.

2.5 Appendix

Appendix 2.5.1 List of the primers used for PCRs and sequencing

Appendix 2.5.2 List of the samples used for phylogenetic and biogeographic analyses

Appendix 2.5.3 Set of trees inferred from BEAST under a strict molecular clock model with a mutation rate of 0.14% per million years

Appendix 2.5.4 Set of trees inferred from BEAST under a strict molecular clock model with a mutation rate of 0.25% per million years

Appendix 2.5.5 Chronogram of the maximum clade credibility tree estimated with a mutation rate of 0.14% per million years

Appendix 2.5.1 List of primers

Primer name	Sequence (5'-3')	Gene
Atp6F_17483	ATTATACCCCCCATTATCCGAACTAC	*Atp6*
Atp6R_16992	GGGTTCGCAATGATTAGTAATGGAATGT	*Atp6*
Atp8F_17634	GAAGTATACTACAGGAAGAGG	*Atp8*
CYTBR_4157	GCTCCCCAAAAGGACATTTGTC	*Cob*
CobOcto-4266F [3]	**TACTACGCTCTATACCGAACA**	***Cob***
COI_smlF	TAATTYTVCCRGGATTTGG	*Cox1*
LIT_1273F	AATMTAACTTTCTTYCCTCAAC	*Cox1*
Cox1R_1123	CATAGTGGAAGTGAGCTACTAC	*Cox1*
COX2F_18105 [1]	**GGTTGAAGGTCACTCGTAGGTATC**	***Cox2***
IGSR_18590 [8]	**GTCAGCAAAGTAACAGGGCTAGAG**	***Cox2***
COX3R_16003 [7]	**GTATTTACTGGTGGGGCTCTTAGC**	***Cox3***
Octo_Cox3r	GTTGGTGTAGAAGTGTTAGA	*Cox3*
12SF_1828 [2]	**GGGTTTCACACTGAGGTCTGTCTA**	**12S rDNA**
12SR_2484 [1]	**GGAACGCTCTACTTCCCGATTAC**	**12S rDNA**
12SF_2457	TGATAGTAATCGGGAAGTAGAG	12S rDNA
Octo_12S	AGGTAAGGTGACACGCGGAT	12S rDNA
16SF_9372	GAGAAAGTACCGTGAGGGAAAGAC	16S rDNA
16SF_10386 [6]	**CACGAGGGTCTTACTGTCTCAAG**	**16S rDNA**
16SR_9395	GTCTTTCCCTCACGGTACTTTCTC	16S rDNA
16S R_10798 [5]	**CACTGTCCTCGATAAGAACTCTCC**	**16S rDNA**
16S-647F [*]	ACACAGCTCGGTTTCTATCTACCA	16S rDNA
NAD1F_3556	GAATGAGATATGACCAACTTATGT	*Nad1*
ND1-56fw	TAGCWTATTTAACWTTRGC	*Nad1*
NAD2F_12137	TGCAGGAATTCCCCCTTTAATT	*Nad2*
ND2-1418R [*]	ACATCGGGAGCCCACATA	*Nad2*
NAD2R_12371	CCCTATTAATATTGCCTTGCCT	*Nad2*

ND3-2126R	CACATTCATAGACCGACACTT	*Nad3*
NAD3R_5855 (2)	**CCACTCTAAGCCTCCTTCTATCCAC**	***Nad3***
NAD4R_14476 (6)	**CAGAGACCACTCTAACGCTTGCTG**	***Nad4***
NAD4F_14772	CAACATTAGATCTGTTAATATT	*Nad4*
ND4Octo-15848	GGTTCAACTCCTGCCTCTAC	*Nad4*
Octo_Nad4r	GCGTCTACCTGTCTGCAAGT	*Nad4*
NAD4lR_6135	GCTCGAACAGCAATTGTACCA	*Nad4L*
ND42599F (4) (^)	**GCCATTATGGTTAACTATTAC**	***Nad4L***
NAD5F_14079 (7)	**GGAAGTTCGGCCATTTTGTGTC**	***Nad5***
NAD5F_12933	ACTTTTGGTTTACGCGTATACA	*Nad5*
NAD5R_13922	AGAGACCATTGTGGGCACAAGC	*Nad5*
ND6-1487F (*)	TTTGGTTAGTTATTGCCTTT	*Nad6*
5' mutSR (3)	**CCGGGTTACTTTGTCCCTGTCCG**	***mtMutS***
3' mutSF (5)	**GCATTAAGCGGGGCTATTGCGG**	***mtMutS***
Sin_mutSF_3'a	GCCCTCTCAATATGGCATTG	*mtMutS*
Sin_mutSF_3'b	TGATTCGCCAGTTCGGTGCT	*mtMutS*
MutSR_8309 (4)	**AAGTAGATTTGCCCGCACCA**	***mtMutS***
tRNA-Met_15925 (8)	**CGTTAGTTGACCCTACAAGCTGAG**	**tRNA^{Met}**

* (McFadden *et al.*, 2004); ^ (France and Hoover, 2002)

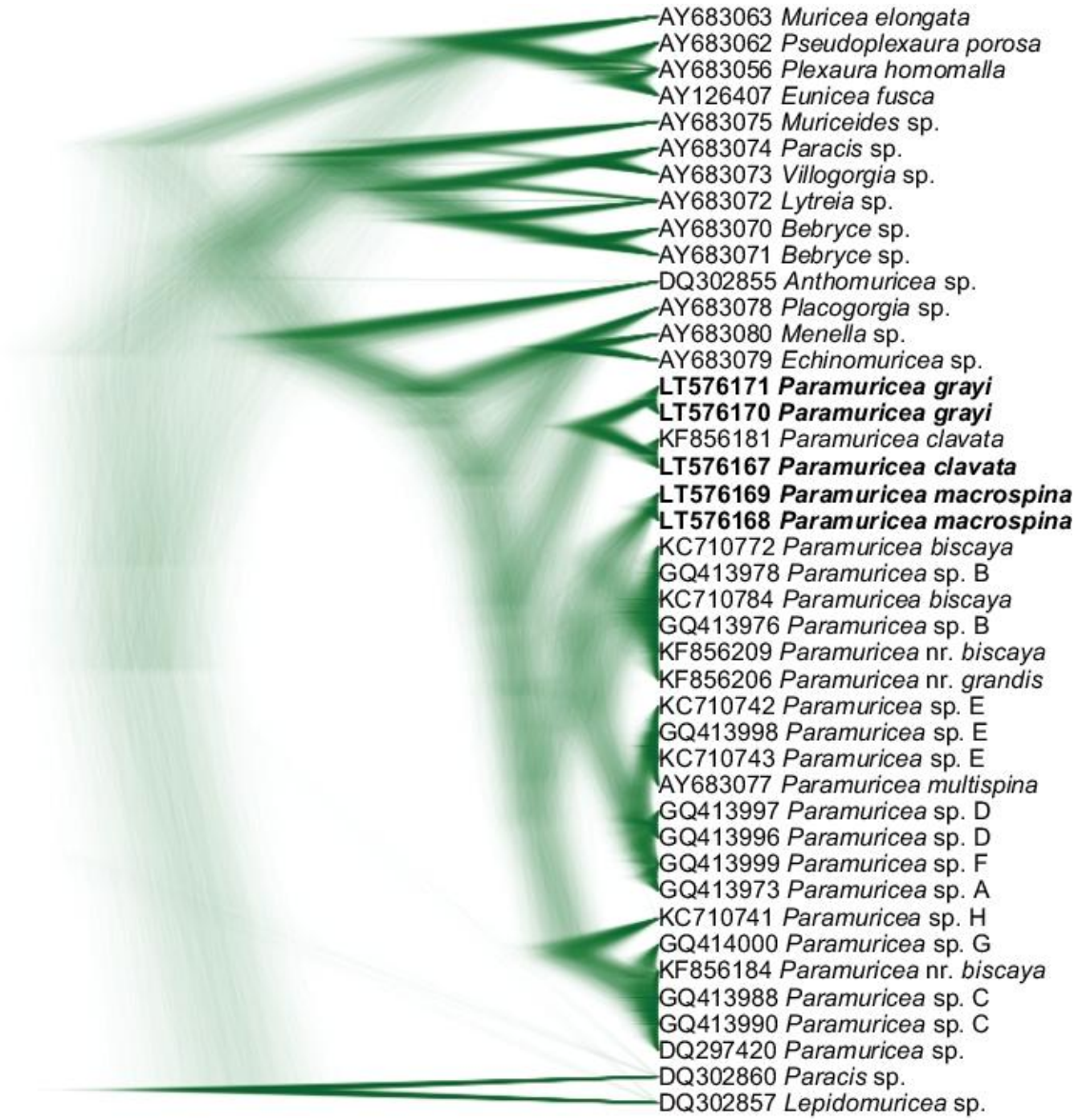
Primers used for long range PCR are in bold, the numbers in brackets indicate those primer pairs used to amplify across gene junctions. The remaining primers were used for sequencing

Supplementary Table 2
List of the specimens used for phylogenetic and biogeographic analyses. Columns from left to right: accession numbers, locality of sampling and distribution. Biogeographic regions: (A) Eastern Pacific (Panama and Galapagos); (B) Caribbean; (C) North West Atlantic; (D) North-Eastern Atlantic; (E) Mediterranean Sea; (F) Central Indo-Pacific (Indonesia, Papua New Guinea, Palau, Western Australia and Philippines) and (G) South-West Pacific

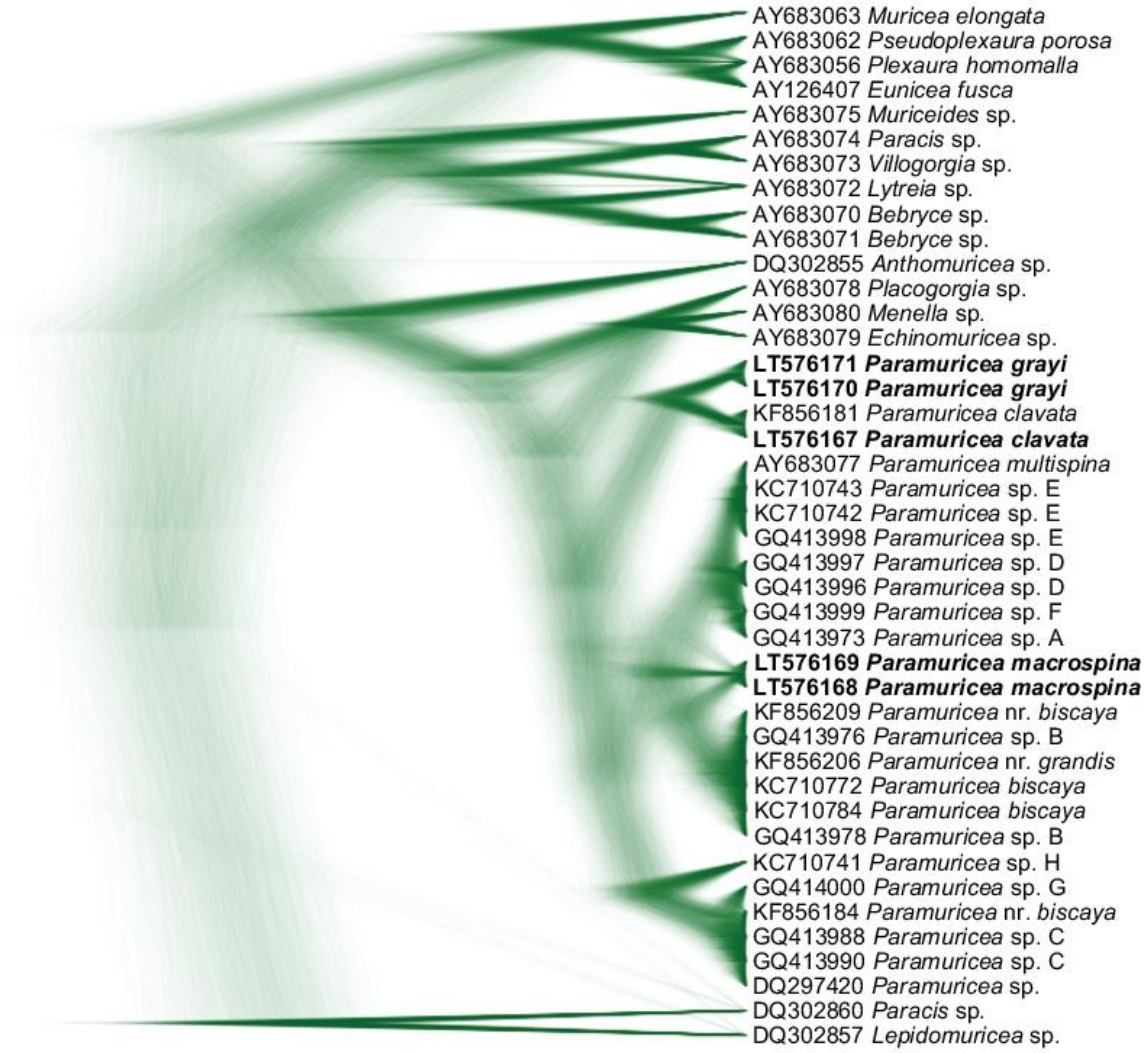
Species	Accession Number	Collection locality	Distribution
Anthomuricea sp.	DQ302855	Tasman Sea	G
Bebryce sp.	AY683070	Bahamas	B
Bebryce sp.	AY683071	Galapagos	A
Echinomuricea sp.	AY683076	Bahamas	B
Echinomuricea sp.	AY683079	Phlippines	F
Lepidomuricea sp.	DQ302857	Tasman Sea	G
Lytreia sp.	AY683072	Jamaica	B
Menella sp.	AY683080	Indonesia	F
Muriceides sp.	AY683075	Florida Keys	B
Paracis sp.	AY683074	Palau	F
Paracis sp.	DQ302860	Tasman Sea	G
Paramuricea biscaya	KC710772	Gulf of Mexico	B
Paramuricea biscaya	KC710784	Gulf of Mexico	B
Paramuricea nr. *biscaya*	KF856184	NW Atlantic	C
Paramuricea nr. *biscaya*	KF856209	NW Atlantic	C
Paramuricea clavata	LT576167*	Mediterranean	E
Paramuricea clavata	KF856181	Mediterranean	E
Paramuricea nr. *grandis*	KF856206	NW Atlantic	C
Paramuricea gayi	LT576170	Galicia	D
Paramuricea gayi	LT576171	Bay of Biscay	D

Paramuricea macrospina	LT576168*	Mediterranean	E
Paramuricea macrospina	LT576169	Mediterranean	E
Paramuricea multispina	AY683077	Curacao	B
Paramuricea sp.	DQ297420	NW Atlantic	C
Paramuricea sp. A	GQ413973	NW Atlantic	C
Paramuricea sp. B	GQ413976	NW Atlantic	C
Paramuricea sp. B	GQ413978	NW Atlantic	C
Paramuricea sp. C	GQ413988	NW Atlantic	C
Paramuricea sp. C	GQ413990	NW Atlantic	C
Paramuricea sp. D	GQ413996	NW Atlantic	C
Paramuricea sp. D	GQ413997	NW Atlantic	C
Paramuricea sp. E	GQ413998	NW Atlantic	C
Paramuricea sp. E	KC710742	Gulf of Mexico	B
Paramuricea sp. E	KC710743	Gulf of Mexico	B
Paramuricea sp. F	GQ413999	NW Atlantic	C
Paramuricea sp. G	GQ414000	NW Atlantic	C
Paramuricea sp. H	KC710741	Gulf of Mexico	B
Placogorgia sp.	AY683078	Panama Pacific	A
Villogorgia sp.	AY683073	Virgin Island	B
Eunicea fusca	AY126407	Bahamas	B
Muricea elongata	AY683063	Florida	B
Plexaura homomalla	AY683056	Bahamas	B
Pseudoplexaura porosa	AY683062	Bahamas	B

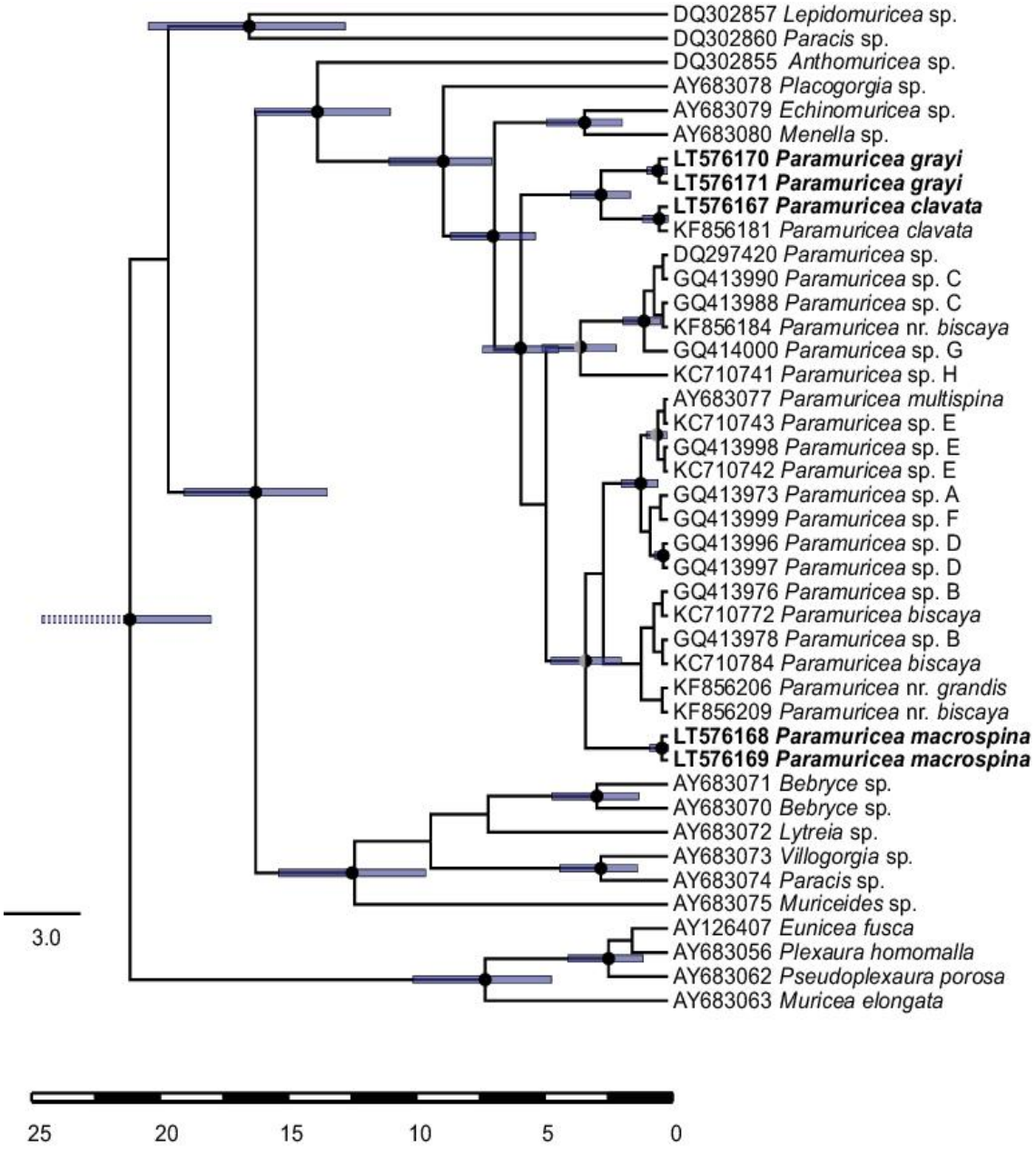
*partial *mtMutS* sequence extracted from the complete mitochondrial genome



