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Molecular phylogenesis of Mediterranean Octocorals

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1. Introduction

1.1. Ecosystem functions and treats of Mediterranean coralligenous

The so-called "coralligène" (Ballesteros 2006) is one of the main Mediterranean hard substratum habitat, which generally occur between 20 and 120 m depth along coasts (Laborel 1987) and seamounts (Clark et al. 2010). The name "coralligenous" could be originate from the findings of red coral branches and calcareous organisms in trawling hauls of semi dark sub littoral bottoms with coarse gravel (Tsounis et al. 2010; Ballesteros 2006). These habitats as the tropical reefs are considered a species diversity ''hot spot''; therefore, these reefs are intrinsically valuable for their biological diversity and for the ecological processes that they can support (Ballesteros 2006; Coll et al. 2010). However, we are still only beginning to understand the principal ecological aspects of coralligenous assemblages, including the environmental factors (temperature, salinity, nutrition) and biological processes (reproductive biology, molecular genetics, predation, parasitism and bioerosion), which regulate their life and distribution (UNEP 2004; UNEP 2010).

Several sources of human disturbance threaten Mediterranean coralligenous assemblages, e.g.: pollution, sediment smothering and deposition, bottom-trawling, deep-sea mining, hydrocarbon extraction, waste disposal, recreational fishing and diving (Airoldi & Beck 2007; Airoldi et al. 2008; see for review). Moreover, environmental changes, leading to mass mortality events (Cerrano et al. 2000), invasions by alien species (Occhipinti-Ambrogi 2007) and acidification (Ramirez-Llodra et al. 2011), are additional sources of disturbance to these habitats. Other causes of mortality for these benthic assemblages are bottom hypoxia/anoxia events and suffocation by mucilaginous aggregates (Ponti et al. 2011 and references therein). Due

to the above mentioned features, the Mediterranean coralligenous should be a priority for several international bodies (e.g. UNEP, IUCN, EU organizations; Fraschetti et al. 2011). In order to support the protection of coralligenous reefs, identification of species cladogenesis and preservation of evolutionarily distinct lineages (as for example endemic species) are important aspects to take in account for their conservation (Fukami et al. 2004).

Among the coralligenous habitat, octocorals (Anthozoa: Octocorallia) are one of the main taxonomic groups with a high number of species. Moreover, some of them have a pivotal role as ecosystem engineers. In fact, they act as refuge or nursery zone, may reduce current flow velocity, can stabilise soft substrata and can experience a sort of ''buffer zone'' where environmental modifications occur slower (Cerrano et al. 2009). Moreover, coral ecosystems also support fisheries (D'Onghia et al. 2011; Soeffker et al. 2011) and have been identified as important sources of marine natural products (Leal et al. 2012). Treats as fishing impacts on octocorals are less well known (Stone, 2006; Edinger et al., 2007). The resilience of such communities is perceived to be very low as some octocorals have slow growth rates and high longevity (Freiwald et al., 2004; Althaus et al., 2009). A significant problem with the identification of vurnerable marine ecosystems (VMEs), such as octocoral habitat, is the lack of information of benthic communities throughout much of the deep sea (Yesson et al 2012).

1.2. Mediterranean octocorals morphological characteristics and classification

Within Anthozoa, the subclass Octocorallia included approximately 3,000 existing species (Daly et al. 2007), the diagnostic apomorphies (an evolutionary trait that is unique to a particular species and all its descendants; Dictionary of Biology 2000) are the eight tentacles and the eight mesenteries of polyps (Daly et al. 2007; Figure 1.1), as suggested by the group name.

The current classification system divides Octocorallia into three orders: Alcyonacea, Pennatulacea, and Helioporacea (Bayer 1981). Only the first two orders live in the Mediterranean Sea. The primary morphological characters that have been used to define the Alcyonacea families are the overall growth forms of colonies, details of the composition of the skeletal axis (if present is characterized by having some form of hard skeletal structure composed of some combination of calcium carbonate and the horn-like, proteinaceous material gorgonin; McFadden et al. 2006) and the shape or the arrangement of sclerites (free skeletal elements embedded in the tissue of polyps and coenenchyme; Daly et al. 2007). The presence of pinnules (lateral extensions) on the tentacles is also considered diagnostic, although this character is absent in several taxa (Daly et al. 2007; Figure 1.1). Differently, the Pennatulacea (sea pens) are recognizable by the unique colony form, in which the single axial polyp differentiates into a proximal peduncle and a distal rachis. This represents a morphological synapomorphy (i.e. the possession of apomorphic features by two or more taxa, which related the group evolutionarily; Dictionary of Earth Science 1999; Figure 1.1) that clearly unites species from the order Pennatulacea and distinguishes its members from all other octocorals (Daly et al. 2007).
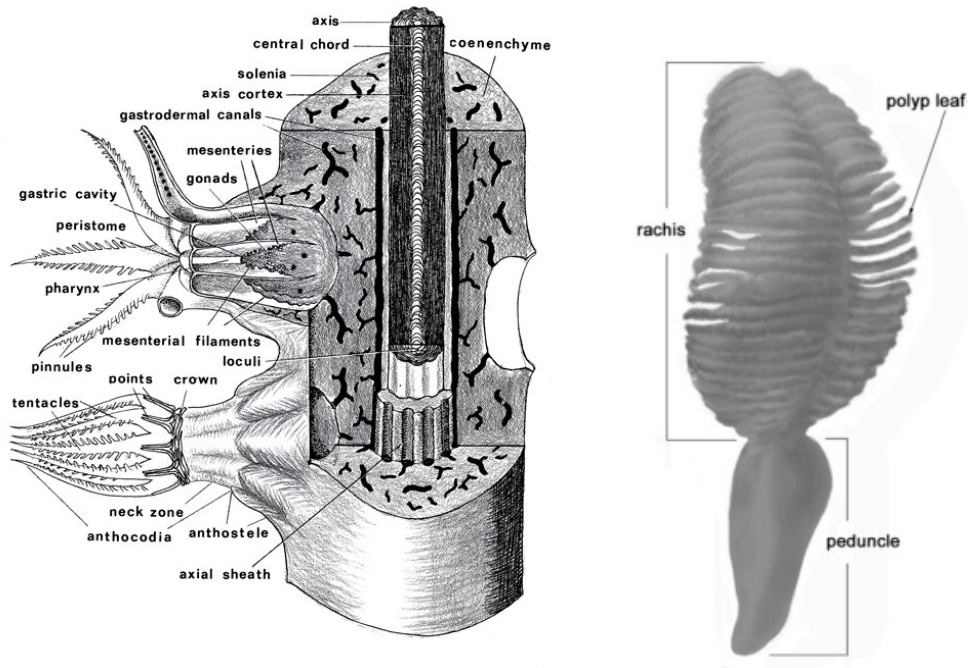
Figure 1.1. On the left, scheme of a gorgonacean ctocoral (from Bayer et al. 1983). On right, picture of a *Pennatula sp.* of Subsessiflorae sub-order (Williams et al. 2011).

Within Anthozoa, the subclass Octocorallia included approximately 3,000 existing species (Daly et al. 2007). In the Mediterranean Sea, diverse scientific sources such as S.I.B.M. Italian species checklist (Morri 2008), or Oceana "Mediterranean corals" reports (Oceana 2007) and several zoological papers recognized 56 Octocorallia species to date (Table 1.1).

Species rank	Papers references
OTTOCORALLIA (SUBCLASS)	
ALCYONACEA (ORDER)	
Sub order Alcyoniina	
Family Alcyonidae	
Alcyonium	
Alcyonium palmatum	Morri 2008
Alcyonium acaule	Morri 2008
Parerythropodium	
Parerythropodium coralloides	Ocàna et al. 2000
Family Paralcyonidae	
Paralcyonium	
Paralcyonium spinulosum	Morri 2008
Maasella	
Maasella edwardsii	Morri 2008
Family Nidaiidae	
Nidalia	
Nidalia studeri	Lòpez-Gonzales 2012
Sub order Calcaxonia	
Family Ellisellidae	
Ellisella	
Ellisella paraplexauroides	Vafidis et al. 1994
Viminella	
Viminella flagellum	Giusti et al. 2011
Family Isididae	
Isidella	
Isidella elongata	Morri 2008
Family Primnoidae	
Callogorgia	
Callogorgia verticillata	Morri 2008
Family Dendrobrachiidae	
Dendrobrachia	
Dendrobrachia bonsai	Sartoretto 2012
Dendrobrachia fallax	Zibrowius & Taviani (2005)
Sub order Holaxonia	
Family Acanthogorgiidae	
Acanthogorgia	
Acanthogorgia hirsuta	Morri 2008
Acanthogorgia armata	Ocàna et al. 2000
Family Gorgoniidae	
Eunicella	
Eunicella cavolini	Morri 2008
Eunicella singularis	Morri 2008
Eunicella verrucosa	Morri 2008

Eunicella filiformis	Gori et al. 2012 and references therein
Eunicella gazella	Gori et al. 2012 and references therein
Eunicella labiata	Gori et al. 2012 and references therein
Filigorgia	
Filigorgia guineensis	Ocàna et al. 2000
Leptogorgia	
Leptogorgia sarmentosa	Morri 2008
Leptogorgia viminalis	Oceana 2007

Family Plexauridae
Bebryce	
Bebryce mollis	Morri 2008
Muriceides	
Muriceides lepida	Morri 2008
Paramuricea	
Paramuricea clavata	Morri 2008
Paramuricea macrospina	Morri 2008
Placogorgia	
Placogorgia coronata	Vafidis et al. 1994
Placogorgia massiliensis	Vafidis et al. 1994
Spinimuricea	
Spinimuricea klavereni	Morri 2008
Spinimuricea atlantica	Vafidis et al. 1994
Swiftia	
Swiftia pallida	Morri 2008
Swiftia dubia	Vafidis et al. 1994
Villogorgia	
Villogorgia bebrycoides	Morri 2008

Sub order Scleraxonia
Family Corallidae
Corallium	
Corallium rubrum	Morri 2008

Sub order Stolonifera
Family Clavulariidae
Clavularia	
Clavularia crassa	Morri 2008
Clavularia marioni	Morri 2008
Clavularia carpediem	Oceana 2007
Rolandia	
Rolandia coralloides	Morri 2008
Sarcodictyon	
Sarcodictyon catenatum	Ocàna et al. 2000
Scleranthelia	
Scleranthelia rugosa	Oceana 2007
Scleranthelia microsclera	Oceana 2007

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Family Cornulariidae
    Cornularia
            Cornularia cornucopiae      Morri 2008
    Cervera
            Cervera atlantica            Oceana 2007