Supplementary Figure 1
Topology uncertainty among the different clades shown by a set of trees (10,000) obtained from BEAST. The analyses were run under a strict molecular clock model with a "conservative" mutation rate of 0.14% per million years



Supplementary Figure 2
Topology uncertainty among the different clades shown by a set of trees (10,000) obtained from BEAST. The analyses were run under a strict molecular clock model with a "speculative" mutation rate of 0.25% per million years



Supplementary Figure 3
Chronogram of the maximum clade credibility tree estimated with a speculative mutation rate of 0.25% per million years. Bars at the nodes indicate 95% highest posterior density intervals. Filled circles indicate high support for both ML (BP ≥70) and Bayesian (PP ≥0.95) analyses. Split circles indicate high support for one analysis only (black: high support; grey: low support); the left and right half of the circles refer to ML and Bayesian analyses, respectively. Samples sequenced in this study are in bold. Scale bar in million years

Chapter 3

Comparative mitogenomics, phylogeny and evolutionary history of *Leptogorgia* (Gorgoniidae)

This chapter will be submitted as a standalone publication to the Journal "Molecular Phylogenetics and Evolution"

Chapter 3

Comparative mitogenomics, phylogeny and evolutionary history of *Leptogorgia* (Gorgoniidae)

3.1 Introduction

The genus *Leptogorgia* includes more than 50 valid species with a wide geographic and bathymetric distribution. One third of the species is distributed in the eastern Pacific, EP (Breedy and Guzman, 2007), more than 30 species were reported in the Atlantic (Grasshoff, 1988) and only two species have been described from the Mediterranean (Carpine and Grasshoff, 1975) and subarctic (Williams and Lindo, 1997). The identification and classification of *Leptogorgia* is extremely difficult due to their morphological homogeneity (Prada *et al.* 2008), the phenotypic plasticity observed in the genus (West et al., 1993; Breedy and Guzman, 2007) and the lack of distinct diagnostic morphological characters (Bayer, 1961; Sánchez, 2007) that can be used to differentiate between species. In addition, the systematic history of the genus is complex. The current concept of *Leptogorgia* includes species formerly classified as *Lophogorgia* (Milne Edwards and Haime, 1857) or *Leptogorgia* (Milne Edwards and Haime, 1857); two genera that have been synonimized and segregated multiple times by different authors (Verrill, Bayer, Grasshoff). According to Bayer (1961), *Leptogorgia* included species with short disk-spindles in the outer portion of coenenchyme and uniformly sculptured spindles in the inner part, mostly restricted to the Caribbean and Western Atlantic. *Lophogorgia* was used to group species with symmetrical sculptured spindles that are not fused into a disk-like form and anthocodial sclerites usually flat rods, distributed in the eastern Atlantic-Mediterranean and the eastern Pacific. However, the morphological distinction between *Lophogorgia* and *Leptogorgia* was later questioned by Grasshoff (1988) based on his observation of transitional forms between the two genera.

Molecular studies on gorgoniid octocorals have shown phylogenetic divergence between eastern Pacific and Caribbean species of *Leptogorgia* (Aguilar and Sánchez, 2007; Vargas *et al.* 2014; Ament-Velásquez *et al.* 2016) pointing towards a possible separation of these species groups in agreement with previous morphological hypotheses. Moreover, analyses including eastern Atlantic (EA) *Leptogorgia* representatives (Sánchez, 2007) clearly indicated the need of a systematic revaluation of the genus. Molecular methods such as DNA barcoding have been widely used to complement octocoral traditional taxonomy (Sánchez, 2007; Breedy *et al.* 2012; McFadden and van Ofwegen, 2012, 2013; Bryce *et al.* 2015) and to provide an independent source of evidence that can be used to test morphology-based systematic hypotheses (see e.g. Wirshing *et al.* 2005; Vargas *et al.* 2010, 2014). Single mitochondrial protein-coding genes, especially partial *mtMutS* sequences, are among the most commonly used molecular markers for phylogenetic inference in octocorals (McFadden *et al.* 2006, 2011). Yet the resolution of these markers is known to be low among members of this group, and in general among non-bilaterian metazoans (Huang *et al.* 2008). Despite the relative simplicity in obtaining complete mitochondrial genomes (mitogenomics) using Next Generation Sequencing (NGS)technologies (Maricic *et al.* 2010; Timmermans *et al.* 2010), the recovery of mitogenomes for phylogenetic research remains only superficially explored in octocorals. Indeed, only 30% of the complete mitochondrial genomes publicly available belong to invertebrates (Bernt *et al.* 2013) and among the ca. 3000 octocoral species described to date only 1% have been considered in mitogenomic analyses (Park *et al.* 2011, 2012; Brockman and McFadden, 2012; Figueroa and Baco, 2015). Here, we present the complete mitochondrial genome of five *Leptogorgia* species from the eastern Pacific, Mediterranean and eastern Atlantic and use them to infer a robust phylogeny of the genus to clarify its systematics. We also use an extended dataset based on the *mtMutS* marker to provide divergence time estimates for the different lineages of *Leptogorgia* and discuss the potential role of different geological events that could have played a role on the diversification of this genus through history.

3.2 Material and Methods

3.2.1 Sampling, DNA extraction, amplification and sequencing

Samples were collected by SCUBA diving from shallow waters in the Mediterranean, eastern Atlantic and eastern Pacific. Genomic DNA was extracted from the tissue of a single individual using a modified CTAB method (Porebski *et al.* 1997). Except for *Eunicella cavolini*, *Muricea crassa* and *Muricea purpurea*, for which complete mitochondrial genomes were obtained from the sequencing of short-insert libraries, the remaining mitogenomes (i.e. *Eugorgia mutabilis*, *Eunicella albicans*, *Pacifigorgia cairnsi*, *Leptogorgia alba*, *Leptogorgia capverdensis*, *Leptogorgia gaini*, *Leptogorgia sarmentosa* and *Leptogorgia palma*) were sequenced using standard primer walking procedures. Depending on the quality and yield of the extracted DNA, amplification was performed using two-step or three-step PCRs. The primers utilised for the amplification of complete mitochondrial genomes are available in the Appendix 2.5.1. Specific primers were designed in order to bridge the gaps in some mitochondrial intergenic spacers (e.g. *Nad5-Nad4*) and protein coding genes (i.e. *Nad2* and *mtMutS*) in *Eunicella albicans* and *Pacifigorgia cairnsi* (see Appendix 3.6.1). For standard three-step PCR, the annealing temperature and the extension time were adjusted according to the primer pairs used and the expected amplicon lengths. PCR was performed in 12.5 µL volumes following the GoTaq® G2 Flexi DNA Polymerase (Promega, Madison, WI, USA) or the Crimson LongAmp™ *Taq* DNA Polymerase (New England BioLabs) protocols. PCR products were purified by precipitation with one volume 20% (w/v) polyethyleneglycol 8000 in 2.5molL^{-1} NaCl and sequenced directly using the BigDye 3.1 chemistry (Applied Biosystems, Foster City, CA, USA) with same primers used for PCR or by primer walking. All mitogenome sequences will be submitted to the European nucleotide archive.

3.2.2 Mitogenomes assembly, annotation and comparison

Sequences were checked, assembled and annotated using GENEIOUS 6.0.5 (Kearse *et al.* 2012). Nucleotide composition, GC content and pairwise genetic distances were calculated in MEGA 6.06 (Tamura *et al.* 2013). Nucleotide diversity in the coding-protein genes was assessed with DnaSP 5.10.1 (Librado and Rozas, 2009). The presence of DNA tandem repeats was detected with the Tandem Repeats Finder server 4.08 (Benson, 1999) available at https://tandem.bu.edu/trf/trf.html.

3.2.3 NGS and mitogenome assembly

Genomic DNA libraries for *Eunicella cavolini*, *Muricea crassa* and *Muricea purpurea* were prepared using standard library preparation procedures for Illumina sequencing. Libraries were multiplexed and sequenced in a single lane of an Illumina HiSeq (100bp pairs end) at the Gene Center of the Ludwig Maximilians Universität. Quality was assessed with FastQC (Andrews, 2010). Low quality reads and Illumina TruSeq adaptors were trimmed with Trimmomatic 0.3.2 using Trinity RNA-Seq 2.0.6 (Grabherr *et al.* 2011) and the same program was utilised for *de novo* assembly of the mitogenomes.

3.2.4 Phylogenetic analyses of the mitogenomic dataset

The complete mitochondrial genomes obtained were aligned with those of other octocorals available in Genbank using MUSCLE with default parameters (Edgar, 2004). Due to the existence of different mitochondrial gene arrangements in Octocorallia, protein-coding genes were extracted from each mitochondrial genome and aligned to produce single gene matrices that were concatenated to produce an alignment including 14 mitochondrial protein-coding genes. Sequence annotation was not consistent within the downloaded mitogenomes and some differences were reported in the Coding DNA Sequences (CDSs). Therefore, before concatenation, all the alignments were visually inspected and in some cases, the mitochondrial protein-coding genes were trimmed at one or both their 5' and 3' ends. The concatenated alignment was used for mito-phylogenetic analyses of the subclass Octocorallia. Maximum Likelihood (ML) and Bayesian analyses were performed in RAxML 7.2.8 and MrBayes 3.2.5 (Ronquist and Huelsenbeck, 2003) respectively. The ML tree was inferred under the GTRGAMMA substitution model, using 1000 bootstrap pseudo-replicates. Under Bayesian analysis the best-fit substitution model (GTR+I+G) was selected using the Akaike Information Criterion (AIC) in jModeltest 2.1.3 (Darriba *et al.* 2012). The Metropolis-coupled Markov chain Monte Carlo (MCMCMC) was run for 10,000,000 generations, sampling every 500 steps, and 25% of the sampled trees were discarded as burn-in. The analysis was allowed to run until the average standard deviation of the split frequencies (ASDSF) was below 0.009. In addition, MCMCMC convergence was assessed with Tracer 1.6 (Rambaut *et al.* 2014). The resulting mitogenomic phylogeny was rooted using the Calcaxonian-Pennatulacea species as outgroup. All the alignments used in this study will be available at OpenDataLMU.

3.2.5 Bayesian analyses, fossil calibration and divergence time estimation

We used a dataset including 64 *mtMutS* sequences (59 gorgoniids and 5 plexaurids) and 725 nucleotide positions (see Appendix 3.6.2 for the list of samples used). All the sequences were aligned with MUSCLE using the default options; the alignment generated will be available at OpenDataLMU. Divergence time estimation was done using an uncorrelated lognormal relaxed clock model as implemented in BEAST 2.3.2 (Bouckaert *et al.* 2014). The best-fit substitution model (HKY+G) was selected with the AIC in jModelTest, while the Yule tree prior speciation model was choose for divergence time estimation analyses using inter-species sequences (Aldous, 2001; Gernhard, 2008). For molecular dating, the age of crown *Eunicella* was constrained using a lognormal prior distribution with an offset of 28.4 Ma, mean = 1 and standard deviation = 1.

Among gorgonians, fossils are rare and those found are difficult to assign to genera with confidence due to the lack of diagnostic characters among sclerites (Kocurko and Kocurko, 1992). Gorgonian fossils have been reported from Texas (Middle Eocene) (Giammona and Robert, 1980), Poland (Upper Cretaceous) (Malecki, 1982), Mississippi (Lower Oligocene) (Kocurko and Kocurko, 1992) and recently from Wales (Lower Ordovician) (Cope, 2005). The fossil assemblages from the Red Bluff Formation in Mississippi —dated to the Lower Oligocene (28.4-33.9 Mya) —represent one of the most diverse collections found from a single location with about 1000 well-preserved sclerites. The majority of the sclerites described belong to Plexauridae and Gorgoniidae genera and among them balloon-club sclerites, belonging the genus *Eunicella*, can be clearly identified (Kocurko and Kocurko, 1992). Fossil of *Eunicella* sclerites was used as a single calibration point in our molecular dating analyses. For molecular dating, the MCMC ran for 10 million generations, sampling every 1000 generations, until the Effective Sample Size (ESS) for all parameters was >200. ESS and convergence onto the posterior distribution were assessed using Tracer 1.6 (Rambaut *et al.* 2014). Among the 10000 trees obtained, 10% were discarded as burn-in and the maximum clade credibility (MCC) tree with mean divergence times for the nodes was summarized in TreeAnnotator.

3.3 Results

3.3.1 Mitogenomes

Mitogenome size in *Leptogorgia* species ranged between 18,722 bp in *L. sarmentosa* and *L. capverdsensis* to 19,681 bp in *L. gaini*. All species investigated have the same gene arrangement including 14 protein-coding genes, 2 ribosomal RNA subunits (12S and 16S) and one methionine tRNA gene ($tRNA^{Met}$). Among the protein-coding genes, four were encoded in the light strand (*Cox2*, *Atp8*, *Atp6* and *Cox3*) and ten in the heavy strand (*Cox1*, *Nad1*, *Cob*, *Nad6*, *Nad3*, *Nad4L*, *MutS*, *Nad2*, *Nad5* and *Nad4*). For *L. sarmentosa* and *L. alba* the CDS span 79.09% and 78.52% of the mitogenome, whereas CDS for *L. capverdensis*, *L. gaini* and *L. palma* were 79.33%, 75.20% and 78.95%, respectively. GC content ranged from 36.8% (*L. alba*, *L. capverdensis* and *L. sarmentosa*) to 37.1% (*L. gaini*) (Figure 3.1A). Only one case of gene overlap was observed, with the loci *Nad2* and *Nad5* overlapping by 13 bp. All the other genes were separated by intergenic regions (IGRs). The length of the non-coding regions was different, for *L. sarmentosa* and *L. alba* the longest IGRs were located between *Cox1* and 12S rRNA (134 bp) and between *Nad5* and *Nad4* (204 bp) respectively. In *L. gaini* the longest IGR was between *Cob* and *Nad6* (1041 bp), this region included a tandem repeat and an unidentified putative open reading frame (ORF) for which the *blastx* search of NCBI did not provide any high score hits. For *L. alba*, *L. sarmentosa* and *L. gaini* the shortest IGR (4 bp) occurred between 12S rRNA and *Nad1*. *L. capverdensis* and *L. palma* had a mitogenomic structure similar to that of *L. sarmentosa* and *L. alba*. In particular, the mitogenomes of *L. capverdensis* and *L. sarmentosa* were almost identical with a genetic distance lower than 0.05%. Polymorphic sites (excluding gaps or missing data) in the mitochondrial coding sequences of the five *Leptogorgia* species were ~5% of the total and the following genes: *Nad5*, *Nad4*, *Cob* and *mtMutS* were the most variable (see Figure 3.1B). The start codon ATG was the same for all the protein-coding genes, stop codons TAA and TAG were predominant with the exception of *Cox1* which terminates with an incomplete stop codon (TA). Amino acids frequencies were consistent across the five *Leptogorgia* species and four amino acids (i.e. Leucine, Isoleucine, Valine, Glycine and Alanine) accounted for about half of the total amino acid composition (Figure 3.1C). The complete mitogenomes of *Eugorgia mutabilis*, *Eunicella albicans*, *Eunicella cavolini* and *Pacifigorgia cairni* are described in the

Appendix 3.6.3. The complete mitogenomes of *Muricea crassa* and *Muricea purpurea* have been communicated elsewhere (Poliseno *et al.* 2016).