PENNATULACEA (ORDER)
Sub order Sessiflorae
Family Funiculinidae
    Funiculina
            Funiculina quadrangularis    Morri 2008
Family Veretillidae
    Veretillum
            Veretillum cynomorium        Morri 2008
    Cavernularia
            Cavernularia pusilla         Oceana 2007
Family Kophobelemnidae
    Kophobelemnon
            Kophobelemnon stelliferum    Morri 2008
            Kophobelemnon leucharti      Oceana 2007
Sub order Subsessiflorae
Family Pennatulidae
    Pennatula
            Pennatula rubra              Morri 2008
            Pennatula phosphorea         Morri 2008
            Pennatula aculeata           Ocàna et al. 2000
    Pteroides
            Pteroides spinosum           Morri 2008
            Pteroides griseum            Oceana 2007
    Crassophyllum
            Crassophyllum thessalonicae  Vafidis et al. 1994
Family Virgulariidae
    Virgularia
            Virgularia mirabilis         Morri 2008
```

Table 1.1. List of Mediterranean octocorals. Species classification was checked by the world register of marine species (WoRMS) and the European marine register of biodiversity and ecosystem functions (MaRBEF).

The Mediterranean Alcyonacea can be divided into five sub-ordinal groups (Alcyoniina, Calcaxonia, Scleraxonia, Holaxonia and Stolonifera; Daly et al. 2007). Alcyoniina comprise two families of soft corals (Alcyonidae and Paralcyonidae) in which polyps are embedded within a fleshy mass of coenenchymal tissue (McFadden

et al. 2010; Figure 1.2-H, I, M, N). Only three Calcaxonia families, characterized by a solid axial skeleton, live in the Mediterranean Sea and most species are distributed in deep water (Watling et al. 2011). In the Scleraxonia sub-order the axis is composed largely of hard structure (McFadden et al. 2010). Just one species of this sub-order is represented in the Mediterranean: *Corallium rubrum* (Figure 1.2-D). The major number of Mediterranean octocorals is represented by the Holaxonia, the typical sea fan or sea whips with soft proteinaceous skeletal. Many families of this group are defined by skeletal axis details, such as the diameter of the hollow central core, and the presence of scleritic or non-scleritic calcite (McFadden et al. 2010; Figure 1.2-A, B, C, E, F and G). Stolonifera includes two families (Clavularidae and Cornularidae; Figure 1.2-L) in which polyps are connected basally by stolons or thin membranes (McFadden et al. 2010). Pennatulacea are distinguished in two sub order by the conspicuous presence of polyp leaves, often strap-shaped wing-like expansions, which can contain numerous (sub-order Subsessiflorae; Figure 1.2-O, P) or less (sub-order Sessiflorae; Williams 2011). Two families of leaf sea pens (Virgularidae and Pennatulidae) and three of Sessiflorae (Funiculinidae, Kophobelemnidae and Veretillidae) were found in the Mediterranean Sea to date.
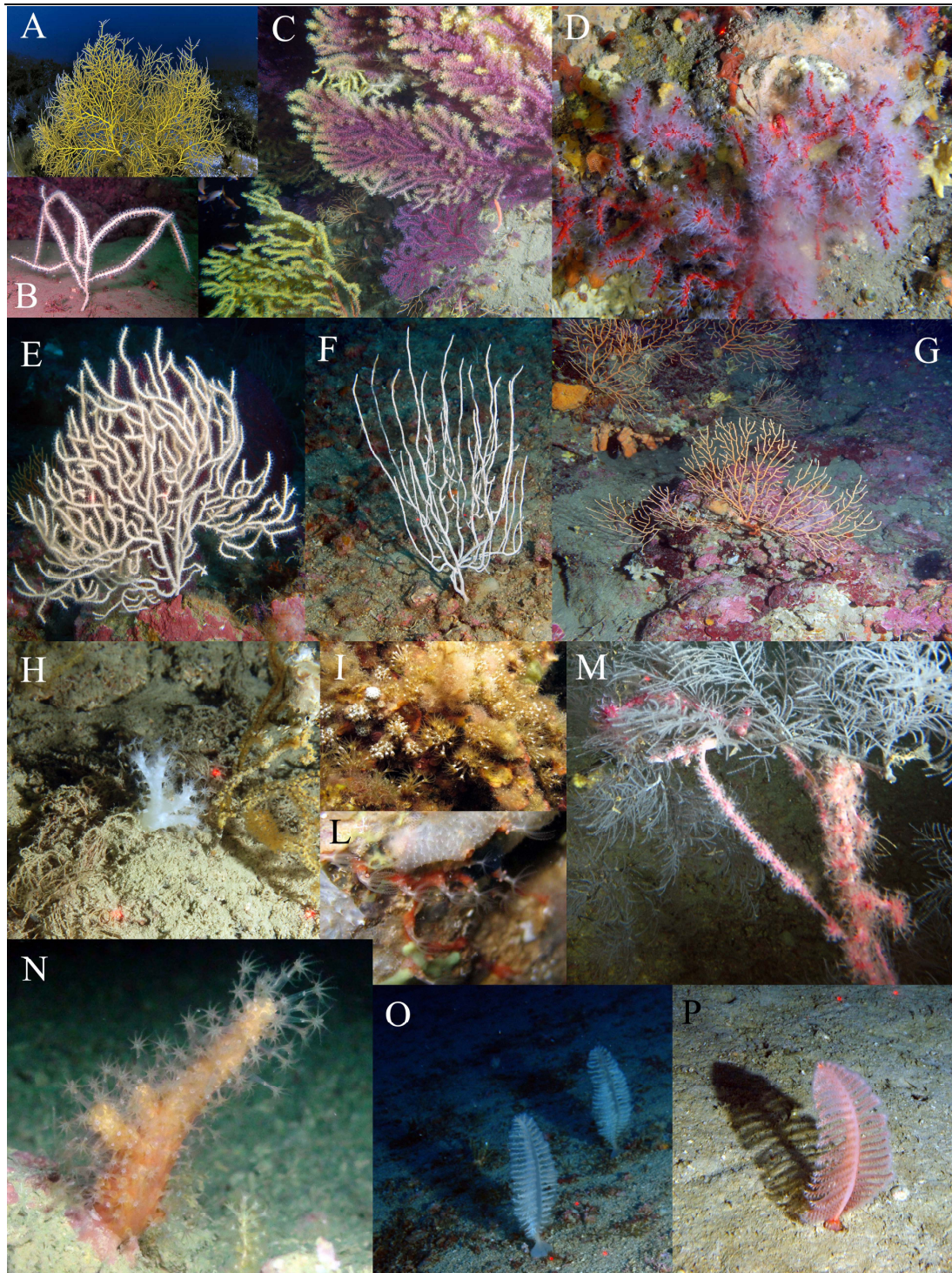
Figure 1.2. Picture of some Mediterranean Octocorals: *Leptogorgia sarmentosa* (A), *Swiftia pallida* (B), *Paramuricea clavata* (C), *Corallium rubrum* (D), *Eunicella verrucosa* (E), *Eunicella singularis* (F) *Eunicella cavolini* (G), *Paralcyonium spinulosum* (H), *Maasella edwardsii* (I), *Sarcodictyon catenatum* (L), *Parerythropodium coralloides* (M), *Alcyonium sp.* (N), *Pteroides spinosum* (O) and *Pennatula rubra* (P).

1.2.1 Mediterranean octocorals distribution

The number of endemic species in the Mediterranean Sea are high, averaging more than one quarter of the whole Mediterranean biota. Compared to world estimates, the Mediterranean apparently harbours somewhat between 4% and 18% of the world marine species, with large differences according to the group considered (Bianchi and Morri 2000). Vicariance (Cunningham & Collins 1998), historical environmental factors related to habitat, currents and glaciations (Roy et al. 1996; Wares 2002) combined with biological species-specific traits such as reproduction (Jackson 2001), play a pivotal role in shaping the pattern of inter- and intra-specific differentiation of many species inhabiting the Mediterranean Sea (see Paternello et al. 2007 and references therein). In fact, the biogeography of this Sea strongly reflects both ancestral and modern discontinuities in marine environment. (Longhurst 1998). For example, Octocorals are important members of a wide variety of marine communities, from shallow tropical coral reefs to the deep sea bottoms and cliffs (McFadden et al. 2006). But their species diversity within Mediterranean Sea is constrained by some factors.

However, among the Mediterranean octocorals species selected for this thesis, only *Eunicella cavolini* two species were was recognized as true endemism, *Maasella edwardsii,* and *Eunicella cavolin*(*i* (Rossi 1959; Table 1.2). Some other species as *Corallium rubrum* (Zibrowius et al. 1984), *Maasella edwardsii* (Parenzan 1977) *and Eunicella singularis* (Rossi 1959*)* were largely present and typical of the Mediterranean Sea, but they have been found also along the South-Eastern coasts of the Atlantic Ocean. The rest of the analysed species, such as *Paramurice clavata* and *Pennatula rubra*, had a widespread distribution generally extended to all the North-Est Atlantic, but with few scientific observation.

Taxa	Species distribution
OTTOCORALLIA (SUB CLASS)	
ALCYONACEA (ORDER)	
SUB ORDER ALCYONIINA	
Family Alcyonidae	
Alcyonium	
Alcyonium palmatum	NE-Atlantic, Mediterranean
Alcyonium acaule	NE-Atlantic, Mediterranean
Parerythropodium	
Parerythropodium coralloides	Wester Mediterranean, N-Europe
Family Paralcyonidae	
Paralcyonium	
Paralcyonium spinulosum	NE-Atlantic, Mediterranean
Maasella	
Maasella edwardsii	Mediterranean
SUB ORDER HOLAXONIA	
Family Gorgoniidae	
Eunicella	
Eunicella cavolini	Mediterranean
Eunicella singularis	Mediterranean
Eunicella verrucosa	N-Atlantic, Mediterranean
Leptogorgia	
Leptogorgia sarmentosa	NE-Atlantic, Mediterranean
Family Plexauridae	
Paramuricea	
Paramuricea clavata	N-Atlantic, Mediterranean
Swiftia	
Swiftia pallida	N-Atlantic, Mediterranean
SUB ORDER SCLERAXONIA	
Family Corallidae	
Corallium	
Corallium rubrum	Mediterranean, N-Africa Atlantic coast
SUB ORDER STOLONIFERA	
Family Clavulariidae	
Sarcodictyon	
Sarcodictyon catenatum	NE-Atlantic, North Sea , Mediterranean
PENNATULACEA (ORDER)	
SUB ORDER SUBSESSIFLORAE	
Family Pennatulidae	
Pennatula	
Pennatula rubra	E-Atlantic, Mediterranean
Pteroides	
Pteroides spinosum	E-Atlantic, Mediterranean, NE-Indian

Table 1.2. Geographical distribution of the species analysed in the present thesis.

1.3. Octocorals phylogenetic analyses

Molecular tools are extremely useful to increase the knowledge on classical taxonomy based just on morphological traits, by comparing the morphological differences among species with their genetic features. Moreover they permit to figure out the evolutionary relationship among species, place them within their evolutionary historical framework and understand the relative timing of lineage splitting (Palumbi 2012). These reconstructions also depend on understanding the temporal framework of species, using the lens of paleontological dating, climate records, human historical records or other links to the past (Palumbi 2012).

Mitochondrial DNA loci represent useful molecular markers for genetic analysis, principally due to features as feasibility, relatively simple genetic structure and straightforward mode of genetic transmission (Avise et al. 1987). The France et al. (1996) work on 16S rDNA revealed little variation among families of octocorals, having values of sequence divergence ranging from 2.7 to 6.3%, compared to those observed among families of Hexacorallia (16.1–26.3%; McFadden et al. 2010). Possible explanations for the reduced rates of divergence observed seems due to the presence of a gene (mtMSH), which may code for a mitochondrial DNA mismatch-repair system (France and Hoover 2002). In fact, nearly 16% of the octocoral mtDNA sequence is occupied by a novel open reading frame (ORF hereafter) that has not been found in any mitochondrial metazoans genome than the Octocorallia group up to now (France and Hoover 2002; McFadden et al. 2010, Bilewitch and Degnan 2011). Based on the octocoral translated sequence 19.7% similarity to the yeast nuclear Mutation Suppressor Homolog 1 (MSH1) gene, it was suggested that the novel ORF might be active in DNA mismatch repair. Accordingly, it was first termed 'mtMSH' as a mitochondrial homolog of the eukaryotic MSH family (Bilewitch and Degnan 2011).

The origin of this gene in the Octocorallia mitochondrial genome has recently been attributed to a horizontal gene transfer from a non-eukaryotic source (Bilewitch and Degnan 2011; Park et al. 2012). The mtMSH gene is approximately twice more polymorphic compared to most other protein-coding regions in the octocoral mitochondrial genome (France and Hoover 2001). It has been widely used for genus-level and species-level phylogenetic studies, but generally has not resulted in better discrimination of congeneric morphospecies (McFadden et al. 2010 and references therein).

Phylogenetic studies obtained using 18S rDNA or 16S mtDNA sequences have supported the monophyletic origin of Octocorallia (Bertson et al. 1999, Sánchez et al. 2003a). Nevertheless, many phylogenetic studies on Octocorallia reveal that traditional taxonomic rank doesn't mirror its molecular phylogenetic structure (McFadden et al. 2010 and references therein). At lower taxonomic levels (family/genus), phylogenetic analyses shown that the classification of species was congruent with taxonomy (Sánchez et al., 2003b; Wirshing et al., 2005; McFadden et al. 2006). Contrarily, at higher levels, the current order/family ranks of taxonomy were not supported by molecular data (McFadden et al. 2006) and reflect grades of morphological construction rather than evolutionary clades (Daly et al. 2007).

The most complete phylogeny of Octocorallia published to date (McFadden et al. 2006; McFadden et al. 2010) sequenced two portions of the mitochondrial protein-coding regions (MSH and ND2) of 115 genera representing 29 of 46 families. Results shown that octocorals were split in three clades:

• Clade 1 (Holaxonia-Alcyoniina): included all members of Holaxonia, the majority of Alcyoniina, and some representatives of Scleraxonia and Stolonifera;

- Clade 2 (Calcaxonia-Pennatulacea): comprised Calcaxonia plus Pennatulacea;

- Clade 3 (*Anthomastus-Corallium*): a small clade including only precious coral genus *Corallium* and several taxa of Alcyoniina (McFadden et al. 2010). Furthermore few taxa of Scleraxonia and Stolonifera occupy unresolved positions near the base of the phylogeny (McFadden et al. 2006). Complete mtDNA genomes studies based on few species (Park et al. 2012) continue to support the two major clades of Octocorallia (Holaxonia–Alcyoniina and Calcaxonia– Pennatulacea) of McFadden et al. (2006).

Despite the increasing amount of molecular information about octocorals (see McFadden et al. 2010 for a review), there are no phylogenetic studies on Mediterrenean species.

1.4. Mediterranean Octocorallia dated phylogeny

The geological history of the Mediterranean Sea has provided sample opportunities for allopatric speciation to the Atlantic species (McFadden et al. 1999). Around 6 millions years ago (MYA hereafter) the connectivity between Mediterranean Sea and any other ocean was lost resulting in the biologically critical Messinian Salinity Crisis (MSC hereafter), during which the vast majority of the Mediterranean Sea dried out (Krijgsman et al. 1999; Donald et al. 2011). The MSC ended at the start of the Pliocene (5.3 Ma) with the Zanclean Flood and the reconnection of the Mediterranean to the Atlantic Ocean via the Strait of Gibraltar (Harzhauser et al., 2007). Many molecular studies analysed biogeographical separation between the Atlantic and Mediterranean biota (see review Paternello et al. 2007, Meynard et al.

2012, Donald et al. 2011) suggesting that the present-day biota is largely the result of colonization, mostly from the Atlantic Ocean after the MSC (Patarnello et al. 2007).

The fossil record has traditionally provided the way to date events in the history of life (Wray 2001).. The anthozoans records were biased and incomplete, since many species lack any solid skeleton, which would be preserved more readily (Berntson 1998)., Information on fossil species principally includes two Paleozoic octocorals documentations up to now, one from the lower Ordovician (471–478 Ma; Cope 2005) and one from the lower Silurian (425–435 Ma; Bengtson 1981). The Ordovician fossil is the earliest gorgonian coral known belonging to the order Alcyonacea. The Silurian fossil had been the earliest representative of the order Alcyonacea known before the Cope (2005) work. Moreover, regarding the genus *Corallium*, Vertino et al. (2010) suggested that populations of *C. rubrum* have been inhabited the Mediterranean basin without interruptions from the early Pleistocene (more than 2 MYA).

Like the fossil record, the genomic record can provide a valuable source of information regarding the timing of evolutionary events whether correctly interpreted (Wray 2001). Estimating divergence time involves calibrating the rate at which protein or DNA sequences evolve, and then estimating when two evolutionary lineages diverged, using the sequences' differences among their living representatives (Wray 2001). A dated phylogeny on Mediterranean Octocorallia and their closely related (cogeners) oceanic species could be useful to identify the evolutionary origin of Mediterranean corals and to test whether recent Mediterranean geological events (such as MSC) were the main force that drove octocorals speciation within the basin.

1.4.1 The red coral

Corallidae family comprises nearly 32 described species which are found throughout the world in tropical, subtropical and temperate oceans. Seven of them live in the Atlantic Ocean: *Corallium niobe, Corallium maderense, Corallium medea, Corallium johnsoni, Corallium tricolor, Corallium bathyrubrum* and *Corallium bayeri* (Watling and Auster 2005, Simpson and Watling 2011). All the other species inhabit the Pacific and Indian Ocean, excepted the just one living in the Mediterranean Sea: the red coral (*Corallium rubrum)*. The Mediterranean red coral (*C. rubrum*) is a branching gorgonian whose colonies could reach 50 cm height (Tsounis et al. 2010). The red coral is one of the most long-lived inhabitants of the coralligenous, in fact it can live for more than 100 years (Tsounis et al. 2010). It is a sciaphilous species that can be found at 5–800 m depth, more commonly at 30–200 m (Costantini et al. 2010). *Corallium rubrum* are currently used for jewellery together with other six species (the so-called "precious corals"): *Corallium secundum, Corallium regale, Corallium elatius, Corallium konojoi, Corallium sp. nov.,* and *Paracorallium japonicum* (Tsounis et al. 2010). Despite many genetic studies focused on red coral (Abbiati et al. 1993, Costantini and Abbiati 2006; Costantini et al. 2007a, b, 2010), phylogenetic hypothesis regarding its speciation event does not exist to date. Consequently, due to its commercial importance, the presence of a great number of species belonging its family together with the fact that it represents the single species inhabiting the Mediterranean Sea, this kind of studies could be useful to individuate which are the main drivers of the *C. rubrum* speciation within the Mediterranean. Moreover, these information could be extremely important to analyse the evolutionary potential of this precious species in a context of global environmental change.

Two main scenarios were investigated in this thesis work. The first hypothesis supposed that the recent (after MSC, late Miocene about 5 millions years ago; Paternello et al. 2007) Mediterranean geological events promoted the allopatric speciation process of Atlantic species within the basin. The alternative scenario proposed the earlier (before MSC) and external *C. rubrum* birth previous the origin of the present-day Mediterranean Sea conformation, followed by secondary colonization within the basin.

1.5. Aims

The aim of this thesis work is to carry out a Mediterranean octocorals species molecular phylogenetic study using two mitochondrial genes with different mutation rate (mtMSH and 16S). Moreover, an extended datasets with Atlanto/Pacific cogeners of Mediterranean Octocorallia species were used to understand their phylogenetic relationships and to estimate the divergence times of Mediterranean species from the cogeners. Finally, the genetic relationships among *Corallium rubrum* and the other *Corallium* species, together with a dated phylogeny were carried out to increase the knowledge about this precious Mediterranean coral. The principal outcome of the work allow to: 1) find differences and/or similarity among molecular phylogeny and morphological taxonomy of octocorals; 2) reconstruct the cladogenesis and 3) evaluate the speciation time of the Mediterranean species.

2. Materials and methods

2.1. Sampling

Molecular analyses were performed on 15 Mediterranean octocoral species (Table 2.1). These specimens were collected along the Mediterranean coasts during oceanographic campaigns (e.g. Monitoring of Calabria Marine Biodiversity; Deep red coral project) and Scuba diving activities. All the species were identified by Marzia Bo (Genova University) following the Bayer (1981) taxonomic keys. Due to difficulties related to the mtMSH region amplification, sequences of *Alcyonium palmatum* and *Swiftia pallida* available in GenBank (http://www.ncbi.nlm.nih.gov /Entrez/) were added to the Mediterranean data set (see Table 2.1).

In order to evaluate the phylogenetic relationship between Mediterranean and oceanic species, Atlantic or closely related species (cogeners) sequences were added from GenBank for both markers (Table 2.2). Within the large amount of sequences published for species belonging to *Alcyonium* (McFadden et al. 2011) and *Leptogorgia* (Lepard 2003) genera, only few widespread and Atlantic distributed species were chosen for the analysis. Unspecific sequences of the analysed genera were also included to extend the sample size. Moreover, four cogeners species were analysed in laboratory. *Alcyonium digitatum* were obtained thanks to the Sven Lovén Centre for Marine Sciences (Göteborg University). *Alcyonium sp.1* were sampled from the oceanographic vessel of I.S.P.R.A "Astrea". *Corallium konojoi, Corallium elatius* and *Paracorallium japonicum* were obtained by Nozomu Iwasaki (Usa Marine Biological Institute, Kochi University). The combined dataset on cogeners species was generated using fewer sequences than single gene tree. The more representative analyses were performed on Corallidae family counting 8 species for both markers. For each species