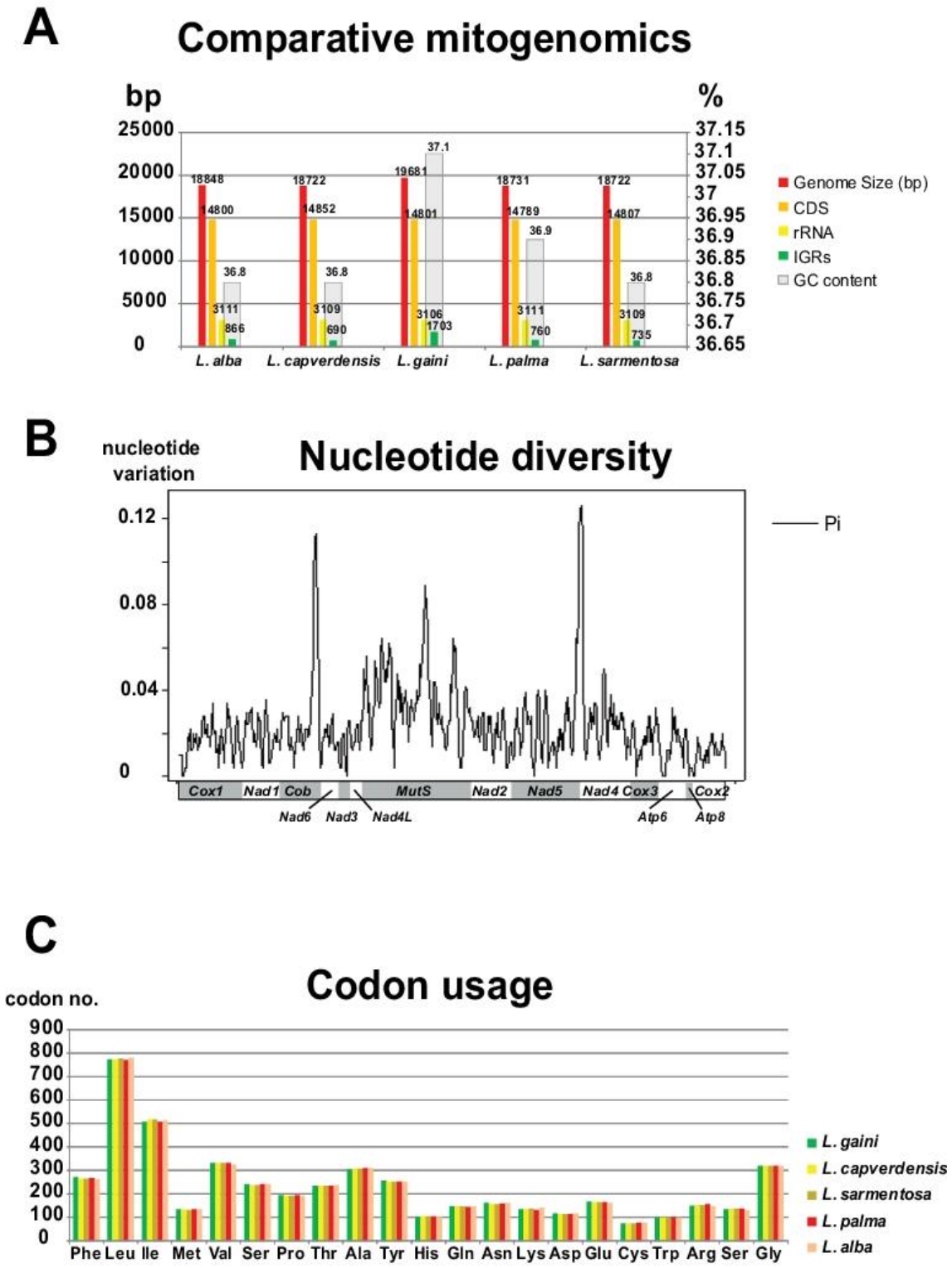


Figure 3.1 Nucleotide and amino acid comparisons between the five *Leptogorgia* mitogenomes. A: Comparative mitogenomics including information on mitogenome size (red) and length (bp) of Coding DNA Sequence (orange), 12S rRNA and 16S rRNA genes (yellow), intergenic regions (green) and GC-content (grey). B: Sliding window analysis on mitochondrial protein-coding genes. The black line indicates nucleotide diversity across the 14 protein-coding genes in a window of 500 bp (10 bp steps). C: Codon usage of the 21 amino acids.

3.3.2 Mito-phylogeny

The trees obtained with ML and Bayesian methods have the same topology and the separation of the species into two main groups is supported. One (I) clade conteins the scleraxonian genera (*Corallium*, *Paracorallium*, *Paragorgia* and *Sibobagorgia*) and the alcyonaceans *Anthomastus* sp. and *Paraminabea aldersladei*. The second one (II) comprised *Briareum asbestimum* and 25 alcyoniina specimens further divided into the Alcyoniidae, Nephtheidae, and "gorgonian" groups (Figure 3.2). The "Gorgonian group" includes members of the families Gorgoniidae and Plexauridae. *Leptogorgia* was split into two clades (1 and 2). *L. sarmentosa* and the eastern Atlantic species (*L. gaini*, *L. capverdensis* and *L. palma*) formed a well-supported clade (1) sister to a clade (2) including *Pseudopterogorgia bipinnata*, *Pacifigorgia cairnsi*, *Eugorgia mutabilis* and *Leptogorgia alba*.

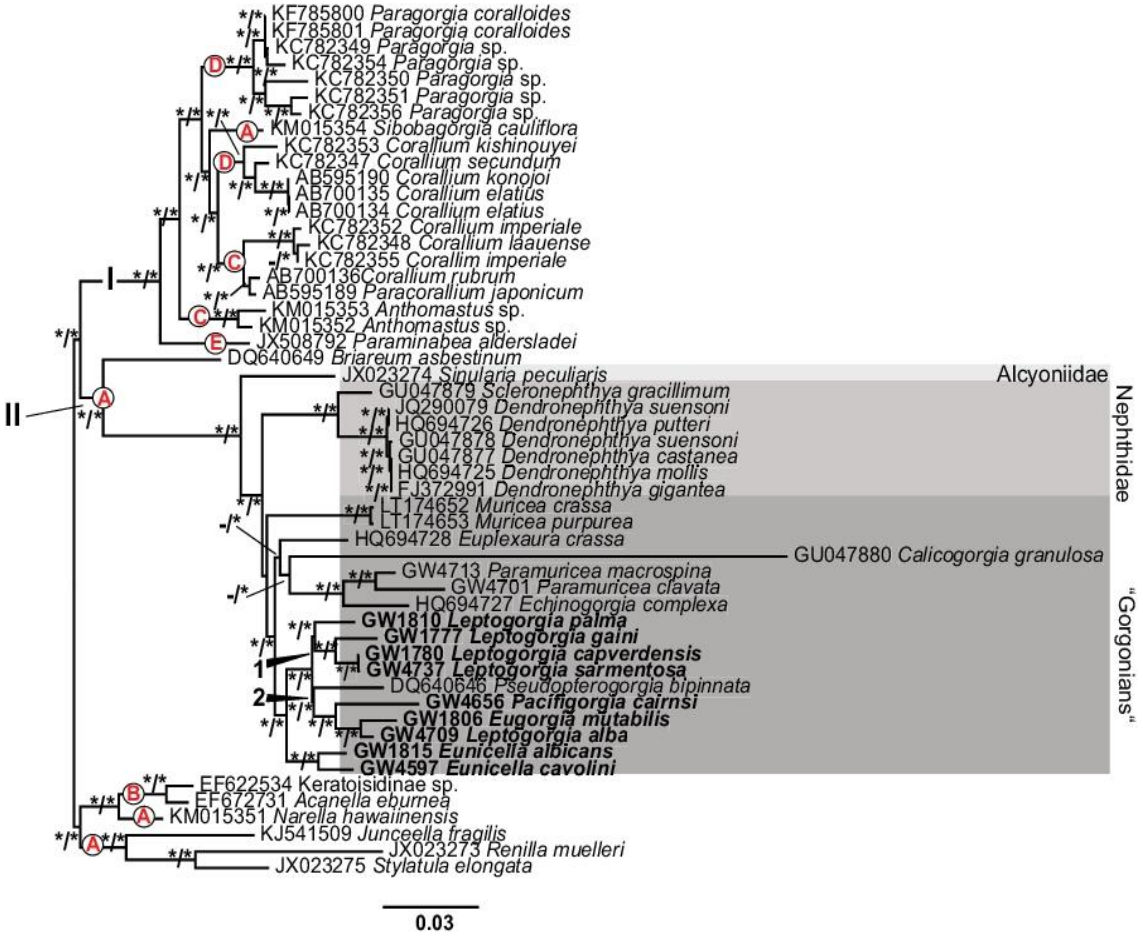


Figure 3.2 Phylogenetic tree of the subclass Octocorallia obtained using complete mitogenomes. Roman numbers indicate the two main clades. Red letters on the branches refer to the mitochondrial gene arrangement according to Brockman and McFadden (2012). Asterisks at the nodes indicate bootstrap values ≥70 (left) and posterior probabilities ≥0.95 (right), respectively. Black arrows and numbers correspond to the two main groups of the family Gorgoniidae. Grey boxes include species belonging different families such as Alcyoniidae and Nephthidae. Gorgoniidae and Plexauridae members have been simplify under the general name of "gorgonians". Newly sequenced samples are in bold.

3.3.3. *MtMutS* phylogeny and pairwise genetic distances

The maximum clade credibility tree obtained from BEAST recovered a polyphyletic *Leptogorgia* divided in four main groups (Figure 3.3).

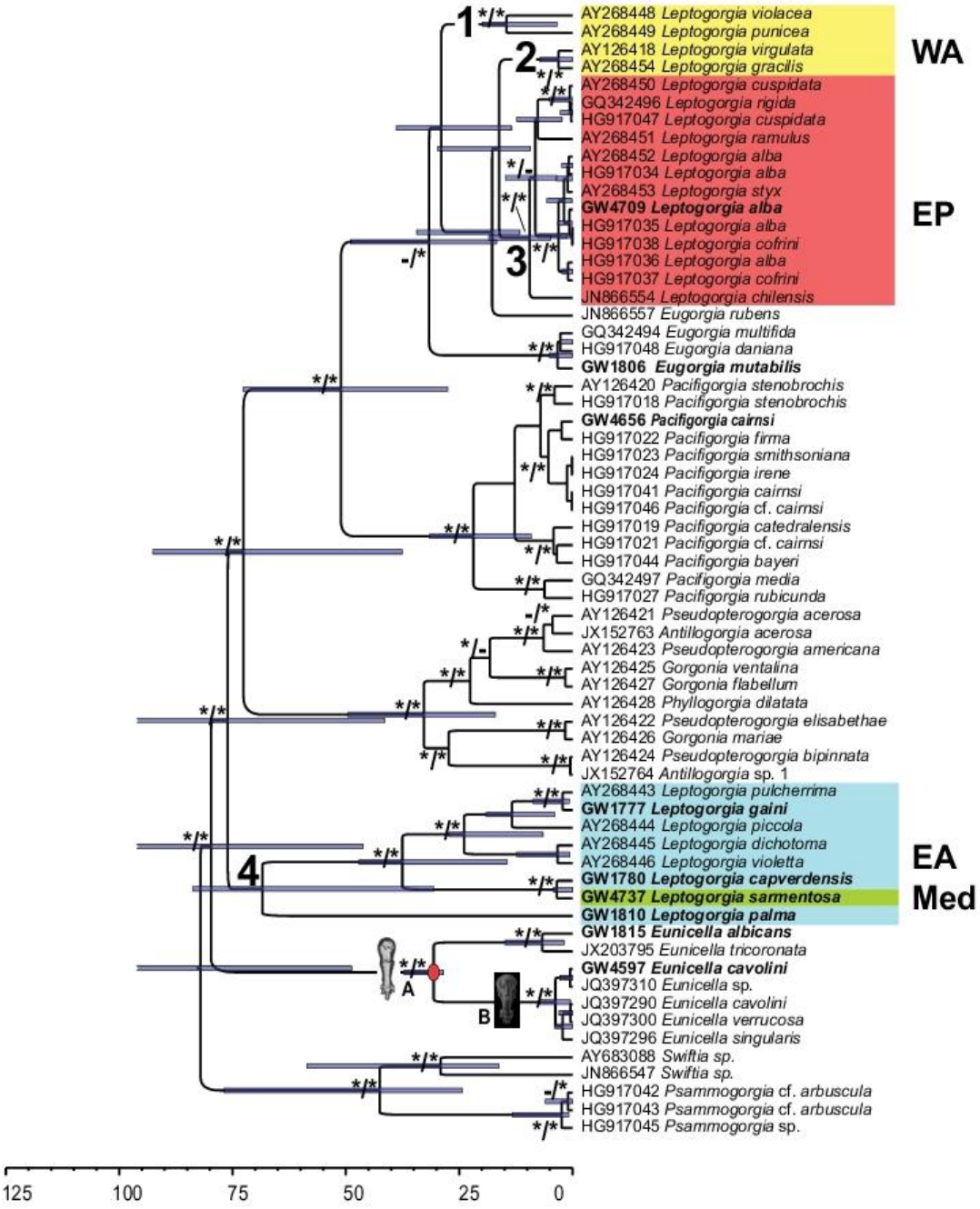


Figure 3.3 Chronogram of the maximum clade credibility tree performed using BEAST. Node bars show 95% highest posterior density intervals (HPD). Asterisks indicate those nodes with posterior probabilities ≥0.95 (right) and bootsrap values ≥70 (left). Numbers from 1 to 4 refer to the main *Leptogorgia* groups. Red circle indicates fossil calibration node. Scale bar on the bottom of the tree refers to million years. Western Atlantic and eastern Pacific *Leptogorgia* are included in yellow and red boxes, respectively. Those collected from eastern Atlantic and Mediterranean are included in light blue and green boxes, respectively. Pictures of sclerites correspond to: A: balloon-club fossil of *Eunicella* sp. (from Kocurko and Kocurko, 1992) and B: balloon-club of *Eunicella singularis* (from Gori *et al.* 2012).

The western Atlantic species (e.g. *L. violacea*, *L. punicea*, *L. virgulata* and *L. gracilis*) were divided into two well-supported clades (1 and 2). All eastern Pacific species (i.e. *L. alba*, *L. cuspidata*, *L. rigida*, *L. ramulus*, *L. styx*, *L. cofrini* and *L. chilensis*) grouped in a well-supported clade (3). Western Atlantic and eastern Pacific *Leptogorgia* species were sister to *Eugorgia*, which was not monophyletic due to the exclusion of *E. rubens*. This clade was sister to *Pacifigorgia*. Eastern Atlantic *Leptogorgia* species formed a clade (4) with *Leptogorgia palma* sister to the remaining species. This branch had a low posterior probability (PP=0.92). Among the remaining species, the Mediterranean *L. sarmentosa* was closely related to *L. capverdensis* and both were sister to a clade including *L. pulcherrima*, *L. gaini*, *L. piccola*, *L. dichotoma* and *L. violetta*.

Pairwise genetic distances among the eastern Pacific *Leptogorgia* species ranged from 0 to 0.01%. The genetic distances between western Atlantic and eastern Pacific *Leptogorgia* species ranged from 0.01 to 0.02 (Appendix 3.6.4). Genetic distances between western and eastern Atlantic species are overall high ranging from 0.04 to 0.06. Among eastern Atlantic species, the greatest genetic distance (0.04%) was reported between *L. palma* and the following species: *L. capverdensis*, *L. pulcherrima*, *L. sarmentosa* and *L. violetta*, whereas *L. sarmentosa* and *L. capverdensis* shared identical haplotypes. The mean pairwise genetic distances within the eastern Atlantic species was 0.02%, five times higher than that within eastern Pacific species, and two time higher than the distance between eastern Pacific and western Atlantic congeners.

3.3.4 Molecular dating

According to our molecular dating analyses, the divergence time between the WA *Leptogorgia* groups, *L. violacea-L. punicea* and *L. virgulata-L. gracilis*, and EP *Leptogorgia* was 28.97 Mya (95% HPD: 13-39 Mya) and 16.17 Mya (95% HPD: 9-30 Mya), respectively (see Fig. 3). Separation within EP *Leptogorgia* species groups occurred 9.46 Mya (95% HPD: 18-54 Mya) and the divergence time between species typically confined to the Central-Pacific America (e.g. *L. alba*, *L. cofrini*, *L. cuspidata*) and the widespread EP *Leptogorgia chilensis* was estimated ~9 Mya. The estimated date for divergence between *Eugorgia* (e.g. *E. daniana*, *E. multifida* and *E. mutabilis*) and *Leptogorgia* from both WA and EP was 31.63 Mya (95% HPD: 17-49 Mya), whereas the separation between these to genera and *Pacifigorgia* was estimated around 51 Mya (95% HPD: 27-73 Mya). When considering the eastern Atlantic *Leptogorgia*, divergence from other gorgonians was ~80 Mya (95% HPD: 46-108 Mya). Within EA

species, separation among *L. palma* and congeners was estimated at ~68 Mya (95% HPD: 31-84 Mya), while divergence time between *L. sarmentosa*, *L. capverdensis* and the other EA species (e.g. *L. pulcherrima*, *L. gaini*, *L. piccola*, *L. dichotoma* and *L. violetta*) was estimated 37.53 Mya (95% HPD: 14-47 Mya). According to our analyses, estimation of divergence time between *L. capverdensis* and *L. sarmentosa* ranges between 0 and 4 Mya. Similarly, separation between *L. gaini* and *L. pulcherrima* occurred 2.18 Mya (95% HPD: 1-9 Mya).

3.4 Discussion

Our mitogenomic analyses on five *Leptogorgia* species revealed the presence of polymorphic and highly variable traits in the non-coding region between *Cob* and *Nad6*. The complete mitochondrial genomes obtained have been also successfully exploited to assess the phylogenetic relationships within *Leptogorgia*, in fact our analysis recovered a fully resolved and highly supported phylogeny which shows divergence between *L. alba* and the Mediterranean and eastern Atlantic congeners. In particular, our results support previously suggested relationships, in which EA *Leptogorgia* species typically characterised by long, spiny and asymmetrical spindles are genetically distinct form the eastern Pacific congeners (Vargas *et al.* 2014; Ament-Velásquez *et al.* 2016). Based on ITS2, Aguilar and Sánchez (2007) demonstrated that some of the western African taxa formerly classified as *Leptogorgia* spp. were split from the WA-Caribbean congeners and Sánchez (2007) proposed for them a taxonomic reallocation into the genus *Filigorgia.* Despite the lack of morphological divergence between EA and EP *Leptogorgia*, our phylogenetic results point the need of a taxonomic revision for the eastern Atlantic species, remarking the possibility of erecting a new genus.

The taxonomic separation into *Leptogorgia* and *Lophogorgia* species according to the presence/absence of symmetrical, asymmetrical and disk-spindle sclerites, as proposed by Bayer (1956, 1961), seems to work only for the West Indies (western Atlantic-Caribbean) taxa but not for the eastern Pacific (Breedy and Guzman, 2007). In addition, the presence of "intermediate" forms in the western Africa fauna (Grassoff, 1988) and the phylogenetic segregation of *Leptogorgia* lineages, may indicate convergent morphological evolution as recently shown for the colony-shape (e.g. whip-like) among eastern Pacific species (Ament-Velásquez *et al.* 2016). Concerning our phylogenetic analyses, the *mtMutS* phylogeny corroborated the polyphyly of

Leptogorgia found in the mitogenomic phylogeny. This analysis also resulted in distinct clades that corresponded to geographical regions (i.e. Mediterranean-eastern Atlantic, eastern Pacific and western Atlantic). Our fossil-calibrated divergence time estimation pointed to a late Cretaceous (~77 Mya) separation of the eastern and western Atlantic gorgonians, likely related to changes in the spreading geometry within the Central and South Atlantic Ocean (see e.g. Cande *et al.* 1988; Bosworth, 1992; Guiraud *et al.* 1992) and a possible decrease in gene flow between the ancestral Atlantic populations. Interestingly, the low genetic distances among the eastern Pacific *Leptogorgia* species is probably due to their recent divergence, estimated between ~3 to 0.8 Mya, which contrasts with the eastern Atlantic fauna long evolutionary history (~68 Mya). We interpret this result as evidence for the existence of multiple old lineages deserving generic status among eastern Atlantic '*Leptogorgia*', something remarkable given the morphological heterogeneity observed among these species and in general in *Leptogorgia*.