just one individual were sampled. All the species were conserved in 80% ethanol at

4°C.

Taxa	GeneBank accession numbers	
	mtMSH	16S
ALCYONACEA (ORDER)		
Sub order Alcyoniina		
Family Alcyonidae		
Alcyonium		
Alcyonium palmatum	GU355971	X
Alcyonium acaule	X	X
Parerythropodium		
Parerythropodium coralloides	X	X
Family Paralcyonidae		
Paralcyonium		
Paralcyonium spinulosum	X	X
Maasella		
Maasella edwardsii	X	X
Sub order Holaxonia		
Family Gorgoniidae		
Eunicella		
Eunicella cavolini	X	X
Eunicella singularis	X	X
Eunicella verrucosa	X	X
Leptogorgia		
Leptogorgia sarmentosa	X	X
Family Plexauridae		
Paramuricea		
Paramuricea clavata	X	X
Swiftia		
Swiftia pallida	JQ241247	X
Sub order Scleraxonia		
Family Corallidae		
Corallium		
Corallium rubrum	X	X
Sub order Stolonifera		
Family Clavulariidae		
Sarcodictyon		
Sarcodictyon catenatum	X	X
PENNATULACEA (ORDER)		
Sub order Subsessiflorae		
Family Pennatulidae		
Pennatula		
Pennatula rubra	X	X
Pteroides		
Pteroides spinosum	X	X

Table 2.1. Taxonomical classification and Genbank accession numbers for the 15 Mediterranean Ottocorallia analysed. The X indicates sequences obtained in this thesis. GU355971 and JQ241247 derived from McFadden et al. (2011) and Quattrini and Cordes (2011) respectively.

Taxa	GeneBank accession	
	ND4-MSH	lsu 16S
ALCYONACEA (ORDER)		
Sub order Alcyoniina		
Family Alcyonidae		
Alcyonium		
Alcyonium digitatum	GU355963	X
Alcyonium glomeratum	GU355965	-
Alcyonium hibernicum	AY607771	-
Alcyonium bocagei	GU355960	-
Alcyonium haddoni	GU355974	-
Alcyonium siderium	GU355973	-
Alcyonium sp, 1	-	X
Alcyonium sp, 2	-	U40297
Sub order Holaxonia		
Family Gorgoniidae		
Leptogorgia		
Leptogorgia dichotoma	AY268445	-
Leptogorgia chilensis	AY268460	U40305
Leptogorgia virgulata	AY268458	U19371
Leptogorgia capverdensis	-	FJ389254
Family Plexauridae		
Paramuricea		
Paramuricea placomus	GU563312	-
Paramuricea sp, 1	GQ414000	-
Paramuricea sp, 2	-	U40304
Swiftia		
Swiftia sp,	-	FJ643598
Sub order Scleraxonia		
Family Corallidae		
Corallium		
Corallium konojoi	AB595190	X
Corallium elatius	X	X
Corallium secundum	GQ293303	GQ293245
Corallium ducale	DQ297416	U40300
Corallium kishinouyei	GQ293300	GQ293242
Corallium laauense	GQ293301	GQ293243
Corallium niobe	EF060051	FJ389250
Corallium sp,	GQ293302	GQ293244
Paracorallium		
Paracorallium japonicum	X	X
Paracorallium sp,	GQ293306	GQ293241
PENNATULACEA (ORDER)		
Sub order Subsessiflorae		
Family Pennatulidae		
Pennatula		
Pennatula sp,	DQ302870	-
Pteroides		
Pteroides sp,	DQ302871	-

Table 2.2. Genbank accession numbers of the Atlanto/Pacific species used for the analysis. The "X" indicates sequences obtained in this thesis, while "-" indicates sequences not available.

2.2. DNA extraction, amplification and sequencing

Total genomic DNA was extracted from two to four polyps per individual colonies using the cetyltrimethyl ammonium bromide protocol (CTAB), a method successfully implemented on many other marine invertebrate (e.i. Coffroth et al. 1992; Winnepenninckx et al. 1993). The CTAB protocol was adapted to laboratory contrains excluding the phenol/chloroform phase due to toxicity, without decreasing the extraction quality. DNA was suspended in 25 µL TE buffer (Tris/EDTA solution) and conserved at -20°C until amplification. Each extraction was checked on 0.8% agarose electrophoresis gel.

Two portion of mitochondrial DNA were amplified: the mitochondrial mutS homolog gene (mtMSH, Pont-Kingdon et al. 1995) 5' end and a portion of the ribosomal large sub-unit (16S). mtMSH and 16S were amplified respectively using the primers ND42599F (5'-GCCATTATGGTTAACTATTAC-3') and MUT3458R (5'-TSGAGCAAAAGCCACTCC-3') designed by France and Hoover (2002); and the primers 16S Octo1L (5'-AGACCCTATCGAGCTTTACTGG-3') and 16 Octo2H (5'-CGATAAGAACTCTCCGACAATA-3') implemented by France et al. (1996). The PCR amplification was performed for 16S and mtMSH in all species applying respectively the France et al. (1996) and France and Hoover (2001) protocols parameters (Figure 2.1.a and 2.1.b). Each PCR product was checked on 1.5% agarose electrophoresis gel. PCR products were sent to Macrogen Europe (NL) for purification and sequencing.
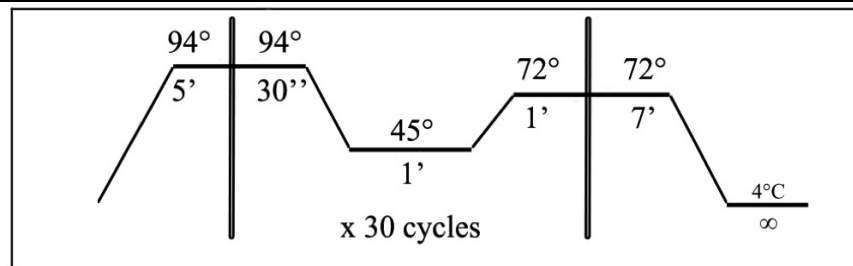
Figure 2.1.a. PCR protocol for 16S region (France et al. 1996).
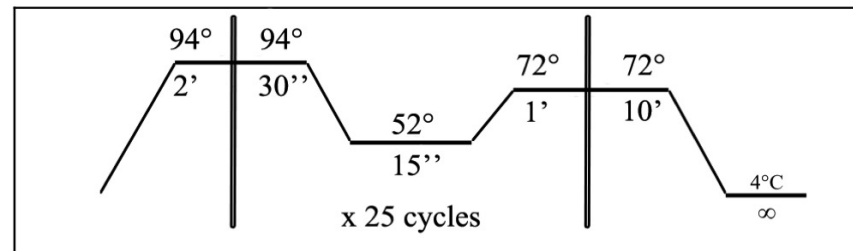
Figure 2.1.b. PCR protocol for mtMSH region (France & Hoover (2001).

2.3. Data analyses

All the mtMSH and 16S sequences were edited to assess the goodness of sequencing using CROMASLITEv2.01 software (Technelysium Pty Ltd 2007). Researches of genetic similarities were performed in BLAST (Altschul et al. 1990) to identify the identity of the obtained sequences. Sequences were aligned using CLUSTALW software (Larkin et al. 2007) implemented in MEGAv.5.05 workspace (Tamura et al. 2011); the final alignment was manually performed. Number of polymorphic sites, haplotype and nucleotide diversity were obtained using DNASPv.5 (Librado et al. 2009). Distance matrixes of sequence divergence were calculated as p-distance (D_p) in MEGAv.5.05. Non-metric multi-dimensional scaling plots of distance matrixes for each marker for the Mediterranean dataset were obtained by MATLABv.7.10.0 (MathWorks 2010) and edited using PHOTOSHOP CS5.

2.4. Alignment methods

It has been repeatedly shown that the alignment quality may have an enormous impact on the final phylogenetic analysis (Tavelara and Castresana 2007). This is particularly true when comparing very divergent and unequal in length sequences, where the introduction of gaps by insertion or deletion (INDELS) in the alignments is necessary (Tavelara and Castresana 2007).

In order to find gaps and homologues sites, algorithms attempt to approximate the INDELS reconstruction allowing lower costs for gap initiation and gap extension (Pons et al. 2006).The INDELS number decrease with higher gap opening costs (GOC), and the gap sizes increase with lower gap extension cost (GEC; Pons et al. 2006). Therefore, in both markers INDELS regions were found imposing high transition cost, small gap opening penalty and little gap extension cost in CLUSTALW algorithm (Tr cost= 1; GOC = 4, GEC= 2 in pairwise and multiple alignment; ratio GEC/GOC=0.5; implemented in MEGA 5.05).

Moreover, length variable DNA sequences such as introns and ribosomal RNA genes require alignment to establish homologous nucleotide positions for phylogenetic analysis (Pons et al. 2006; Sànchez et al. 2003a) using some highly conserved or coding sequences. Concerning the mtMSH alignment, sequences were traduced using the coelenterate mitochondrial code implemented in MEGAv.5.05. Open Reading Frames were checked by ORF-FINDER (www.ncbi.nlm.nih.gov/projects/gorf/) adding a start codon at the beginning of each sequence. Improvement of the mtMSH alignment was achieved implementing the protein coding sequence of *Sarcophyton glaucum* AAC16386 (Pont-Kingdom et al. 1998) according to McFadden et al. (2005). Biases in the open reading frame were visually inspected and correct by hand in MEGAv.5.05. Backward from translation,

INDELS were localized in the mtDNA alignment using *S. glaucum* nucleotide

sequence AB665479 (Aratake et al. 2012), which traduces the same peptide sequence

of the nucleotide sequences AAC16386. In this alignment, octocoral mtMSH

sequences identified protein functional sites (Bilewitch e Degnan 2011).

Counterpart, Ottocorallia 16S sequences were aligned using the *Escherichia*

coli homologous molecule (GenBank accession number: J01695, included in the

operon sites 1268-2809) to identify the conserved motifs (Sànchez et al. 2003a).

Likewise the mtMSH alignment, the same restricted algorithm was applied to find

INDELS (Tr cost= 1; gap opening penalty = 4, gap extension cost= 2 in pairwise and

multiple alignment; GEC/GOC=0.5).

The aligned nucleotide sequences of both regions were used as markers for

octocoral phylogeny. The included INDELS were used as missing data in all input files.

2.5. Substitution model test and gene partition

Phylogenetic trees were developed both using each gene alignment

independently and creating a concatenated dataset of both genes. Phylogenetic

inference depends on the underlying evolutionary model (Guindon et al. 2009). The

models that better fits the data were implemented to obtain the higher confidence in

inferences (Posada et al. 2005). The best-fit substitution model selection for each

dataset was calculated using JMODELTESTv.1.1 (Posada 2008) Mac software,

considering 88 substitution models by hLRT calculator with 4-gamma category. The

resulting models were applied to both each single gene trees and combined genes and

in both trees built using the partitioned prior substitution model.

However, the combined genes maximum-likelihood analysis actually does

not allow the partition of the substitution models for combined dataset, so is it requires

the single new substitution model calculated on combined sequences (Swofford 2002). The combined analysis of genes or gene-complexes (i.e., linked genes such as the mitochondrial ones) with incongruent evolutionary histories can produce misleading results by increasing the wrong phylogenetic trees support (Kubatko and Degnan 2007; Herrera et al. 2010). To quantify the conflicts that can occur between sets of characters from different data sources (e.g. the two regions mtMSH + 16S), Incongruence Length Difference test (ILD test, Farris et al. 1995) was performed. ILD test statistically compares the trees length in the parsimony context. It was computed after 1000 replicates without invariant sites estimate, and *p-value* < 10 % indicates that the combined dataset is not allowed (Darlu and Lecointre 2002). Moreover, the combined dataset (mtMSH + 16S) best-fit substitution model was calculated independently of the IDL results. PARTITIONFINDER (Lanfear et al. 2012) software was used to find the best partition scheme and substitutions models for the combined dataset accounting the partition data (Lanfear et al. 2012)

2.6. Maximum likelihood analyses

Maximum likelihood tree estimation (ML) consists in finding the phylogenetic features (expressed as in relevance order: tree topology, branch lengths and parameters that describe substitution) that maximises the likelihood, i.e. the probability that the data were generated according to the selected (a priori) evolutionary model (Guindon et al. 2009). The sites of the alignment were expected to evolve independently and under the same phylogeny (Guindon et al. 2009). The analysis produces a statistic for each tree, the maximum likelihood score (Lscore) that is the probability of that tree given the data set and the model. This score can be used

to statistically compare two different trees (i.e. two evolutionary hypotheses; Bertson 1998).

Because ML gives point-estimates of the tree (Nylander et al. 2004), non-parametric bootstrap is the most popular approach to reach the statistical support on ML tree (Guindon et al. 2009). It easily gives information about the stability of the tree topology (the branching order), and it helps in assessing whether the sequence data is adequate to validate the topology (Holmes 2003). Maximum likelihood analyses were executed on PAUPv.4.0B10 (Swofford 2002) using the heuristic search of random addition sequence to stepwise addition procedure and TBR branch-swapping with 20 random-addition-sequence replications. Node supports were calculated through 150 bootstrap replicates.

The sea pens species included in the analyses joined the same family, showed clear morphological synapomorphies (Williams 2011) and appear to be part of a dinstictive clade in many octocorals phylogenetic analyses of all anthozoans (rRNA 16S: France et al., 1996; Sanchez et al. 2003 and mtMSH: McFadden et al 2006). Consequently, Pennatulacea were used as out-group in all ML analyses and to root all ML trees.

2.7. Bayesian analyses

In phylogenetic analysis, an alternative method to searching for the single highest point in the "parameter landscape" (e.i. heuristic search in ML) is to use what is called " marginal estimation" by Bayesian inference (Nylander 2004). Bayesian analyses make interference about the posterior probability (BP), branch lengths and other parameters, given the data, a model of character evolution and prior probabilities on parameters in the model (Nylander 2004). Metropolis-coupled Markov chain Monte

Carlo method (MCMC) is used to estimate the posterior probability of phylogenies when runs appear to have "converged" to a stationary value (Nylander 2004). Here, Bayesian analyses were carried out using MRBAYESv.3.2 (Ronquist et al. 2011; Mac software). The MCMC analyses in this thesis run over $1*10^7$ generations and were replicate for 2 runs. The sample frequencies parameter was set to 1000. The BP was calculated on $1*10^4$ samples ($1*10^7/1*10^3$) less the first 25% of burn-in ($2.5*10^3$ samples). The obtained trees were summarized into 50% majority-rule consensus tree. Pennatulacea were used as out-group to root all Bayesian trees.

Moreover, extensive post-run analyses of MCMC for assessing convergence are seldom seen in phylogenetic literature (Nylander et al., 2008). Frequently used post-run method involves examining trace plots of the likelihood scores for trees sampled using the Markov chain method (Nylander et al., 2008). However, judging the success of a MCMC from the likelihood trace alone might lead to inaccurate and misleading results (Nylander et al., 2008). The convergence of trees parameters was evaluated by TRACERv.1.5 (Rambaut and Drummond 2007). Convergence was indicated as the ''straight hairy caterpillar'' (Drummond et al., 2007) shape of the stationary posterior-distribution trace (generations vs. LnL) of each parameter and it was supported from high value of the effective sample sizes ESS (>200). Other convergence tests and mixing diagnostics were executed in the program AWTY (http://ceb.csit.fsu.edu/awty; Nylander et al., 2008)

Finally, the phylogenetic trees obtained both from ML and Bayesian analyses were edited by FIGTREEv.1.3.1 (Rambaut 2012) and corrected by hand with PHOTOSHOP CS5.

2.8. Estimation of divergence time

A Bayesian statistics method was implemented to estimate the divergence time between Mediterranean species to the other Atlanto-Pacific Ottocorallia species. An estimation of gene genealogy and divergence times was performed in BEAST 1.7.1 (Drummond et al. 2012) for the two mitochondrial marker assuming the substitution model of previous analyses. It was calculated using BEAST (Drummond and Rambaut 2007) analyses package with an uncorrelated relaxed lognormal molecular clock model was assumed. It allows for the variation in mutation rates among branches, implementing as the tree priors the Yule model of constant speciation rate (Yule 1925; Gernhard 2008) and the coalescent model of constant population size (Kingman 1982). MCMC analyses run over $5*10^7$ generations sampling every $5*10^3$ before to construct 50% consensus trees by TREEANNOTATORv.1.7.1 (Rambaut and Drummond 2007).

TRACERv.1.5 (Rambaut and Drummond 2007) was used as post analyses to evaluate the convergence of trees parameters. Other examined convergence and mixing diagnostics included the standard deviation of partition frequencies (<0.01), the potential scale reduction factor (PSRF) (ca. 1.00), the effective sample sizes (EES) (>200) and the posterior probabilities of specific nodes similitude between different runs in the program AWTY (http://ceb.csit.fsu.edu/awty) (Nylander et al. 2008).

Two ways to estimate the divergence time was used. First, the estimated mutation rates found in bibliography was used to calibrate the dated phylogeny. The 16S mutations rate was set to 0.1% substitution every million year (Palumbi 2012), while in the case of mtMSH it was set to 0.25% (Lepard 2003). Addionally, some of the few fossils available for Octocorallia, such as Coralliidae, were implemented as calibrating point. The Coralliidae node was calibrated implementing a normal prior distribution for the time to the most recent common ancestor (TMRCA) with a mean of

83.5 million years before present (Myr BP) and a standard deviation of 0.7, corresponding to the Campanian age stratum, in which the oldest known fossil in this family has been found (Schlagintweit & Gawlick 2009). Then trees were edited by FIGTREEv.1.3.1 (Rambaut 2012) and fine-tuned with PHOTOSHOP CS5.

3. Results

3.1. Mediterranean MtMSH genetic variability

The obtained MtMSH fragments length ranged from 594 to 612 base pairs (bp hereafter) in the 15 Mediterranean species analysed. The alignment length was 627 bp including the INDELS (see the following paragraph), with 591 homologues sites excluding gaps and missing data. This alignment presented 215 polymorphic sites (36.4%), with values of haplotype diversity of 0.971 and nucleotide diversity of 0.105.

Differences in sequences length were attributable both to two large insertion/deletion regions (INDELS) localized between the sites 90-115 and 306-316, and to two small regions (405-409 and 573-577) of the nucleotide alignment (Figure 3.1.a). *Corallium rubrum* and *Pennatula rubra* had the longest sequences (612 bp) and presented the first (90-115) and the second (306-316) big INDELS respectively. The other two smaller INDELS regions were due to the three nucleotide bases deletion in *Sarcodyction catenatum, Corallium rubrum, Pteroides spinosum* and *Pennatula rubra*.

The three *Eunicella* species sequences were the same length of 606 bp (D_p=0; Table 3.1 and Figure 3.1.b). The main differences among sequences (as *p*-distance D_p> 0.15) were observed between the two order Pennatulacea and the Alcyonacea. Specifically, *Paramuricea clavata* and *Maasella edwardsii* versus *Pennatula rubra* presented the biggest pairwise genetic *p*-distances (D_p>=0.18; Table 3.1 and Figure 3.1.b). *Corallium rubrum* also shows great genetic distance compared to all the other species ranging between D_p=0.13 and D_p=0.16, particularly against *P. clavata* and *M. edwardsii* (D_p=0.16; Figure 3.1.b).

```
[                                1111111111222222222233333333334444444444455555555556]
[                      12345678901234567890123456789012345678901234567890123456789 0]
A. acaule             CAAATTGGTAAGTTCTATGAACTTTGGCACGAGCCCGATACTTCTAGTAGGCAACAAGCA
A. palmatumGU355971   ............................................................
P. coralloides        ..G.............................T......A...................
M. edwardsii          ..G.............................T.....T.....C....C.........
P. spinulosum         ..G..................G..........T.....T.....C..............
E. cavolini           ................................T.....TA....C.............
E. singularis         ................................T.....TA....C.............
E. verrucosa          ................................T.....TA....C.............
L. sarmentosa         ..G.............................T.....T.....CG.............
S. pallidaJQ241247    ..G.............................T.....T....................
P. clavata            ..G.............................T.....T.CC...C............
C. rubrum             ..G...........................TA....G..GT.C....GTA.........
S. catenatum          ..G.........T...................G....T...GT...G....A.......
P. rubra              ..G.............................T.....T...G.C..T...TA..G....
P. spinosum           ..G.............................T.....T...GT.C..T...TA..G....

[                                                  1111111111111111111111]
[                      6666666666777777777788888888889999999999900000000001111111111 2]
[                      12345678901234567890123456789012345678901234567890123456789 0]
A. acaule             TACTCTCAAGCTGAGCTATTAGCTGAATCATCCATG-----------CGAAGTCAGCCT
A. palmatumGU355971   .......................................----------............
P. coralloides        .......................T.............................C......
M. edwardsii          ....T.....C..T......T...G...........----------........CG.T..
P. spinulosum         ....T.....C..T......T...G...........----------........CG.T..
E. cavolini           ..........C..T......T...G...........----------.........CG...
E. singularis         ..........C..T......T...G...........----------.........CG...
E. verrucosa          ..........C..T......T...G...........----------.........CG...
L. sarmentosa         C..A....G.C..T......T...GC..........----------.........AG...
S. pallidaJQ241247    ..........C..T......T...G...........----------..........G...
P. clavata            ..........C..T......T...G...........----------.........CG...
C. rubrum             ................T......T...G..CC.T..ACCAATTTACATA......AG...
S. catenatum          ..............T..G..T.A.G..G....A-----------------.....CG...
P. rubra              C.T..........T......T..TG.CGG.CCCCCCGTG--------..CGG...
P. spinosum           ..T.A........T.....A......G--GCCCCT--------------CG...