Based on shape and size of the colony, and on sclerite composition, Grasshoff (1992) divided the eastern Atlantic species of *Leptogorgia* into seven main groups. These groups were not supported in our phylogenetic analyses. For instance, *L. piccola* was also included by Grasshoff (1992) in the *L. sarmentosa* group, in our phylogeny *L. piccola* was found sister to the *L. gaini*-*L. pulcherrima* clade. These last two species also belong to different morphological groups with *L. pulcherrima* assigned to its own group and *L. gaini* forming part, together with *L. dichotoma*, of the *L. vimminalis* group. *Leptogorgia violetta* was included in the *L. dakarensis* group. Our phylogenetic results clearly show that these morphological groups are artificial. Interestingly, molecular analyses using the *mtMutS* marker have offered support for morphologically defined groups among eastern Pacific *Leptogorgia* species (Vargas *et al.* 2014, Ament-Velásquez *et al.* 2016). In the case of the eastern Atlantic fauna, the discrepancies detected here between Grasshoff's morphological grouping scheme, mainly based on colony branching pattern and sclerome composition, and the results of our molecular analyses provide another example of the homoplasic nature of these characters at shallow level (i.e. intragenus; see Ament-Velásquez *et al.* 2016). In agreement with Grasshoff (1992), *L. sarmentosa* and *L. capverdensis* were found to be sister with high support in our analyses. Our molecular dating analysis suggests a recent divergence (~3 Mya) for *L. sarmentosa* and *L. capverdensis* postdating the re-opening of the Gibraltar strait after the Messinian Salinity Crisis (5.96-5.33 Mya) (Krijgsman *et al.* 1999;

Garcia-Castellanos and Villaseñor, 2011). The separation between *L. capverdensis-L. sarmentosa* and their central-south African congeners (~16 Mya) roughly correspond with intense volcanism associated to the formation of the Cape Verdean archipelago (see Mitchell *et al.* 1983; Pim *et al.* 2008).

Finally, it the only representative of the south-African '*Leptogorgia*' species included in our phylogenies (i.e. *L. barnardi*, *L. capensis*, *L. gilchristi* and *L. palma*; Grasshoff, 1992) was sister to other eastern Atlantic *Leptogorgia* species and the divergence time estimation for this split was inferred at ~68 Mya (95% HPD 31-84 Mya). Although we acknowledge that the poor taxon sampling among these species, and in general among African '*Leptogorgia*' may lead to biased phylogenetic relationships and branch lengths, the younger age (~30 Mya) coincides with the enhancement of the Antarctic Circumpolar Current (ACC) an event that caused climate fluctuations and changes in the sea-surface temperatures (Chase and Meadows, 2007) that could be speculated to be responsible for the separation between the south and north African '*Leptogorgia'*. A better taxon sampling will allow a more accurate divergence time estimation for the separation of south and north African *'Leptogorgia'* species and will allow to test whether in fact this divergence can be related to the enhancement of the ACC in the early Oligocene.

3.5 Conclusions

This study showed a deep divergence among morphological similar but geographically restricted octocoral lineages currently included in the genus *Leptogorgia.* East Atlantic and West Atlantic octocoral clades diverged in the late Cretaceous, likely due to the spreading of the Atlantic ocean. Eastern Atlantic-Mediterranean species of *'Leptogorgia'* represent an old lineage not closely related to western Atlantic-eastern Pacific '*Leptogorgia*', which diverged and diversified recently. The levels of sequence divergence observed within the eastern Atlantic '*Leptogorgia*' match divergence levels observed between eastern Pacific genera suggesting the existence of multiple lineages deserving generic status in the eastern Atlantic. A revision and reappraisal of these fauna is thus necessary.

3.6 Appendix

Appendix 3.6.1 List of specific primers used for PCR and/or Sanger sequencing in *Eunicella albicans* and *Pacifigorgia cairnsi*
Appendix 3.6.2 List of species used for phylogenetic and molecular dating analyses
Appendix 3.6.3 Description of the complete mitogenome of *Eugorgia mutabilis*, *Eunicella albicans*, *Eunicella cavolini* and *Pacifigorgia cairnsi*
Appendix 3.6.4 Matrix of pairwise genetic distances

Appendix 3.6.1 List of specific primers used for PCR and/or Sanger sequencing in *Eunicella albicans* and *Pacifigorgia cairnsi*

Primer name	**Sequence (5'-3')**	**Gene(s)**
NAD3_7813F	CTGGGCTGTTACATATATGGGCT	*Nad3*
MutS_10383R	GCCTGCTCCATTTACCGAGA	*mtMutS*
MutS_10364F	TCTCGGTAAATGGAGCAGGC	*mtMutS*
16S_13089R	GCATCGCTGAGACCATTCCT	16S
NAD2_14268F	ATCTTGGCAGCTGTTGGTCA	*Nad2*
NAD4_17062R	CCGAGCTCCCCATACTCCTA	*Nad4*
IGR10R	ATACATAATTAGCTAGTAAGC	*Nad5*-igr-*Nad4*

Appendix 3.6.2 List of species used for phylogenetic and molecular dating analyses

Taxon	Geographic Area	*mtMutS*	Voucher # - ID number	References
Gorgoniidae				
Antillogorgia acerosa	WA	JX152763	CAS 097785	Williams and Chen, 2012
Antillogorgia sp.	Car	JX152764	CAS 100661	Williams and Chen, 2012
Eugorgia daniana	EP	HG917048	HMG93	Vargas *et al.* 2014
Eugorgia multifida	N/A	GQ342494	N/A	unbablished
Eugorgia mutabilis	EP		GW1806	this study
Eugorgia rubens	N/A	JN866557	34213-094	unbablished
Eunicella albicans	EA		GW1815	this study
Eunicella cavolini	Med	JQ397290	Gori41	Gori *et al*. 2012
Eunicella cavolini	Med		GW4597	this study
Eunicella singularis	Med	JQ397296	Gori36	Gori et al., 2012
Eunicella sp.	Med	JQ397310	Gori49	Gori et al., 2012
Eunicella tricoronata	EA	JX203795	RMNH Coel.40814	McFadden and van Ofwegen, 2012
Eunicella verrucosa	Med	JQ397300	Gori3	Gori *et al.* 2012
Gorgonia flabellum	Car	AY126427	16[1]	Sánchez *et al.* 2003
Gorgonia mariae	Car	AY126426	1007505	Sánchez *et al.* 2003
Gorgonia ventalina	Car	AY126425	1007421	Sánchez *et al.* 2003
Leptogorgia alba	N/A	AY268452	N/A	unpublished
Leptogorgia alba	EP		GW4709	Vargas *et al.* 2014
Leptogorgia alba	EP	HG917034	HMG07	Vargas *et al.* 2014
Leptogorgia alba	EP	HG917035	HMG35	Vargas *et al.* 2014
Leptogorgia alba	EP	HG917036	HMG71	Vargas *et al.* 2014
Leptogorgia capverdensis	EA		GW1780	this study
Leptogorgia chilensis	EP	JN866554	N/A	unpublished
Leptogorgia cofrini	EP	HG917038	HMG32	Vargas *et al.* 2014

Leptogorgia cofrini	EP	HG917037	HMG17	Vargas *et al.* 2014
Leptogorgia cuspidata	EP	AY268450	N/A	Unpublished
Leptogorgia cuspidata	EP	HG917047	HMG97	Vargas *et al.* 2014
Leptogorgia dichotoma	EA	AY268445	N/A	Unpublished
Leptogorgia gaini	EA		GW1777	this study
Leptogorgia gracilis	WA	AY268454	N/A	Unpublished
Leptogorgia palma	EA		GW1810	this study
Leptogorgia piccola	EA	AY268444	N/A	Unpublished
Leptogorgia pulcherrima	EA	AY268443	N/A	Unpublished
Leptogorgia punicea	WA	AY268449	N/A	Unpublished
Leptogorgia ramulus	EP	AY268451	N/A	Unpublished
Leptogorgia rigida	EP	GQ342496	N/A	Unpublished
Leptogorgia sarmentosa	Med		GW4737	this study
Leptogorgia styx	EP	AY268453	N/A	Unpublished
Leptogorgia violacea	WA	AY268448	N/A	Unpublished
Leptogorgia violetta	EA	AY268446	N/A	Unpublished
Leptogorgia virgulata	WA	AY126418	1007414	Sánchez *et al.* 2003
Pacifigorgia bayeri	EP	HG917044	HMG77	Vargas *et al.* 2014
Pacifigorgia cairnsi	EP	HG917041	HMG23	Vargas *et al.* 2014
Pacifigorgia cairnsi	EP		GW4656	this study
Pacifigorgia cf. *cairnsi*	EP	HG917021	HMG20	Vargas *et al.* 2014
Pacifigorgia cf. *cairnsi*	EP	HG917046	HMG106	Vargas *et al.* 2014
Pacifigorgia catedralensis	EP	HG917019	HMG109	Vargas *et al.* 2014
Pacifigorgia firma	EP	HG917022	HMG53	Vargas *et al.* 2014
Pacifigorgia Irene	EP	HG917024	HMG10	Vargas *et al.* 2014
Pacifigorgia media	EP	GQ342497	N/A	Unpublished
Pacifigorgia rubicunda	EP	HG917027	HMG74	Vargas *et al.* 2014
Pacifigorgia stenobrochis	EP	AY126420	27[1]	Sánchez *et al.* 2003

Pacifigorgia stenobrochis	EP	HG917018	HMG04	Vargas *et al.* 2014
Pacifigorgia smithsoniana	EP	HG917023	HMG59	Vargas *et al.* 2014
Phyllogorgia dilatata	WA	AY126428	4336[2]	Sánchez *et al.* 2003
Pseudopterogorgia acerosa	Car	AY126421	1007413	Sánchez *et al.* 2003
Pseudopterogorgia americana	Car	AY126423	1007391	Sánchez *et al.* 2003
Pseudopterogorgia bipinnata	Car	AY126424	1007374	Sánchez *et al.* 2003
Pseudopterogorgia elisabethae	WA	AY126422	1007390	Sánchez *et al.* 2003
Plexauridae				
Swiftia pacifica	N/A	JN866547	N/A	Unpublished
Swiftia sp.	N/A	AY683088	NSUOC-JSL I 3685	Wirshing *et al.* 2005
Psammogorgia cf. *arbuscula*	EP	HG917042	HMG15	Vargas *et al.* 2014
Psammogorgia sp.	EP	HG917045	HMG84	Vargas *et al.* 2014
Psammogorgia cf. *arbuscula*	EP	HG917043	HMG38	Vargas *et al.* 2014

[1] J.A. Sánchez, personal collection; [2] Museu Nacional do Rio de Janeiro (MNRJ)

List of species used for phylogenetic and molecular dating analyses. Additional information such as geographic area, Genbank accession numbers, sample vouchers and references are also reported. Car: Caribbean; EA: Eastern Atlantic; EP: Eastern Pacific; Med: Mediterranea and WA: Western Atlantic. N/A: not available.

Appendix 3.6.3 Description of the complete mitogenome of *Eugorgia mutabilis*, *Eunicella albicans, Eunicella cavolini* and *Pacifigorgia cairnsi*

Complete mitogenomes of *Eugorgia*, *Eunicella* and *Pacifigorgia*

Eugorgia mutabilis and *Pacifigorgia cairnsi* had a mitogenome size of 19,157 bp and 19,156 bp respectively, whereas the complete mitochondrial genome of the two *Eunicella* species ranged from 19,175 bp (*E. albicans*) to 19,316 bp (*E. cavolini*). All species have the ancestral gene arrangement (type A) and include 14 protein-coding genes, 2 ribosomal RNA subunits (12S and 16S) and one methionine tRNA gene ($tRNA^{Met}$). GC-content ranged from 37.2% of *E. mutabilis* to 37.4% of *E. albicans* and *E. cavolini*. The protein-coding genes were identified spanning 76.61% and 77.25% of the complete mitochondrial genomes in *E. cavolini* and *E. albicans*, respectively. The percentage of protein-coding genes in the mitogenome of *E. mutabilis* and *P. cairnsi* was 77.30% and 77.41%, respectively. The nucleotide composition and length of the different intergenic regions (IGRs) were variable among species. For instance the longest IGR, for all species, was located between *Nad5* and *Nad4* but its length ranged from 515 bp (*Pacifigorgia cairnsi*) to 699 bp (*Eunicella cavolini*). The shortest IGRs (4 bp), for all species, were those between 12S rRNA and *Nad1* and between 16S rRNA and *Nad2*. The start codon ATG was the same for all the protein-coding genes. Except for *Cox1*, which had an incomplete stop codon (TA) the other termination codons were either TAA or TAG.

Appendix 3.6.4 Pairwise uncorrected genetic distance (P) matrix

Species	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	21.	22.	23.	24.	25.	26.	27.	28.	29.	30.	31.
1. JQ397290_*Eunicella cavolini*																															
2. JQ397296_*Eunicella singularis*	0,00																														
3. JQ397300_*Eunicella verrucosa*	0,00	0,00																													
4. JQ397310_*Eunicella* sp.	0,00	0,00	0,00																												
5. AY126422_*Pseudopterogorgia elisabethae*	0,07	0,07	0,07	0,07																											
6. AY126426_*Gorgonia mariae*	0,07	0,07	0,07	0,07	0,00																										
7. AY126424_*Pseudopterogorgia bipinnata*	0,06	0,06	0,06	0,06	0,02	0,02																									
8. JX152764_*Antillogorgia* sp.	0,06	0,06	0,06	0,06	0,02	0,02	0,00																								
9. AY126428_*Phyllogorgia dilatata*	0,07	0,07	0,07	0,07	0,04	0,04	0,03	0,03																							
10. AY126425_*Gorgonia ventalina*	0,07	0,07	0,07	0,07	0,03	0,03	0,03	0,03	0,02																						
11. AY126427_*Gorgonia flabellum*	0,07	0,07	0,07	0,07	0,03	0,03	0,03	0,03	0,02	0,00																					
12. JX152763_*Antillogorgia acerosa*	0,07	0,07	0,07	0,07	0,03	0,03	0,02	0,02	0,02	0,01	0,01																				
13. AY126421_*Pseudopterogorgia acerosa*	0,06	0,06	0,06	0,06	0,02	0,02	0,02	0,02	0,02	0,01	0,01	0,00																			
14. AY126423_*Pseudopterogorgia americana*	0,06	0,06	0,06	0,06	0,02	0,02	0,02	0,02	0,02	0,01	0,01	0,00	0,00																		
15. HG917019_*Pacifigorgia catedralensis*	0,05	0,05	0,05	0,05	0,04	0,04	0,03	0,03	0,04	0,04	0,04	0,04	0,03	0,03																	
16. HG917021_*Pacifigorgia* cf. *cairnsi*	0,05	0,05	0,05	0,05	0,04	0,04	0,03	0,03	0,04	0,04	0,04	0,04	0,03	0,03	0,00																
17. HG917044_*Pacifigorgia bayeri*	0,05	0,05	0,05	0,05	0,04	0,04	0,03	0,03	0,04	0,04	0,04	0,04	0,03	0,03	0,00	0,00															
18. AY126420_*Pacifigorgia stenobrochis*	0,06	0,06	0,06	0,06	0,04	0,04	0,04	0,04	0,05	0,04	0,04	0,04	0,04	0,04	0,01	0,01	0,01														
19. HG917018_*Pacifigorgia stenobrochis*	0,06	0,06	0,06	0,06	0,04	0,04	0,04	0,04	0,05	0,04	0,04	0,04	0,04	0,04	0,01	0,01	0,01	0,00													
20. HG917022_*Pacifigorgia firma*	0,06	0,06	0,06	0,06	0,04	0,04	0,03	0,03	0,05	0,04	0,04	0,04	0,04	0,04	0,01	0,01	0,01	0,01	0,01												
21. HG917023_*Pacifigorgia smithsoniana*	0,05	0,05	0,05	0,05	0,04	0,04	0,03	0,03	0,04	0,04	0,04	0,04	0,03	0,03	0,00	0,00	0,00	0,01	0,01	0,00											
22. HG917024_*Pacifigorgia irene*	0,05	0,05	0,05	0,05	0,04	0,04	0,03	0,03	0,04	0,04	0,04	0,04	0,03	0,03	0,00	0,00	0,00	0,01	0,01	0,00	0,00										
23. HG917041_*Pacifigorgia cairnsi*	0,05	0,05	0,05	0,05	0,04	0,04	0,03	0,03	0,04	0,04	0,04	0,04	0,03	0,03	0,00	0,00	0,00	0,01	0,01	0,00	0,00	0,00									
24. GW4656_*Pacifigorgia cairnsi*	0,06	0,06	0,06	0,06	0,04	0,04	0,03	0,03	0,04	0,04	0,04	0,04	0,03	0,03	0,01	0,01	0,01	0,01	0,01	0,00	0,00	0,00	0,00								
25. HG917046_*Pacifigorgia* cf. *cairnsi*	0,05	0,05	0,05	0,05	0,04	0,04	0,03	0,03	0,04	0,04	0,04	0,04	0,03	0,03	0,00	0,00	0,00	0,01	0,01	0,00	0,00	0,00	0,00	0,00							
26. GQ342497_*Pacifigorgia media*	0,05	0,05	0,05	0,05	0,04	0,04	0,03	0,03	0,04	0,04	0,04	0,04	0,03	0,03	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01						
27. HG917027_*Pacifigorgia rubicunda*	0,05	0,05	0,05	0,05	0,04	0,04	0,03	0,03	0,04	0,04	0,04	0,04	0,03	0,03	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,00					
28. GQ342494_*Eugorgia multifida*	0,07	0,07	0,07	0,07	0,06	0,06	0,06	0,06	0,07	0,06	0,06	0,06	0,06	0,06	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,04	0,05	0,05	0,05				
29. HG917048_*Eugorgia daniana*	0,07	0,07	0,07	0,07	0,06	0,06	0,06	0,06	0,07	0,06	0,06	0,06	0,06	0,06	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,04	0,05	0,05	0,05	0,00			
30. GW1806_*Eugorgia mutabilis*	0,07	0,07	0,07	0,07	0,06	0,06	0,06	0,06	0,07	0,06	0,06	0,06	0,06	0,06	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,04	0,05	0,05	0,05	0,00	0,00		
31. AY268448_*Leptogorgia violacea*	0,06	0,06	0,06	0,06	0,06	0,06	0,05	0,05	0,06	0,06	0,06	0,05	0,05	0,05	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,03	0,03	0,03	
32. AY268449_*Leptogorgia punicea*	0,06	0,06	0,06	0,06	0,05	0,05	0,04	0,04	0,05	0,05	0,05	0,05	0,04	0,04	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,01
33. AY126418_*Leptogorgia virgulata*	0,05	0,05	0,05	0,05	0,05	0,05	0,04	0,04	0,05	0,05	0,05	0,04	0,04	0,04	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02
34. AY268454_*Leptogorgia gracilis*	0,05	0,05	0,05	0,05	0,05	0,05	0,04	0,04	0,05	0,05	0,05	0,04	0,04	0,04	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02
35. JN866557_*Eugorgia rubens*	0,06	0,06	0,06	0,06	0,05	0,05	0,04	0,04	0,05	0,05	0,05	0,04	0,04	0,04	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02

…continue

Species	32.	33.	34.	35.	36.	37.	38.	39.	40.	41.	42.	43.	44.	45.	46.	47.	48.	49.	50.	51.	52.	53.	54.	55.	56.	57.	58.	59.	60.	61.	62.
1. JQ397290_*Eunicella cavolini*																															
2. JQ397296_*Eunicella singularis*																															
3. JQ397300_*Eunicella verrucosa*																															
4. JQ397310_*Eunicella* sp.																															
5. AY126422_*Pseudopterogorgia elisabethae*																															
6. AY126426_*Gorgonia mariae*																															
7. AY126424_*Pseudopterogorgia bipinnata*																															
8. JX152764_*Antillogorgia* sp.																															
9. AY126428_*Phyllogorgia dilatata*																															
10. AY126425_*Gorgonia ventalina*																															
11. AY126427_*Gorgonia flabellum*																															
12. JX152763_*Antillogorgia acerosa*																															
13. AY126421_*Pseudopterogorgia acerosa*																															
14. AY126423_*Pseudopterogorgia americana*																															
15. HG917019_*Pacifigorgia catedralensis*																															
16. HG917021_*Pacifigorgia* cf. *cairnsi*																															
17. HG917044_*Pacifigorgia bayeri*																															
18. AY126420_*Pacifigorgia stenobrochis*																															
19. HG917018_*Pacifigorgia stenobrochis*																															
20. HG917022_*Pacifigorgia firma*																															
21. HG917023_*Pacifigorgia smithsoniana*																															
22. HG917024_*Pacifigorgia irene*																															
23. HG917041_*Pacifigorgia cairnsi*																															
24. GW4656_*Pacifigorgia cairnsi*																															
25. HG917046_*Pacifigorgia* cf. *cairnsi*																															
26. GQ342497_*Pacifigorgia media*																															
27. HG917027_*Pacifigorgia rubicunda*																															
28. GQ342494_*Eugorgia multifida*																															
29. HG917048_*Eugorgia daniana*																															
30. GW1806_*Eugorgia mutabilis*																															
31. AY268448_*Leptogorgia violacea*																															
32. AY268449_*Leptogorgia punicea*																															
33. AY126418_*Leptogorgia virgulata*	0,02																														
34. AY268454_*Leptogorgia gracilis*	0,02	0,00																													
35. JN866557_*Eugorgia rubens*	0,02	0,01	0,01																												

…continue

Species	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	21.	22.	23.	24.	25.	26.	27.	28.	29.	30.	31.
36. AY268452_*Leptogorgia alba*	0,06	0,06	0,06	0,06	0,05	0,05	0,04	0,04	0,05	0,05	0,05	0,04	0,04	0,04	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02	0,02	0,02	0,02
37. AY268453_*Leptogorgia styx*	0,06	0,06	0,06	0,06	0,05	0,05	0,04	0,04	0,05	0,05	0,05	0,04	0,04	0,04	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02	0,02	0,02	0,02
38. GW4709_*Leptogorgia alba*	0,06	0,06	0,06	0,06	0,05	0,05	0,04	0,04	0,05	0,05	0,05	0,04	0,04	0,04	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02	0,02	0,02	0,02
39. HG917034_*Leptogorgia alba*	0,06	0,06	0,06	0,06	0,05	0,05	0,04	0,04	0,05	0,05	0,05	0,04	0,04	0,04	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02	0,02	0,02	0,02
40. HG917035_*Leptogorgia alba*	0,06	0,06	0,06	0,06	0,05	0,05	0,04	0,04	0,05	0,05	0,05	0,04	0,04	0,04	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02	0,02	0,02	0,02
41. HG917036_*Leptogorgia alba*	0,06	0,06	0,06	0,06	0,05	0,05	0,04	0,04	0,05	0,05	0,05	0,04	0,04	0,04	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02	0,02	0,02	0,02
42. HG917038_*Leptogorgia cofrini*	0,06	0,06	0,06	0,06	0,05	0,05	0,04	0,04	0,05	0,05	0,05	0,04	0,04	0,04	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02	0,02	0,02	0,02
43. HG917037_*Leptogorgia cofrini*	0,06	0,06	0,06	0,06	0,05	0,05	0,04	0,04	0,05	0,05	0,05	0,04	0,04	0,04	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02	0,02	0,02	0,02
44. JN866554_*Leptogorgia chilensis*	0,05	0,05	0,05	0,05	0,04	0,04	0,04	0,04	0,05	0,04	0,04	0,04	0,04	0,04	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02	0,03	0,03	0,03	0,02	0,02	0,02	0,02
45. AY268451_*Leptogorgia ramulus*	0,05	0,05	0,05	0,05	0,05	0,05	0,04	0,04	0,05	0,04	0,04	0,04	0,04	0,04	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02	0,02	0,02	0,02
46. AY268450_*Leptogorgia cuspidata*	0,06	0,06	0,06	0,06	0,05	0,05	0,04	0,04	0,05	0,05	0,05	0,05	0,04	0,04	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02	0,02	0,02	0,02
47. GQ342496_*Leptogorgia rigida*	0,06	0,06	0,06	0,06	0,05	0,05	0,04	0,04	0,05	0,05	0,05	0,05	0,04	0,04	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02	0,02	0,02	0,02
48. HG917047_*Leptogorgia cuspidata*	0,06	0,06	0,06	0,06	0,05	0,05	0,04	0,04	0,05	0,05	0,05	0,05	0,04	0,04	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02	0,02	0,02	0,02
49. GW4737_*Leptogorgia sarmentosa*	0,07	0,07	0,07	0,07	0,05	0,05	0,05	0,05	0,06	0,06	0,06	0,05	0,05	0,05	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,07	0,07	0,07	0,06
50. AY268443_*Leptogorgia pulcherrima*	0,07	0,07	0,07	0,07	0,06	0,06	0,05	0,05	0,06	0,06	0,06	0,06	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,04	0,05	0,04	0,04	0,07	0,07	0,06
51. AY268446_*Leptogorgia violetta*	0,07	0,07	0,07	0,07	0,05	0,05	0,05	0,05	0,06	0,05	0,05	0,05	0,05	0,05	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,06	0,06	0,06
52. AY268444_*Leptogorgia piccola*	0,07	0,07	0,07	0,07	0,05	0,05	0,04	0,04	0,06	0,05	0,05	0,05	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,06	0,06	0,06	0,06
53. AY268445_*Leptogorgia dichotoma*	0,06	0,06	0,06	0,06	0,05	0,05	0,04	0,04	0,05	0,05	0,05	0,05	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,03	0,04	0,04	0,04	0,06	0,06	0,06	0,06
54. JN866547_*Swiftia pacifica*	0,06	0,06	0,06	0,06	0,06	0,06	0,06	0,06	0,07	0,06	0,06	0,06	0,06	0,06	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,06	0,06	0,06	0,06
55. AY683088_*Swiftia* sp.	0,05	0,05	0,05	0,05	0,06	0,06	0,05	0,05	0,06	0,05	0,05	0,05	0,05	0,05	0,04	0,04	0,04	0,05	0,05	0,05	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,06	0,06	0,06	0,05
56. HG917042_*Psammogorgia* cf. *arbuscula*	0,05	0,05	0,05	0,05	0,05	0,05	0,04	0,04	0,05	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,05	0,05	0,05	0,05
57. HG917045_*Psammogorgia* sp.	0,04	0,04	0,04	0,04	0,05	0,05	0,04	0,04	0,05	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,03	0,04	0,04	0,04	0,04	0,04	0,04	0,05
58. HG917043_*Psammogorgia* cf. *arbuscula*	0,04	0,04	0,04	0,04	0,05	0,05	0,04	0,04	0,05	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,03	0,04	0,04	0,04	0,04	0,04	0,04	0,05
59. GW1810_*Leptogorgia palma*	0,06	0,06	0,06	0,06	0,05	0,05	0,04	0,04	0,05	0,05	0,05	0,05	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,03	0,04	0,04	0,04	0,06	0,06	0,06	0,05
60. GW1815_*Eunicella albicans*	0,03	0,03	0,03	0,03	0,06	0,06	0,06	0,06	0,06	0,06	0,06	0,06	0,06	0,06	0,05	0,05	0,05	0,06	0,06	0,06	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,07	0,07	0,07	0,06
61. GW1777_*Leptogorgia gaini*	0,06	0,06	0,06	0,06	0,05	0,05	0,05	0,05	0,06	0,05	0,05	0,05	0,05	0,05	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,06	0,06	0,06	0,06
62. GW1780_*Leptogorgia capverdensis*	0,07	0,07	0,07	0,07	0,05	0,05	0,05	0,05	0,06	0,06	0,06	0,05	0,05	0,05	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,07	0,07	0,07	0,06
63. JX203795_*Eunicella tricoronata*	0,02	0,02	0,02	0,02	0,06	0,06	0,05	0,05	0,06	0,06	0,06	0,06	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,06	0,06	0,06	0,06
64. GW4597_*Eunicella cavolini*	0,00	0,00	0,00	0,00	0,07	0,07	0,06	0,06	0,07	0,07	0,07	0,07	0,06	0,06	0,05	0,05	0,05	0,06	0,06	0,06	0,05	0,05	0,05	0,06	0,05	0,05	0,05	0,07	0,07	0,07	0,06

…continue

	32.	33.	34.	35.	36.	37.	38.	39.	40.	41.	42.	43.	44.	45.	46.	47.	48.	49.	50.	51.	52.	53.	54.	55.	56.	57.	58.	59.	60.	61.	62.
36. AY268452_*Leptogorgia alba*	0,02	0,01	0,01	0,01																											
37. AY268453_*Leptogorgia styx*	0,02	0,01	0,01	0,01	0,00																										
38. GW4709_*Leptogorgia alba*	0,02	0,01	0,01	0,01	0,00	0,00																									
39. HG917034_*Leptogorgia alba*	0,02	0,01	0,01	0,01	0,00	0,00	0,00																								
40. HG917035_*Leptogorgia alba*	0,02	0,01	0,01	0,01	0,00	0,00	0,00	0,00																							
41. HG917036_*Leptogorgia alba*	0,02	0,01	0,01	0,01	0,00	0,00	0,00	0,00	0,00																						
42. HG917038_*Leptogorgia cofrini*	0,02	0,01	0,01	0,01	0,00	0,00	0,00	0,00	0,00	0,00																					
43. HG917037_*Leptogorgia cofrini*	0,02	0,01	0,01	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00																				
44. JN866554_*Leptogorgia chilensis*	0,01	0,01	0,01	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00																			
45. AY268451_*Leptogorgia ramulus*	0,01	0,01	0,01	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00																		
46. AY268450_*Leptogorgia cuspidata*	0,02	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,00																	
47. GQ342496_*Leptogorgia rigida*	0,02	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,00	0,00																
48. HG917047_*Leptogorgia cuspidata*	0,02	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,00	0,00	0,00															
49. GW4737_*Leptogorgia sarmentosa*	0,06	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05														
50. AY268443_*Leptogorgia pulcherrima*	0,06	0,06	0,06	0,06	0,06	0,06	0,06	0,06	0,06	0,06	0,06	0,06	0,05	0,05	0,06	0,06	0,06	0,03													
51. AY268446_*Leptogorgia violetta*	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,02	0,02												
52. AY268444_*Leptogorgia piccola*	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,04	0,05	0,05	0,05	0,05	0,02	0,01	0,01											
53. AY268445_*Leptogorgia dichotoma*	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,04	0,04	0,05	0,05	0,05	0,02	0,02	0,00	0,01										
54. JN866547_*Swiftia pacifica*	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,04	0,05	0,05	0,05	0,05	0,05	0,06	0,05	0,05	0,04									
55. AY683088_*Swiftia* sp.	0,05	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,05	0,05	0,05	0,05	0,04	0,03								
56. HG917042_*Psammogorgia* cf. *arbuscula*	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,03	0,04	0,04	0,04	0,04	0,05	0,05	0,04	0,04	0,04	0,03	0,02							
57. HG917045_*Psammogorgia* sp.	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,03	0,03	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,03	0,02	0,00						
58. HG917043_*Psammogorgia* cf. *arbuscula*	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,03	0,03	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,03	0,02	0,00	0,00					
59. GW1810_*Leptogorgia palma*	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,03	0,03	0,04	0,04	0,04	0,03	0,03				
60. GW1815_*Eunicella albicans*	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,04	0,05	0,05	0,05	0,05	0,06	0,06	0,06	0,05	0,06	0,04	0,04	0,04	0,04	0,05			
61. GW1777_*Leptogorgia gaini*	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,03	0,00	0,01	0,01	0,01	0,05	0,05	0,04	0,04	0,04	0,04	0,06		
62. GW1780_*Leptogorgia capverdensis*	0,06	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,00	0,03	0,02	0,02	0,02	0,05	0,05	0,05	0,04	0,04	0,04	0,05	0,03	
63. JX203795_*Eunicella tricoronata*	0,05	0,04	0,04	0,05	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,05	0,05	0,05	0,05	0,06	0,06	0,05	0,05	0,05	0,04	0,04	0,04	0,04	0,05	0,00	0,05	0,05
64. GW4597_*Eunicella cavolini*	0,06	0,05	0,05	0,06	0,06	0,06	0,06	0,06	0,06	0,06	0,06	0,06	0,05	0,05	0,06	0,06	0,06	0,07	0,07	0,07	0,07	0,06	0,06	0,05	0,05	0,04	0,04	0,06	0,03	0,06	0,07

Pairwise genetic distances. Western Atlantic and eastern Pacific *Leptogorgia* are highlighted in yellow and red, respectively. Those collected from eastern Atlantic and Mediterranean are highlighted in light blue and green, respectively.

Chapter 4

Complete mitochondrial genome of *Muricea crassa* and *Muricea purpurea* (Anthozoa: Octocorallia) from the eastern tropical Pacific

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Chapter 4

Complete mitochondrial genome of *Muricea crassa* and *Muricea purpurea* (Anthozoa: Octocorallia) from the eastern tropical Pacific

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Muricea crassa (Verrill, 1869) and *Muricea purpurea* (Verrill, 1864) are two shallow water gorgonians of the family Plexauridae. Their distribution is limited to the eastern tropical Pacific where they are abundant members of coral communities and littoral zones (Guzman et al., 2004). Samples were collected as part of an ecological and biodiversity survey undertaken in the Coiba National Park (Panama). Genomic DNA was extracted from ethanol-preserved samples and was used to construct genomic libraries using the Accel-NGS 1S DNA Library kit (Swift Biosciences, Ann Arbor, MI, USA) following the manufacturers instructions. These libraries were sequenced (100bp PE) on an Illumina HiSeq (Illumina Inc., San Diego, CA, USA). The quality of the reads obtained was assessed with FastQC (Andrews, 2010), low quality reads and Illumina adaptors were trimmed using Trimmomatic 0.3.2 called from Trinity RNA-Seq 2.0.6 (Grabherr *et al.* 2011). Despite its original purpose, the Trinity RNA-Seq assembler was used after normalization to 50X coverage for *de-novo* mitogenome assembly. The assembly resulted in a single mitochondrial contig in both species. Initial annotation was performed with the ORF finder function implemented in Geneious 8.1.7 (Kearse *et al.* 2012) and was corroborated by comparison with published octocoral mitogenomes. The presence of DNA repeats was assessed with the tandem repeats finder server 4.08 available at https://tandem.bu.edu/trf/trf.html (Benson, 1999). The complete mitogenomes of *M. crassa* (LT174652) and *M. purpurea* (LT174653) were 19,586 bp and 19,358 bp long, with a GC-content of 36.0% (*M. purpurea*) and 36.1% (*M. crassa*), respectively. Both mitogenomes had gene arrangement of type "A" (see Brockman and McFadden, 2012). In total, the Coding DNA Sequences (CDSs) spanned about 76% of the mitogenome in both species. Among protein-coding genes, the highest level of nucleotide diversity (0.4%) was found in

NAD1, *NAD6* and *COX2*, whereas no nucleotide substitutions were found in *NAD3*, *ATP6* and *ATP8*. Except for *NAD2* and *NAD5* (13bp overlap), the other protein-coding genes were separated by intergenic regions (IGRs) of different lengths. In both species, the shortest IGRs were those located between 12S rRNA and *NAD1* and between 16S rRNA and *NAD2*, while the longest was found between *NAD5* and *NAD4*. The latter IGR was also the most diverse region with a nucleotide diversity of 6.2%. Length polymorphism was found in the *COB-NAD6* IGR, which was 184bp shorter in *M. purpurea* than in *M. crassa*. Sequencing of indel-rich IGRs such as that between *COB* and *NAD6* may result useful for molecular species-identification in the genus *Muricea*. Finally, we found a 37 bp tandem repeat in the IGR between *NAD4* and tRNA of *M. crassa*.

The two newly sequenced complete mitogenomes were used to assess the phylogenetic relationships among 26 different octocoral species. A concatenated nucleotide alignment of 14 protein-coding genes (15,249 bp in total) for 41 taxa was generated with MUSCLE (Edgar, 2004) using the default options provided in Seaview (Gouy *et al.* 2010). The maximum likelihood tree was inferred in RaxML 7.2.8 (Stamatakis, 2006) under the GTRGAMMA substitution model. Node support was estimated using 1000 bootstrap pseudoreplicates.