[                                1111111111111111111111111111111111111111111111111111111111]
[                      2222222222333333333344444444445555555555566666666667777777777 8]
[                      12345678901234567890123456789012345678901234567890123456789 0]
A. acaule             TTGGGGGTAACGCCCCCCATTGAACAAGTTGCCTCATTACTTGATATGAGAATAATATTG
A. palmatumGU355971   ............................................................
P. coralloides        ............................................................
M. edwardsii          ........G....A..T................G...................CG.C.
P. spinulosum         ........G....A..T................G...................CG.C.
E. cavolini           ....A........T..................G....................C.
E. singularis         ....A........T..................G....................C.
E. verrucosa          ....A........T..................G....................C.
L. sarmentosa         ....A.....A............A.............................C.
S. pallidaJQ241247    .............T........A...G.........................C.
P. clavata            ....A........T................A..............C..CA
C. rubrum             ..........A.T.......G.......G......C...A........CA
S. catenatum          ..........A...............G.........................CA
P. rubra              ..A.AAA....T..T..T................T.................G......CC
P. spinosum           ..A.......A..A..................T.................G..G..CA
```

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                         [1111111111111111111122222222222222222222222222222222222222222222]
                         [8888888889999999999000000000011111111112222222222233333333334]
                         [1234567890123456789012345678901234567890123456789012345678901234567890]
A. acaule                CCCGGTAAAAGATCTTTGCTTCAAATGGGATTTCCAATTTATTCCCTTACTACTCATCTA
A. palmatumGU355971      ....................................................................
P. coralloides          ............................G..................................T..
M. edwardsii             ............C..........G............G.C.....
P. spinulosum            ............C..........G............G.C.....
E. cavolini              ...........CC..........G.......................
E. singularis            ...........CC..........G.......................
E. verrucosa             ...........CC..........G.......................
L. sarmentosa            .....C.......C..........G.......................
S. pallidaJQ241247       .....C....A...C..........G.......................
P. clavata               ...........CC..........G....CG....................G
C. rubrum                ............C..........G....T.A.....T...........A.
S. catenatum             .....C.......CC..........G....TG...................T..
P. rubra                 ...............T..........G....T.......T..CG....T..T..
P. spinosum              ..T........C..........G....T.C........AC....T..T..

                         [2222222222222222222222222222222222222222222222222222222222223]
                         [44444444455555555555666666666667777777777888888888899999999990]
                         [1234567890123456789012345678901234567890123456789012345678901234567890]
A. acaule                AGCACCCTATTGGATAAAGGTTGGACTGTTATAGTTATCGATGAATTAGTCACTGGTAAA
A. palmatumGU355971      ....................................................................
P. coralloides          ......T....................A.......................
M. edwardsii             ..T..TT.G.........................................
P. spinulosum            ..T..TT.G..............................AA.................
E. cavolini              ..T...T.G.................T.....T.................
E. singularis            ..T...T.G.................T.....T.................
E. verrucosa             ..T...T.G.................T.....T.................
L. sarmentosa            ..T..TT.G.................T.................C..
S. pallidaJQ241247       ..T..TT.G.................T.................
P. clavata               ..T...T.GC................T.........GA..........
C. rubrum                ..T..T..G......G....TA..A......C..T................
S. catenatum             .....TT.G.................A.C......T.........T.........
P. rubra                 ...CT.T.G.................A..........T......C....T.........
P. spinosum              G...TT.G.................A......C..T......C..........C..

                         [3333333333333333333333333333333333333333333333333333333333333]
                         [00000000001111111111222222222233333333334444444444455555555556]
                         [1234567890123456789012345678901234567890123456789012345678901234567890]
A. acaule                TCCGGG--------CCAAAACAACGTGCAGTATCTCAGGTTTATTCTCCTAGTTGTAAT
A. palmatumGU355971      ......----------...............................
P. coralloides          ......----------...............................
M. edwardsii             ..A..----------......C.....................
P. spinulosum            ..A..----------......C.....................
E. cavolini              ..T..----------......C...........C...........
E. singularis            ..T..----------......C...........C...........
E. verrucosa             ..T..----------......C...........C...........
L. sarmentosa            ..T..----------......C.....................
S. pallidaJQ241247       ..T..----------......C.....................
P. clavata               ......----------......C.....................
C. rubrum                ..G..----------.G.....C...............G...C..
S. catenatum             ..A..----------.C.....C.....................
P. rubra                 ....A.CCCAAACAG..C.....C.....G...AA..........T.........
P. spinosum              .....A----------.T.....C..........................C
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33

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[                     33333333333333333333333333333333333333333444444444444444444444]
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[                     12345678901234567890123456789012345678901234567890123456789 0]
A.acaule              TTAGAGGACTGTTCGGAGTTATCTTATGTATTATCAATTTATTTTTCTCAAGACGACTTA
A.palmatumGU355971    ..........................................................
P.coralloides         .........................G................................
M.edwardsii           ................A...C.C.....G.........................T.....
P.spinulosum          ................A...C.C.....G.........................T.....
E.cavolini            ................A...G.C.....G..............................
E.singularis          ................A...G.C.....G..............................
E.verrucosa           ................A...G.C.....G..............................
L.sarmentosa          ......A......T...A.....C.....G...............C.............
S.pallidaJQ241247     ................A.....C.....G..............................
P.clavata             .........G......A...G.C.....G.....CG...............A.......
C.rubrum              .....A.....C.....A.....C.....GA.............---A....T......
S.catenatum           .....A..T.......A.....C.....A..............---A....T......
P.rubra               .....A..T....T..A.....C.....GA.............---A....T......
P.spinosum            .....A..T...A.T..A.....C.....GA.............---A....T..T...

[                     44444444444444444444444444444444444444444444444444444444444444]
[                     22222222233333333334444444444455555555555666666666677777777778]
[                     123456789012345678901234567890123456789012345678901234567890]
A.acaule              CTAGGTATTACTTTATTTTCAGCCATGAGTGGGCATAGTGTAATGTTTCCTGTCTCTTGG
A.palmatumGU355971    .....................T....AC........A......................
P.coralloides         .............C.........T....A........A....................
M.edwardsii           T.G.........................A........C.A.....C............
P.spinulosum          T.G.........................A........A.....C............
E.cavolini            T.....................................................C.....
E.singularis          T.....................................................C.....
E.verrucosa           T.....................................................C.....
L.sarmentosa          T...............................A......A..................
S.pallidaJQ241247     T...............................A......A.................A
P.clavata             A.G..A...GT.........G......A......A.....................A
C.rubrum              .........................A...C...A................A....
S.catenatum           ..............................A......A................A....
P.rubra               ..G...G.C....................A...A................A....
P.spinosum            ..T..A......A................A...A......A.........T.A....

[                     44444444444444444445555555555555555555555555555555555555555555]
[                     88888888899999999990000000000011111111112222222222233333333334]
[                     123456789012345678901234567890123456789012345678901234567890]
A.acaule              ACGGACAGGGACAAAGTAGCCCGGTTATTAATCAGTTATCGTATCAGAGAGATAGTAATT
A.palmatumGU355971    ...........T.............A.....................T.............
P.coralloides         G..........T.............A.....................T.....A.....
M.edwardsii           ........A...............C..C.................T.....A...C.....
P.spinulosum          ........A...............C.....................T.A...A.........
E.cavolini            ........A.................................A.........
E.singularis          ........A.................................A.........
E.verrucosa           ........A.................................A.........
L.sarmentosa          G......A.........G....C................A...T.A...A.........
S.pallidaJQ241247     ........A.........G.G..................T.....A.........
P.clavata             ........A...........T..................T.....A.........
C.rubrum              GTC..T..A...........T..A........T.....T.A....G.....
S.catenatum           GTT.....A...........T..A..G............T.A....G.....
P.rubra               GAA.....A...........T..A...........C..C.....T.A....G.......A
P.spinosum            GAA.....A...........T..A........A..C.....T.A....G.G.....
```