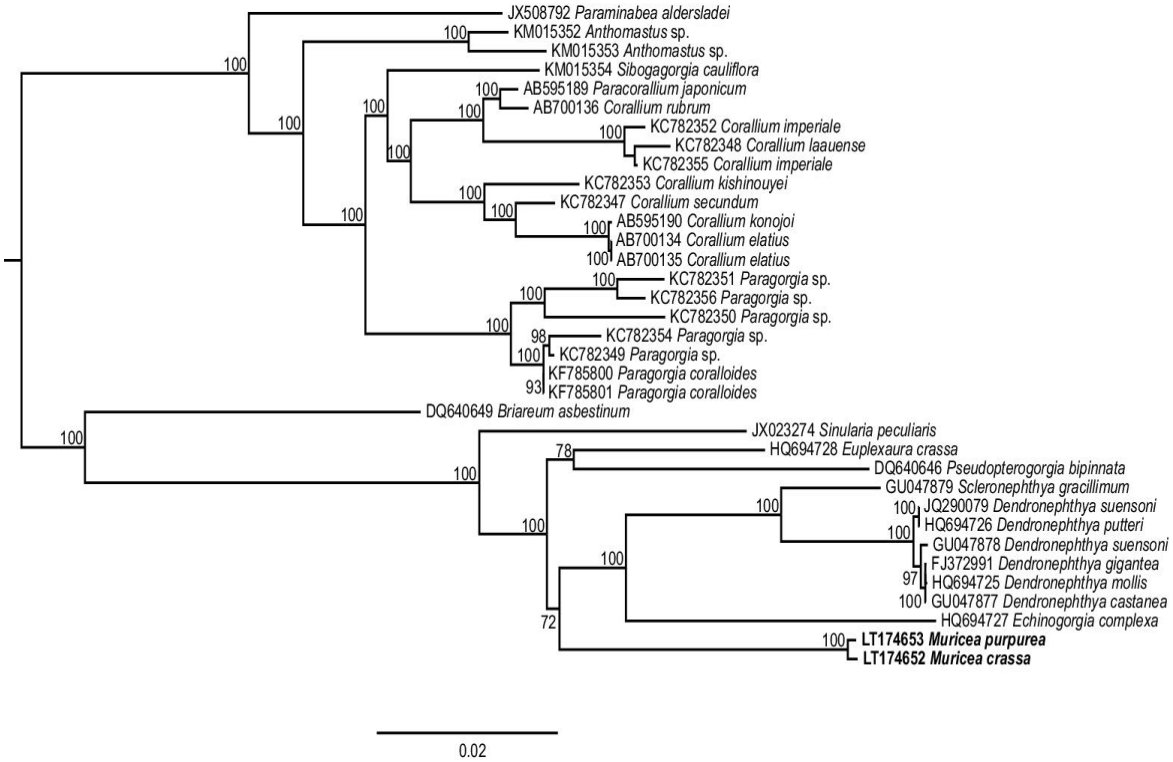

Figure 4.1 Phylogenetic tree of 41 octocorals based on a concatenated alignment of 14 mitochondrial protein-coding genes. The calcaxonians (Keratoisidinae sp., *Acanella eburnea*, *Narella hawaiinensis* and *Junceella fragilis*) and pennatulaceans (*Renilla muelleri* and *Stylatula elongata*) were used to re-root the tree but are not shown here. Numbers at the nodes indicate bootstrap values.

The phylogenetic tree (Figure 4.1) was re-rooted using three calcaxonians and two pennatulaceans as outgroup (not shown in Figure 4.1). Tree topology is consistent with recently published studies (Figueroa and Baco, 2015), however the phylogenetic placement of *Muricea* spp. sister to Nephtheidae (*Dendronephthya* spp. and *Scleronephthya* spp.) is likely an artefact caused by poor taxon sampling in the family Plexauridae.

Chapter 5

Species delimitation and phylogeny of the tropical eastern Pacific gorgonian *Pacifigorgia* using Single Nucleotide Polymorphisms (SNPs)

This chapter will be submitted as a standalone publication to the Journal "Molecular Ecology Resources"

Chapter 5

Species delimitation and phylogeny of the tropical eastern Pacific gorgonian *Pacifigorgia* using Single Nucleotide Polymorphisms (SNPs)

5.1 Introduction

Delimitation of species boundaries is a challenging process, which often requires multidisciplinary information including taxonomical, ecological and molecular data (Dayrat, 2005; Ruiz-Sanchez & Sosa, 2010; Cornetti *et al.* 2015). The number of studies on species delimitation has increased over the last few years and new methods and programs were recently developed for the analysis of morphological and molecular datasets (see e.g. Yang & Rannala 2010; Fujita *et al.* 2011; Zapata & Jiménez 2012; Zhang *et al.* 2013). Although DNA sequences have largely been used for species identification (Hebert *et al.* 2003), their application for species delimitation is still controversial. Delimitation of species based on DNA sequences is sometimes influenced by the number of loci analysed (Dupuis *et al.* 2012) and the methods applied (Carstens *et al.* 2013). On the other hand, integrated approaches based on different morphological characters and molecular data may also lead to divergent results (see e.g. Wiens & Penkrot 2002; Debiasse & Hellberg 2015). This incongruence could stem from hybridisation events and interspecific gene flow that might occur among closely related species (Linnen & Farrell 2007; Arnolg & Fogarty 2009). About 10% of the animal species are involved in hybridisation events (Mallet 2005) and it has been shown that introgression of a few loci can speed-up the speciation processes (Abbott *et al.* 2010). Although hybridisation was considered one of the possible processes underpinning octocoral diversification (McFadden & Hutchinson 2004; Prada *et al.* 2008; Wirshing & Baker 2015), its real influence in speciation is difficult to estimate.

The genus *Pacifigorgia* includes 35 valid species, mainly distributed in the eastern Pacific (Breedy & Guzman 2002, 2003). A high number of species is distributed in the Panamic province —from ~16° N to ~3° N— and six species are endemic of the Gulf of Chiriquí off Panama (Guzman *et al.* 2004; Vargas *et al.* 2008). Species delimitation and taxonomy in *Pacifigorgia* is typically based on the diversity of small calcium carbonate components (sclerites) that are embedded in the soft tissues (Vargas *et al.* 2010a; Carlo *et al.* 2011). Additional morphological traits such as branching thickness, mesh pattern and form of the colony have been also considered, resulting, however, in a lack of resolution for species-level identification (Vargas *et al.* 2010b). Similarly, the use of mitochondrial (*mtMutS*) and high-variable nuclear markers such as SRP54 failed to resolve the phylogenetic relationships at species level (Vargas *et al.* 2014). The systematic uncertainty faced during the last decade raises important evolutionary questions which may be briefly summarized as follows: (1) is the high endemism of the Gulf of Chiriquí the result of adaptive genetic diversity? (2) is hybridisation contributing to speciation, due to reproductive isolation? (3) is there any correlation between the geological formation of the Gulf of Chiriquí and the specieation events occurred in the eastern Pacific? In order to address these questions and to explore the diversity among different *Pacifigorgia* species we analysed wide-genome regions using Next Generation Sequencing (NGS) technologies. We investigate the phylogenetic relationships within nine *Pacifigorgia* species based on single nucleotide polymorphisms (SNPs) using Genotyping by Sequencing (GBS). We assess species boundaries using Marginal Likelihood Estimation (MLE) and a Bayes Factor Delimitation (BFD) method and we also survey the genetic diversity comparing 30 short-DNA sequences (<300 bp) spread over the mitochondrial genome of different species. The present study represents the first attempt to delimit, through a NGS approach, morphologically ambiguous species and to shed light on the evolutionary processes that shaped the diversity of a taxonomically complicated genus.

5.2.Material and methods

5.2.1 Sampling and morphological identification

Eighty-two (82) *Pacifigorgia* specimens belonging nine species (*P. bayeri*, *P. cairnsi*, *P. eximia*, *P. ferruginea*, *P. firma*, *P. pumila*, *P. rubicunda*, *P. smithsoniana* and *P.*

stenobrochis) were collected by SCUBA from six different locations off Panama in the National Park of Coiba (Appendix 5.5.1). Sampling depths range from 8 m to 24 m. All samples were preliminary sorted in the field and were morphologically identified based on sclerites. For each of the specimens, different sub-samples were preserved in 75% ethanol, RNA-later, or silica gel and were further stored at -20°C for molecular investigations.

5.2.2 DNA extraction and library preparation

Genomic DNA of ethanol-preserved sub-samples was extracted with a modified CTAB protocol (Porebski *et al.* 1997). DNA quality was visually checked on 1.5% agarose gel, while DNA yield and purity were assessed using a NanoDrop™ spectrophotometer. RNA contaminants were removed from the DNA extracts by digestion with ribonuclease A (RNase A), followed by precipitation with 1/10 volume of 3M sodium acetate pH 5.2. Samples with degraded DNA were excluded from the analyses and, among the 82 specimens originally considered, only 40 samples —at least two specimens per species — were further used for Illumina sequencing. Multiplexed GBS libraries were prepared following Elshire *et al.* (2011). Briefly, for each sample ~150ng of gDNA were digested with ApekI (cut site CWGC) for two hours at 75°C and were ligated with a common adapter (CWGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG) and one barcode adapter (see Appendix 5.5.2) for one hour at 22°C, followed by half hour at 65°C. PCR amplification of the restriction fragments was performed for 15 cycles with annealing temperature of 65°C and extension time of 30s using the following primers: GBS_PrimerA (AATGATACGGCGACCACCGAGATCTACACTCTTTC-CCTACACGACGCTCTTCCGATCT) and one of the twelve Index primers Illumina compatible (see Appendix 5.5.2 for a detailed list of the "Ilmn_Id" primers used). These primers contain complementary sequences for amplifying restriction fragments with ligated adapters, compatible with the Illumina terminator chemistry (Bentley *et al.* 2008). In order to remove primer and adapter dimmers, PCR products were purified with the Agencourt AMPure XP PCR-purification kit (Beckman Coulter, Inc.) using 1.1 volume of AMPure XP reagent. The purified PCR products were quantified with a QUBit® 2.0 using a dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA) and were pooled in a final library, whose quality was assessed using a Bioanalyzer 2100 (Agilent, Santa Clara, CA). The library was sequenced twice in a 100bp single-end run on Illumina HiSeq 2000 (Illumina, San Diego, CA) at the Gene Center of the Ludwig Maximilians Universität.

5.2.3 Data assembly, sequence reads filtering and clustering

Raw data of the two independent runs were pooled resulting in a total of ~300x10^6 sequence reads obtained. Sequence reads were de-multiplexed with Stacks 1.35 (Catchen *et al.* 2013) and the same program was used to trim the barcode (6 bp) and Illumina adapters, reads with ambiguities were excluded. Four different programs implemented in the Stacks pipeline (*ustacks*, *cstacks*, *sstacks* and *populations*) were utilised for additional filtering and clustering of the de-multiplexed reads using default options. The unique stacks program (*ustacks*) was used to align a set of short read-sequences into a so-called "stacks" and to generate a set of loci by comparing the different stacks. SNPs detection at each of the loci recovered was performed using the Maximum Likelihood framework (Hohenlohe *et al.* 2010). *Cstacks* and *sstacks* were used to build a catalogue of loci, and for matching every sample against the catalogue, respectively. The *populations* module was run assuming that the 40 specimens analysed belong to a single population and considering a minimum of 80% of individuals per population (-r 0.80) and a minimum stack depth per individuals at locus of 10 (-m 10). The final output file was generated in a Variant Call Format (VCF) and consisted of ~30,000 SNPs. In order to infer a phylogeny (see below), we converted our output file into nexus format using a pearl script available in the Supplementary material. The same script was also used to retain only those SNPs that occurred in a percentage of samples *a priori* decided. For our analyses we used a 12.5% threshold, which means that SNPs with less than 35 taxa were excluded. The final dataset consisted of 538 SNPs.

5.2.4 Phylogenetic and species delimitation analyses

A set of 538 SNPs was analysed with a multispecies coalescent program called SNAPP (Bryant *et al.* 2012) which, using a MCMC sampler, estimates the species tree and delimits species using biallelic data. For species delimitation we used a modified Bayes Factor Delimitation method (Grummer *et al.* 2013) that can handle genome-wide SNP data (BFD*). The BFD* method uses a marginal likelihood estimation approach and was run through the program SNAPP (Leaché *et al.* 2014). Although species number and sample assignments can be predefined, our delimitation analysis (BFD*) was run without taxonomic constraints. Unlike BFD*, the species tree estimation analysis was run in SNAPP including species assignments (see Appendix 5.5.1 for a complete list of the samples). The program SNAPP requires a lot of computational memory and time, hence for both analyses a reduced dataset consisting of 27 taxa was generated. Except for *P.*

firma, for which only two specimens were considered, for all other species at least three specimens were considered. SNAPP analyses were performed using default prior options for the coalescent and mutation rates. The Markov chain Monte Carlo ran for 1,000,000 generations or until Effective Samples Size (ESS) reached values >150, with sampling trees at 100 generations interval. ESS and convergence for all runs were checked in Tracer 1.6 (Rambaut *et al.* 2014). The final set of trees generated for each analysis was visualised in DensiTree and a Maximum Clade Credibility Tree (MCC) was summarised in TreAnnotator with 10% of the trees discarded as burn-in.

5.2.5 Mitogenomic reads and phylogeny

The sequence reads obtained for each of the 27 specimens used in the BFD* and species tree estimation analyses, were mapped to the complete mitogenome of *Pacifigorgia cairnsi* (GW4656) using Geneious 8.1 (Kearse *et al.* 2012). Overall, the percentage of the mitochondrial reads was <1%, the average length of the reads was ~70 bp and the mapped reads spanned over 25 regions of the mitochondrial genome. The mean depth of coverage varied according to the specimens and the mitogenomic regions considered, however only those reads occurring in at least 50% of the specimens (14) and with a coverage depth of 10X or higher were retained for further analyses. For each mitochondrial region a consensus sequence was extracted and used for the mitochondrial alignment. Twenty-seven (27) sets of sequences were concatenated and aligned with MUSCLE using default options in Seaview 4.5.3 (Gouy *et al.* 2010) and the resulting alignment, consisting of ~3,800 nucleotide positions and 27 taxa, was utilised to infer a phylogeny. The maximum likelihood tree was inferred in RaxML 7.2.8 (Stamatakis, 2006) under GTRGAMMA substitution model and the branch support was assessed with a rapid bootstrap analysis (Stamatakis *et al.* 2008) using 1000 pseudoreplicates.

5.3 Results

5.3.1 Bayes factor delimitation

Species delimitation and phylogenetic reconstruction based on 538 SNPs, showed the 27 *Pacifigorgia* specimens divided into seven main groups (Figure 5.1). The phylogenetic relationships within these groups were poorly resolved as highlighted by the topological uncertainty. Except for the group including *Pacifigorgia eximia* (1), which had high

posterior probability value (pp=1), all others were poorly supported and most of the support values were well below 50%. In spite of being poorly resolved, our analysis grouped *P. stenobrochis* and *P. rubicunda* into two different groups (2 and 3). Two of the specimens assigned to *P. cairnsi* (GW4649, GW4662) and *P. pumila* (GW4661, GW4671) were split into two different groups (4 and 5). GW4696 (*P. pumila*) and GW4631 (*P. stenobrochis*) nested within the *P. cairnsi* and *P. pumila* groups, respectively.

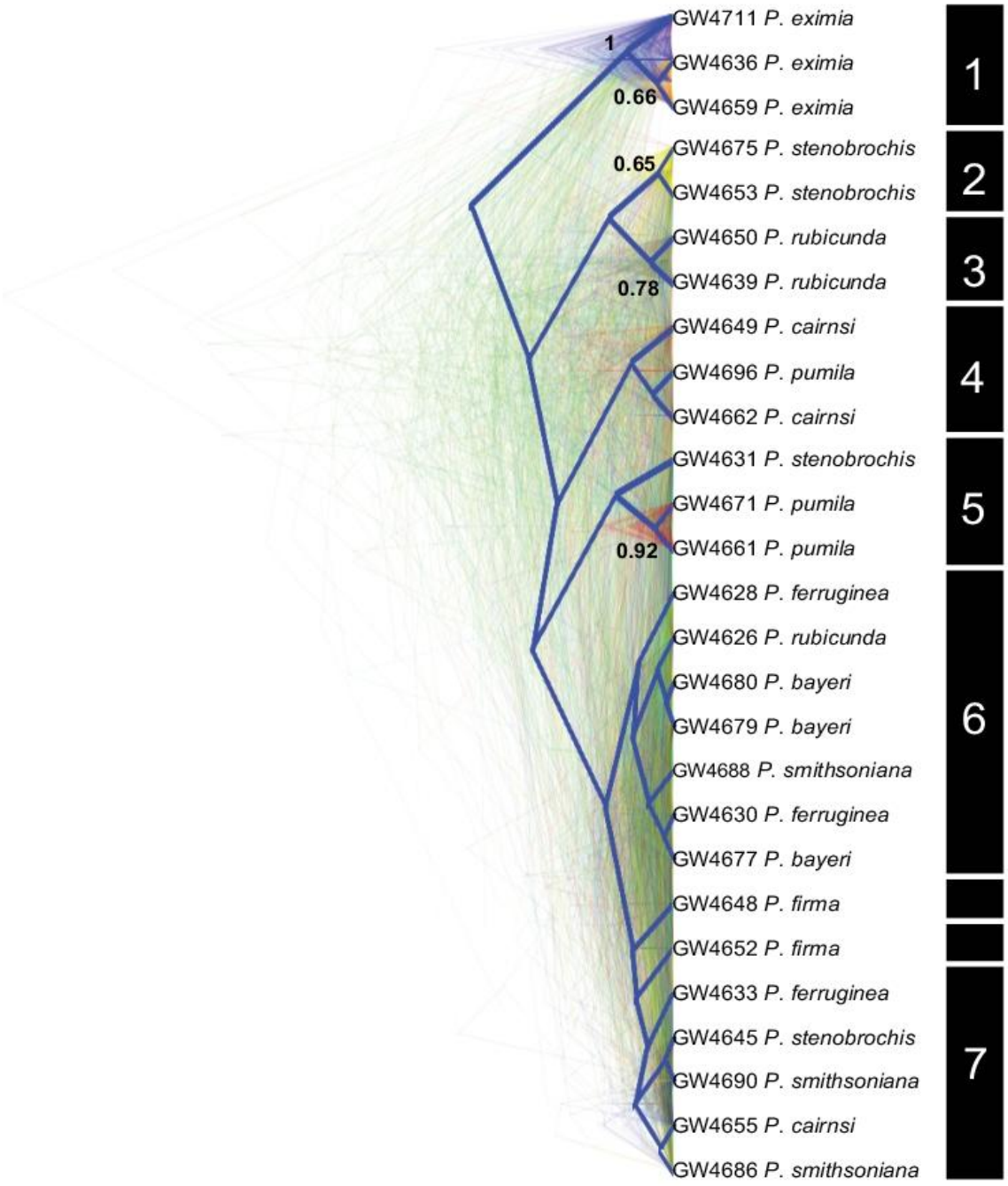

Figure 5.1 Set of trees obtained using a set of 538 SNPs with the Bayes Factor Delimitation (BFD*) method implemented in SNAPP. Numbers on the right side of the trees represent the putative species groups as inferred by the BFD*. The consensus tree is shown in blue and the numbers at the nodes indicate the posterior probability values.

Surprisingly, the two *P. firma* specimens were separated from the rest of the samples and were included in a "mixed" group, which comprises *P. bayeri*, *P. ferruginea* and *P. smithsoniana*, among others.

5.3.2 SNPs and mito-phylogeny

The phylogenetic tree obtained with a set of 538 SNPs, using predefined taxonomic assignments, recovered *P. eximia* sister to all the other species (Figure 5.2). *P. pumila* was sister of *P. stenobrochis* with a fairly low support value (pp=0.84), while the phylogenetic relationships between *P. cairnsi* and *P. rubucanda* were unresolved. *P. bayeri* and *P. ferruginea* were sister species (pp=1) and were further included in a well-supported group (pp=1) which also included *P. smithsoniana* and P. *firma*.

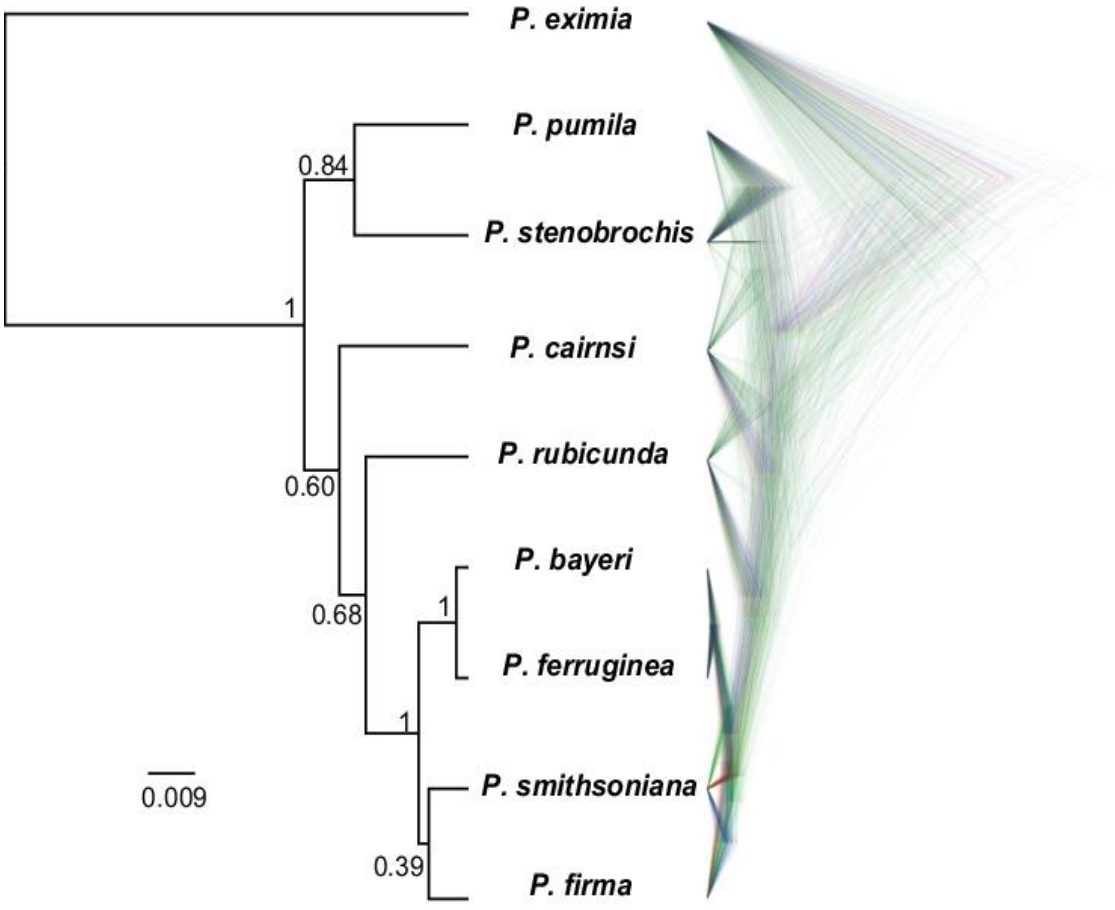

Figure 5.2 Species tree estimation obtained using a set of 538 SNPs. Consensus tree (left) and set of trees (right) inferded with SNAPP are shown on the left and right side, respectively. Number of species and species assignments were *a priori* defined based on preliminary morphological predictions (see Appendix 5.5.1). The numbers at the nodes indicate posterior probability values.

Concerning the mitochondrial phylogeny, the maximum likelihood tree obtained separated 18 specimens into four main groups (see Figure 5.3).

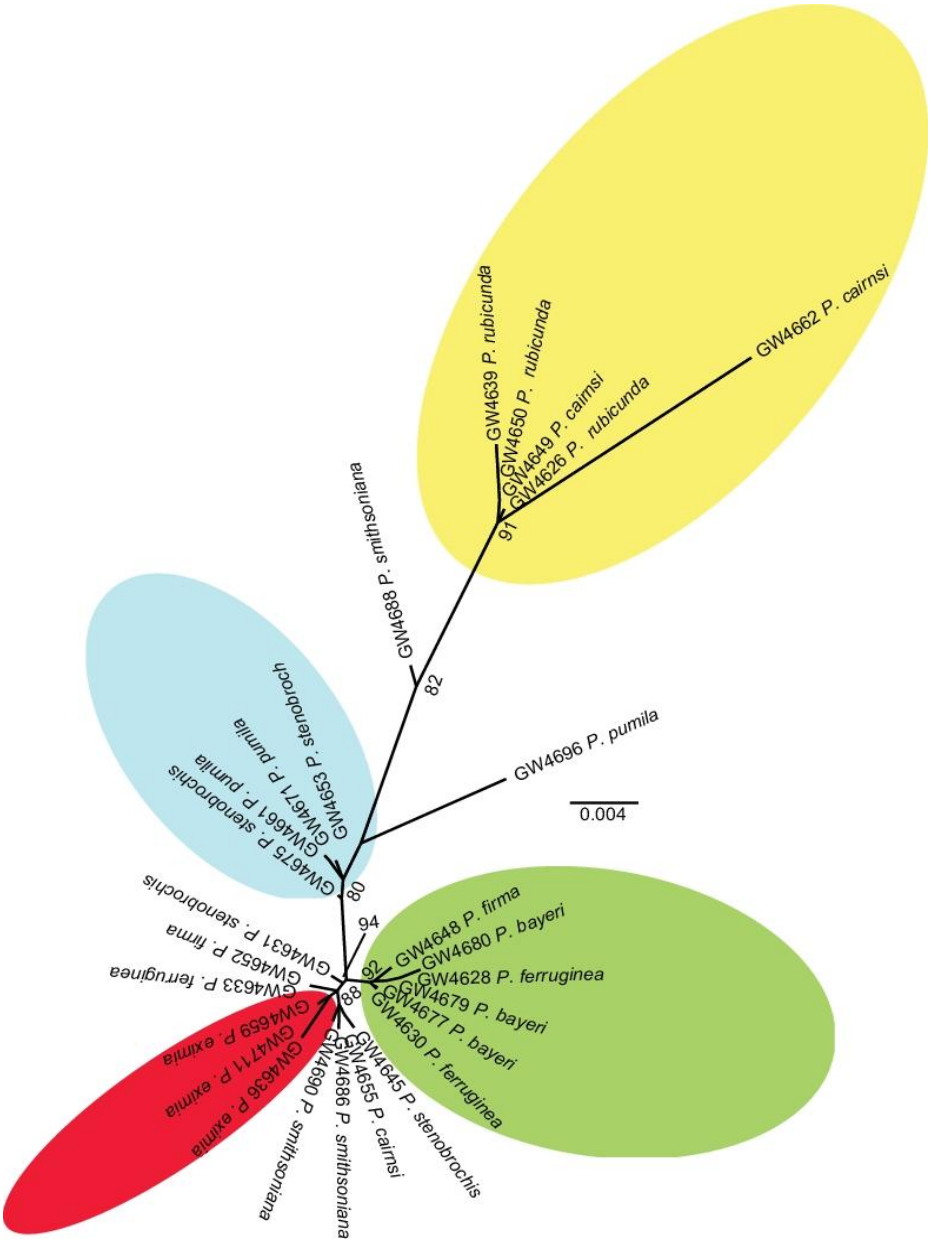

Figure 5.3 Unrooted maximum likelihood tree obtained from a datset consisting of ~3,800 mitochondrial nucleotide positions and 27 taxa. The different colours highlight the four main clades recovered.

Among these, only one group included a single species (e.g. *P. eximia*), whereas the others comprised either two or three different species. Two *P. rubicunda* and two *P. cairnsi* specimens were grouped together (BP=91) and another group comprised three *P. bayeri*, two *P. ferrugina* (GW4628, GW4630) and one *P. firma* sample (GW4648) with high a bootstrap value (BP=92). Similarly, *P. stenobrochis* and *P. pumila* (two specimens each) were included in a single group (BP=80). The remaining specimens were distributed across the tree and some of them grouped in poorly supported clades. For instance, two of

the *P. smithsoniana* samples (GW4686, GW4690) grouped with *P. stenobrochis* (GW4645) and *P. cairnsi* (GW4655) and GW4688 (P. *smithsoniana*) and GW4696 (*P. pumila*) were not closely related to their conspecifics.

Overall, the phylogenies obtained using the mitochondrial and SNPs datasets were consistent and only slight discrepancies were observed (see Figure 5.1 and Figure 5.3). For example, in the mitochondrial phylogeny, *P. rubicunda* (GW4626) was sister to *P. cairnsi* (GW4649, GW4662) and two conspecific species (GW4650, GW4639). In the SNPs phylogeny *P. rubicunda* (GW4626) was included in a clade consisting of *P. ferruginea* (GW4628, GW4630), *P. bayeri* and *P. smithsoniana* (GW4688). *Pacifigorgia stenobrochis* (GW4631) and *P. pumila* (GW4661, GW4671), which grouped together in the SNPs phylogeny, were splint into different subclades in the mitchondrial tree (see Figure 5.1 and Figure 5.3). In the mitochondrial phylogeny *Pacifigorgia ferruginea* (GW4633) was sister to *P. firma* (GW4652), whereas in the tree obtained using SNPs was included into a different clade (Figure 5.1) with low support (posterior probability <0.60).

5.4 Discussion

Single Nucleotide Polymorphisms (SNPs) were recently proposed as a promising marker for species delimitation due to the low level of homoplasy (Brumfield *et al.* 2003). SNPs were also considered a valid and alternative molecular marker for delimiting closely related and recently derived species (Shaffer & Thomson 2007). However, our species delimitation and phylogenetic analyses performed on a pool of different *Pacifigorgia* species using a set of ~500 SNPs showed an overall uncertainty and incongruence with the morphological predictions. Using both SNPs and mitochondrial loci, we found *P. eximia* to be the only species phylogenetically distinct from the others. In terms of morphology, *P. eximia* can be recognised from congenerics by the shape of the spindle sclerites, which typically have acute ends, and by the colony network and its mesh (Breedy & Guzman 2003). In contrast, most of the recently described species (e.g. *P. bayeri*, *P. cairnsi*, *P. ferruginea*, *P. firma*, *P. rubicunda* and *P. smithsoniana*) did not show any phylogenetic relatedness, in agreement with morphology, which revealed remarkably low interspecific variation and high similarities in the branching pattern of the colony and sclerite composition.

For instance, *P. smithsoniana* and *P. ferruginea* can be only identified based on the shape and size of the anthocodial rods (see Breedy & Guzman 2004). Although a high

morphological affinity of both species has been only partially confirmed by our species delimitation based on SNPs analyses, the low support values recovered suggest that our outcomes should be considered with caution. Due to the high plasticity and taxonomic difficulties caused by the lack of defined morphological characters, some of the specimens may have been wrongly identified, therefore further morphological investigations are necessary, but beyond the scope of this study. The phylogenetic placement in the mitochondrial tree of *P. ferruginea* together with *P. bayeri* showed a certain degree of incongruence compared to the phylogeny obtained using SNPs. Similarly, Pante *et al.* (2014) and Herrera & Shank (2015) documented incongruence between the phylogenetic trees inferred from RAD-Seq and mitochondrial data in the octocoral genera *Crysogorgia* and *Paragorgia*, respectively. The phylogenetic discrepancy between mitochondrial and biallelic markers (e.g. AFLP, SNPs) has been associated with to the hybridisation events that occurred among species coexisting in the same habitat (see e.g. Koblmüller *et al.* 2007).

The mitochondrial phylogeny showed high affinity between three *P. rubicunda* and two *P. cairnsi* specimens. These species are commonly found along vertical walls of rocky reefs and they often occur in the same habitat (Breedy & Guzman 2003). Indeed, the samples here analysed were collected from the Jacaron Island and Pta. Catedral in the Southern-West region of the Chiriquí National Park, where the two species coexist. In order to better explore the evolutionary processes that (do not) shape the morphological and genetic variation of these organisms, additional population level studies are required. Concerning *P. stenobrochis*, our results showed remarkable intra-specific variation, which was in agreement with recent molecular studies (Vargas *et al.* 2014). In terms of morphology, taxonomists suggested that many *P. stenobrochis* specimens are usually characterised by diverse sclerite-forms, different colony shapes and variable colours (Breedy & Guzman 2004). The high plasticity observed in the colony shape can be due to the process of adaptation to the different environmental niches, as already shown for other gorgonians exposed to different environmental conditions (see e.g. *Pseudopterogorgia* in Sánchez *et al.* 2007). We also found phylogentic affinity between *P. stenobrochis* and *P. pumila*, confirming what has been recently proposed on the taxonomic re-assignment of *Leptogorgia pumila* to the genus *Pacifigorgia* (Ament-Velásquez *et al.* 2016).

The low genetic variation found among the *Pacifigorgia* species could be the consequence of recent radiation events that occurred in the eastern Pacific as suggested by Vargas *et al.* (2014). Kolarsky & Mann (1995) demonstrated that between the Late

Pliocene (3.6-2.58 Mya) and Early Pleistocene (2.58-0.78 Mya) a subduction and collision between the Cocos Ridge and Costa Rica caused the detachment of the "Chiriquí block" from the Panama Arc. During the same period, the region encontered sea-level oscillations (Cortés 1986) and temperature changes (Glynn & Stewart 1973), which have probably shaped the diversity and distribution of several sessile organisms, including cnidarians. These geological and environmental changes occurred in the Pleistocene and are consistent with the hypothesis here proposed, according to which the high species richness found in the Coiba National Park would be the result of recent speciation. However, as stated by D'Croz & O'Dea (2007, 2009) the exceptional and unique hydrological and environmental conditions of the Gulf of Chiriquí —surface waters constantly warm and poor of nutrient with short seasonal upwelling promoted by thermocline migrations and advection— contribute to make this an hotspot of diversity, with several endemic and ecologically restricted species. The different ecological conditions experienced by the diverse populations may have generated reproductive isolation (e.g. ecological speciation), thereby accelerating speciation (Schluter, 2001; Rundle & Nosil, 2005). Laland *et al.* (2014) in a recent study claimed that: "…plasticity not only allows organisms to cope in new environmental conditions but to generate traits that are well-suited to them. In other words, often it is the trait that comes first; genes that cement it follow, sometimes several generations later…" The fact the many *Pacifigorgia* species have been only described based on the colony shape rather than their molecular variation points out the need to reconsider plasticity. Although this study provides preliminary insights for a better understanding of the genetic diversity and speciation processes in a taxonomic difficult genus, additional studies, including molecular dating and population genetics, are necessarily to shed light on the evolution of these organisms.

5.5 Appendix

Appendix 5.5.1 List of specimens

Appendix 5.5.2 List of the barcode and Illumina adapters used for GBS

Appendix 5.5.1 List of *Pacifigorgia* specimens collected in the National Park of Coiba (Panama) with information on sampling locations and depths. All the samples used for Genotyping by Sequencing (GBS) are in bold. The barcode and Ilumina adapters used for each of the specimens analysed are reported on the right side of the table.