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[                        5555555555555555555555555555555555555555555555555555555556]
[                        4444444445555555555566666666666777777777788888888889999999999990]
[                        12345678901234567890123456789012345678901234567890123456789 0]
A. acaule                TGGGCCAACTCAGGGGTTGGCTCAGATATTTTAATAAATAAAATATATAATTTATTAATT
A. palmatumGU355971      . . . . . . . . . . . G. . A. CC. . . . . . . . G. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. coralloides           . . . . . . . . . . . G. . A. CC. A. . . . . . G. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
M. edwardsii             . . . . TA. . . . TG. . . . . . . . . C. . . . . . . G. C. . . . . . . . G. . . . . . . . . . . . . C. . . .
P. spinulosum            . . . . TA. . . . TG. . . . . . . . . C. . . . G. . . . . . . . . . . . G. . . . . . . . . . . . . . . . . .
E. cavolini              . . . . TA. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
E. singularis            . . . . TA. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
E. verrucosa             . . . . TA. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
L. sarmentosa            . . . . TAG. . . . . . . . G. . . . . . . . . G. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
S. pallidaJQ241247       . . . . GAG. . . . . . . . . . . . . . . . . . G. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. clavata               . . . . . A. . . G. . . . . A. . . . . . . . . G. . . . . . . . . . . . . . . . . . GG. . . . . C. . . . . . .
C. rubrum                . . . . . GG. . CTG. . . . CCAAT. . . . GA. . AC. . - - - . . . . G. . . . . GG. . . . . . .
S. catenatum             . . . . . GG. . . . G. . . . . CC. AT. . . . GG. . AC. . - - - . . . . G. . . . . GG. . . . . . .
P. rubra                 . . . . . GG. . . . G. . . . CC. ATC. . . GG. . AC. . - - - . . . . G. . . . . GG. . . . . . .
P. spinosum              . . . . TGG. . . . G. . . CC. ATC. . . GA. . A. . . - - - . . . . G. . . . . GG. . . . . . .