Sample ID number	Species	Location	Depth	Barcode Adapter	Illumina Adapter
GW4711	*Pacifigorgia eximia*	Twin Peaks, Pta. Cirilo	12-17 m	A1	Ilmn_Id10
GW4710	*Pacifigorgia cairnsi*	Twin Peaks, Pta. Cirilo	12-17 m	A6	Ilmn_Id11
GW4700	*Pacifigorgia rubicunda*	Twin Peaks, Pta. Cirilo	12-17 m		
GW4699	*Pacifigorgia rubicunda*	Twin Peaks, Pta. Cirilo	12-17 m		
GW4698	*Pacifigorgia stenobrochis*	Twin Peaks, Pta. Cirilo	12-17 m		
GW4697	*Pacifigorgia stenobrochis*	Twin Peaks, Pta. Cirilo	12-17 m		
GW4696	*Pacifigorgia pumila*	Twin Peaks, Pta. Cirilo	12-17 m	A7	Ilmn_Id8
GW4695	*Pacifigorgia rubicunda*	Twin Peaks, Pta. Cirilo	12-17 m	A8	Ilmn_Id12
GW4694	*Pacifigorgia rubicunda*	Twin Peaks, Pta. Cirilo	12-17 m	A6	Ilmn_Id10
GW4693	*Pacifigorgia stenobrochis*	Twin Peaks, Pta. Cirilo	12-17 m		
GW4692	*Pacifigorgia smithsoniana*	Twin Peaks, Pta. Cirilo	12-17 m		
GW4691	*Pacifigorgia smithsoniana*	Twin Peaks, Pta. Cirilo	12-17 m		
GW4690	*Pacifigorgia smithsoniana*	Twin Peaks, Pta. Cirilo	12-17 m	A2	Ilmn_Id11
GW4689	*Pacifigorgia stenobrochis*	Twin Peaks, Pta. Cirilo	12-17 m	A8	Ilmn_Id8
GW4688	*Pacifigorgia smithsoniana*	Twin Peaks, Pta. Cirilo	12-17 m	A3	Ilmn_Id11
GW4687	*Pacifigorgia smithsoniana*	Twin Peaks, Pta. Cirilo	12-17 m		

GW4686	*Pacifigorgia smithsoniana*	Twin Peaks, Pta. Cirilo	12-17 m	A3	Ilmn_Id10
GW4685	*Pacifigorgia rubicunda*	Twin Peaks, Pta. Cirilo	12-17 m		
GW4684	*Pacifigorgia cairnsi*	Twin Peaks, Pta. Cirilo	12-17 m		
GW4683	*Pacifigorgia cairnsi*	Twin Peaks, Pta. Cirilo	12-17 m		
GW4682	*Pacifigorgia cairnsi*	Twin Peaks, Pta. Cirilo	12-17 m		
GW4681	*Pacifigorgia bayeri*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m		
GW4680	*Pacifigorgia bayeri*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m	A7	Ilmn_Id10
GW4679	*Pacifigorgia bayeri*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m	A4	Ilmn_Id10
GW4678	*Pacifigorgia bayeri*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m		
GW4677	*Pacifigorgia bayeri*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m	A5	Ilmn_Id10
GW4676	*Pacifigorgia eximia*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m		
GW4675	*Pacifigorgia stenobrochis*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m	A4	Ilm_Indx9
GW4674	*Pacifigorgia rubicunda*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m		
GW4673	*Pacifigorgia cairnsi*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m		
GW4672	*Pacifigorgia rubicunda*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m	A8	Ilmn_Id10
GW4671	*Pacifigorgia pumila*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m	A5	Ilmn_Id9
GW4670	*Pacifigorgia firma*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m		
GW4669	*Pacifigorgia rubicunda*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m		
GW4668	*Pacifigorgia cairnsi*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m		
GW4667	*Pacifigorgia cairnsi*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m		
GW4666	*Pacifigorgia firma*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m		

GW4665	*Pacifigorgia cairnsi*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m		
GW4664	*Pacifigorgia eximia*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m		
GW4663	*Pacifigorgia eximia*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m	A1	Ilmn_Id12
GW4662	*Pacifigorgia cairnsi*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m	A4	Ilmn_Id8
GW4661	*Pacifigorgia pumila*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m	A6	Ilmn_Id9
GW4660	*Pacifigorgia pumila*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m	A7	Ilmn_Id9
GW4659	*Pacifigorgia eximia*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m	A1	Ilmn_Id11
GW4658	*Pacifigorgia stenobrochis*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m		
GW4657	*Pacifigorgia stenobrochis*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m		
GW4656	*Pacifigorgia cairnsi*	Pta. Catedral	10-20 m	A4	Ilmn_Id11
GW4655	*Pacifigorgia cairnsi*	Pta. Catedral	10-20 m	A3	Ilmn_Id9
GW4654	*Pacifigorgia cairnsi*	Pta. Catedral	10-20 m		
GW4653	*Pacifigorgia stenobrochis*	Pta. Catedral	10-20 m	A5	Ilmn_Id11
GW4652	*Pacifigorgia firma*	Pta. Catedral	10-20 m	A6	Ilmn_Id8
GW4651	*Pacifigorgia cairnsi*	Pta. Catedral	10-20 m	A2	Ilmn_Id12
GW4650	*Pacifigorgia rubicunda*	Pta. Catedral	10-20 m	A6	Ilmn_Id12
GW4649	*Pacifigorgia cairnsi*	Pta. Catedral	10-20 m	A5	Ilmn_Id8
GW4648	*Pacifigorgia firma*	Pta. Catedral	10-20 m	A3	Ilmn_Id12
GW4647	*Pacifigorgia stenobrochis*	La Nevera, Islas Copntreras	11-18 m	A4	Ilmn_Id12
GW4646	*Pacifigorgia stenobrochis*	La Nevera, Islas Copntreras	11-18 m	A5	Ilmn_Id12
GW4645	*Pacifigorgia stenobrochis*	La Nevera, Islas Copntreras	11-18 m	A8	Ilmn_Id9

GW4644	*Pacifigorgia cairnsi*	La Nevera, Islas Copntreras	11-18 m		
GW4643	*Pacifigorgia cairnsi*	La Nevera, Islas Copntreras	11-18 m		
GW4642	*Pacifigorgia cairnsi*	La Nevera, Islas Copntreras	11-18 m		
GW4641	*Pacifigorgia rubicunda*	La Nevera, Islas Copntreras	11-18 m		
GW4640	*Pacifigorgia rubicunda*	La Nevera, Islas Copntreras	11-18 m		
GW4639	*Pacifigorgia rubicunda*	La Nevera, Islas Copntreras	11-18 m	A7	Ilmn_Id12
GW4638	*Pacifigorgia eximia*	La Nevera, Islas Copntreras	11-18 m	A2	Ilmn_Id10
GW4637	*Pacifigorgia eximia*	La Nevera, Islas Copntreras	11-18 m	A3	Ilmn_Id8
GW4636	*Pacifigorgia eximia*	La Nevera, Islas Copntreras	11-18 m	A2	Ilmn_Id9
GW4635	*Pacifigorgia eximia*	La Nevera, Islas Copntreras	11-18 m		
GW4634	*Pacifigorgia ferruginea*	Montaña rusa, Bricano Norte	12-24 m		
GW4633	*Pacifigorgia ferruginea*	Montaña rusa, Bricano Norte	12-24 m	A1	Ilmn_Id8
GW4632	*Pacifigorgia stenobrochis*	Montaña rusa, Bricano Norte	12-24 m		
GW4631	*Pacifigorgia stenobrochis*	Montaña rusa, Bricano Norte	12-24 m	A7	Ilmn_Id11
GW4630	*Pacifigorgia ferruginea*	Dedo, Islas Contreras	10-18 m	A2	Ilmn_Id8
GW4629	*Pacifigorgia smithsoniana*	Dedo, Islas Contreras	10-18 m		
GW4628	*Pacifigorgia ferruginea*	Dedo, Islas Contreras	10-18 m	A1	Ilmn_Id9
GW4627	*Pacifigorgia stenobrochis*	Dedo, Islas Contreras	10-18 m		
GW4626	*Pacifigorgia rubicunda*	Piedra Hacha, zona Jicarita/Jicaron	8-20 m	A8	Ilmn_Id11

Appendix 5.5.2 List of the barcode and Illumina adapters used for Genotyping by Sequencing (GBS). The barcode and index sequences are reported for each of the adapters.

Barcode Adapter		**Illumina Adapter**	
Adapter name	**Barcode sequence***	**Adapter name**	**Index sequence°**
GBS_Apek1_A1	AACAGT	Ilmn_Id1	ATCACG
GBS_Apek1_A2	CCAGTA	Ilmn_Id2	CGATGT
GBS_Apek1_A3	TTGCAC	Ilmn_Id3	TTAGGC
GBS_Apek1_A4	GGTTCA	Ilmn_Id4	TGACCA
GBS_Apek1_A5	AACGTC	Ilmn_Id5	ACAGTG
GBS_Apek1_A6	CCAAGT	Ilmn_Id6	GCCAAT
GBS_Apek1_A7	TTGTCA	Ilmn_Id7	CAGATC
GBS_Apek1_A8	GGTCAC	Ilmn_Id8	ACTTGA
		Ilmn_Id9	GATCAG
		Ilmn_Id10	TAGCTT
		Ilmn_Id11	GGCTAC
		Ilmn_Id12	CTTGTA

Barcode Adapter ACACTCTTTCCCTACACGACGCTCTTCCGATCT(*****)

Illumina Adapter CAAGCAGAAGACGGCATACGAGAT(°)GTGACTGGAGTTCAGACGTGTGC

Conclusive discussion

One of the goals of this study was to identify and delimit octocoral species in taxonomically difficult alcyonacean genera using several cases as examples. Although our analyses highlighted the overall limitations of *COI* and *mtMutS* for species delimitation in *Lobophytum* and *Sarcophyton*, the remarkable diversity found within the genus *Sarcophyton* using 28S rDNA indicates the need of considering a multi-locus approach. While the polymorphism detected in terms of sequence length makes 28S rDNA a suitable alternative to the standard barcodes (e.g. *COI*), the presence of indel-rich regions, probably due to the existence of pseudogenes, may significantly distort the phylogenetic signal. Therefore, before considering 28S rDNA for molecular identifications of *Lobophytum* and *Sarcophyton* species, the presence of putative pseudogenes should be in future explored. The number of hypothetical *Lobophytum* and *Sarcophyton* species estimated in the Kimberley region varied according to the marker and method used, however the large number recovered is most likely an artefact caused by the low inter-specific variation. Despite the fact that *mtMutS* demonstrated a general lack of resolution for species level identification, rapid assessment of species richness and diversity in the Kimberley was possible. The obtained results showed that using standard molecular procedures is possible, without specific taxonomic skills, in a short time and with reduced costs, to preliminary identify a restricted number of putative species.

The recent developments in library preparation methodologies and Next Generation Sequencing (NGS) technologies have made genomic studies more accessible for the analysis of non-model organisms such as octocorals. In this study, beside the use of partial mitochondrial protein-coding genes, were tested genome-wide Single Nucleotide Polymorphisms (SNPs) to explore the phylogenetic relationships among morphologically cryptic species. The species discrimination and the phylogeny inferred with a set of SNPs showed uncertainty and incongruence when compared to the morphological predictions based on the colony and sclerite morphology. A degree of divergence was also detected between the mitochondrial and SNP phylogenies, suggesting that hybridisation events may have contributed to the diversification of these organisms. The data generated demonstrated that traditional taxonomy could benefit from integrative approaches based on molecular methods, especially in presence of morphological homogeneus taxa and species with high penotypic plasticity. The high number of *Pacificorgia* species recently described in the eastern Pacific and the morphological diversity shown by the specimens collected in

the Panamic province, is only partly corroborated by the molecular results. In particular, the low inter-specific variation found could be related to recent speciation, which may be associated to the Pleistocenic tectonic events that shaped the geological profile of the central eastern Pacific. A better taxon sampling along with population level studies will be essential to better understand the evolutionary processes that lead to the diversification of these organisms.

Concerning the Mediterranean soft corals, this study represented the first attempt to reconstruct the phylogeny, biogeography and evolutionary history of some, among the most abundant, Mediterranean gorgonians. In particular, the unexpected phylogenetic divergence detected between the two endemic Mediterranean *Paramuricea* species has been here associated to independent speciation events that, according to the molecular clock estimates, may be linked to the geological changes occurred in the Mediterranean during the Messinian (~5.2 Mya) and Gelasian (~2.6 Mya) crises. In addition, the biogeographic analyses performed using an extended *mtMutS* dataset including species distributed in the Atlantic and Mediterranean, pointed to the importance of vicariance for faunal diversification across these basins. Similar to the red gorgonian (*P. clavata*), which was sister to the Atlantic *P. grayi* rather than its Mediterranean congeners, *Leptogorgia sarmentosa* was found to be closely related to Atlantic members and molecular dating analyses suggested for these organisms a recent divergence (~3 Mya). These findings support the hypothesis for the Mediterranean gorgonians *L. sarmentosa* and *P. clavata* of an Atlantic ancestor in agreement with a post-Mesinian Crisis model of colonization as proposed for several Mediterranean marine invertebrates.

Using either traditional molecular methods (PCR, Sanger sequencing) or NGS technologies 13 complete mitogenomes were sequenced, triplicating the number of complete mitochondrial genomes available for gorgonians. The mitogenomic comparison between closely related species showed novel genomic features (e.g. ORF of unknown function) and high nucleotide variation. The highest mutation rate was that found in the intergenic spacers suggesting that these regions can be used in future for molecular identification at species-level. In agreement with previous studies, mainly based on a handful of mitochondrial loci, the mitogenome phylogeny clearly supported the paraphyly of the family Plexauridae and within Gorgoniidae, the polyphyly of the genus Leptogorgia. In particular, the phylogenetic segregation among eastern Atlantic and eastern Pacific *Leptogorgia* species, which was also confirmed by the *mtMutS* phylogeny, indicated the existence of multiple lineages in need of taxonomic revision.

This study has broaden our understanding on the evolution and systematics of ecologically important soft corals providing new and additional insights on the phylogenomics of these organisms.

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Declaration

I hereby confirm that my thesis entitled “Speciation, Evolution and Phylogeny of some Shallow-water Octocorals (Cnidaria: Anthozoa)”, is the result of my own original work. Furthermore, I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any University and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any University or other tertiary institution without the prior approval of the Ludwig-Maximilians-University, Munich.

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School and academic education

2013 – Phd in Geobiology at the Molecular Geo- and Palaeobiology Lab - Department of Earth & Environmental Sciences, Palaeontology & Geobiology of the Ludwig Maximilians Universität (LMU) in Munich (Germany). Research project: Speciation evolution and phylogeny of some shallow water octocorals. Supervisor: Prof. Gert Wörheide; Advisor: Dr. Sergio Vargas.

2010 M.Sc. in Marine Biology, Università Politecnica delle Marche, Ancona (Italy). Thesis: Molecular phylogeny of black corals (Hexacorallia: Antipatharia). Supervisor: Prof. Adriana Canapa & Dr. Marco Barucca.

2007 B.Sc. in Biological Sciences, Alma Mater Studiorum, Bologna (Italy). Thesis: Molecular phylogeny of Mediterranean octocorals. Training at the Department of Environmental Science, Ravenna (Italy). Supervisor: Prof. Marco Abbiati & Dr. Federica Costantini.

Internships

2012 Short term scientific visit at the NCB Naturalis Biodiversity Center, Leiden (The Netherlands). Subject: Taxonomy of zoanthids collected from different geographical area. Supervisor: Dr. James Davis Reimer.

2012 Internship at the Università Politecnica delle Marche, Ancona (Italy). Research project: Molecular phylogeny of black coral (Antipatharia). Advisor: Prof. Adriana Canapa.

2008 Training at the Marine Research Center, Cesenatico (Italy). Subject: Extraction of biotoxin compounds from Adriatic algae. Supervisor: Dr. Anna Milandri.

Diving license

2009 Advanced Open Water Diver CMAS.

2004 Open Water Diver PADI.

Scholarships and fellowships

2015 Fellowship for Highly Dedicated International Doctoral Students under the auspices of the STIBET and support program of the DAAD (German Academic Exchange Service).

2013 Short term research Scholarship financed by (DAAD. Research project: Molecular identification of Australian shallow-water Alcyoniina (Octocorallia: Alcyonacea) using mitochondrial (MutS) and nuclear (ITS2, SRP54) markers. Supervisor: Prof. Gert Wörheide.

2011 "Leonardo da Vinci" Project financed by E.U. and Emilia-Romagna (Italy). Training at O.A.DY.K. (Organization for the Development of Western Crete) on scleractinian fossils. Project entitled: Late Miocene Fossils: Paleoecological and taxonomic consideration on coral fossils from Prasses Formation in Rethymno (Crete – Greece).

2010 Scholarship "Campus World" Università Politecnica delle Marche (Italy). Work activities: Antipatharia and *Zoanthidea.* Further investigations on the ecology and taxonomy of gorgonians in the National Park of Machalilla (Ecuador).

Oceanographic cruises

2013 Cruise "RECORD" aboard the Research Vessel *Urania.* The cruise was under the umbrella of RITMARE, COCONET and MISTRAL/PALEOMEX/COFIMED projects. Mediterranean deep-sea ecosystems. (ISMAR-CNR in Bologna Co-Chief Scientist Dr. Paolo Montagna and Dr. Marco Taviani).

2012 Cruise "ALTRO" aboard the Research Vessel *Urania.* The cruise was under the umbrella of EU 'CoCONET' programme and was a further step to promote the knowledge on the genesis, build-up and demise of the middle-southern Adriatic deep-water corals and to evaluate the connectivity between the Adriatic and the extra-Adriatic deep-water coral stocks. (ISMAR-CNR in Bologna Co-Chief Scientist Dr. Marco Taviani and Dr. Lorenzo Angeletti).

2011 Cruise "MARMARA 2011" Gulf of Saros and Marmara Sea aboard the Research Vessel *Urania*. (ISMAR-CNR in Bologna Chief Scientist Dr. Luca Gasperini).

2011 Cruise "DECORS" in the Mediterranean Sea aboard the Research Vessel *Urania*. The cruise was under the umbrella of EU Hermione programme. (ISMAR-CNR in Bologna Co-Chief Scientist Dr. Marco Taviani and Dr. Lorenzo Angeletti).

Publications

Journals SCI, peer-reviewed

2015 Monika Bryce, **Angelo Poliseno**, Philip Alderslade, Sergio Vargas. Digitate and capitate soft corals (Cnidaria: Octocorallia: Alcyoniidae) from Western Australia with reports on new species and new Australian geological records. *Zootaxa* 3963(2): 160-200.

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Journals non-SCI, peer-reviewed

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Books and book chapters

2016 **Angelo Poliseno**. Tassonomia e Filogenesi. In: Egidio Trainito, Rossella Baldacconi (Eds), Coralli del Mediterraneo. Il Castello Editore, Milan, Italy. ISBN: 978886520786, pp. 176. (In Italian).

Conferences

2016 Monika Bryce, **Angelo Poliseno**, Gert Wörheide, Sergio Vargas. Nidaliidae (Cnidaria: Octocorallia) of the Kimberley region, Western Australia: diversity, distribution and molecular systematic. Abstract presentation at the *Invertebrate Biodiversity and Conservation Conference* - Fremantle, Australia.

2013 **Angelo Poliseno**, Monika Bryce, Sergio Vargas, Gert Wörheide. *Lobophytum, Sarcophyton*: molecular identification of two widespread alcyoniids genera

(Octocorallia: Alcyonacea) from western Australian shallow-water. Oral presentation at the *106th Annual Meeting of the German Zoological Society* - Munich, Germany.

2013 Monika Bryce, Clay Bryce, **Angelo Poliseno**, Sergio Vargas, Gert Wörheide. Soft Coral life of the Kimberley bioregion, Western Australia: Diversity, Distribution, and molecular systematic". Abstract presentation at the *Systematics Without Borders Conference* - Sydney, Australia.

Languages

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