[                        6666666666666666666666666666]
[                        0000000001111111111122222222]
[                        12345678901234567890123 4567]
A. acaule                GGTTGGAATTTATTCCCCCCTGAACCC
A. palmatumGU355971      . . . . . . . . . . . . . . . . . . T. . . . . . . .
P. coralloides           . . . . . . . . . . . . . T. . . T. . . . . . . .
M. edwardsii             . A. . . . . . . . . . . . . . . . C. . . . . .
P. spinulosum            . A. . . . . . . . . . . . . . . . . . . . . . .
E. cavolini              . . . . . . . . . . . . . . . . . . . . . . . . .
E. singularis            . . . . . . . . . . . . . . . . . . . . . . . . .
E. verrucosa             . . . . . . . . . . . . . . . . . . . . . . . . .
L. sarmentosa            . . . . . A. . . . . . . . . . . T. . . . G. .
S. pallidaJQ241247       . . . . . . . . . . . . . . . . . T. . . . G. .
P. clavata               . . . . . . . . CC. . . . . . . . . . . . G. .
C. rubrum                . . C. . . . . . . . . . . . TT. . . . G. T
S. catenatum             . . . . . . . . . . . . . . . . . T. . . . G. T
P. rubra                 . . C. . . . . . . . G. . . . TT. . . . . . T
P. spinosum              . . . . . . . . . . . . . . . . . . T. . . . . T
```

Figure 3.1.a. Alignment of MtMSH sequences of the 15 Mediterranean species analysed. Points mean identical bases to those of *Alcyonium acaule* sequence; dashes mean deletions.

A.acaule	P.coralloides	M.edwardsii	P.spinulosum	E.cavolini	E.singularis	E.verrucosa	L.sarmentosa	S.pallidaJQ241247	P.clavata	C.rubrum	S.catenatum	P.rubra	P.spinosum	
0.02	0.03	0.11	0.10	0.08	0.08	0.08	0.10	0.07	0.11	0.15	0.12	0.17	0.16	A.palmatumGU355971
	0.04	0.10	0.09	0.06	0.06	0.06	0.09	0.07	0.11	0.15	0.13	0.17	0.17	A.acaule
		0.10	0.10	0.08	0.08	0.08	0.09	0.07	0.11	0.14	0.12	0.16	0.15	P.coralloides
			0.02	0.07	0.07	0.07	0.09	0.07	0.10	0.16	0.14	0.18	0.17	M.edwardsii
				0.06	0.06	0.06	0.08	0.06	0.09	0.15	0.13	0.17	0.16	P.spinulosum
					0.00	0.00	0.06	0.04	0.07	0.14	0.12	0.16	0.16	E.cavolini
						0.00	0.06	0.04	0.07	0.14	0.12	0.16	0.16	E.singularis
							0.06	0.04	0.07	0.14	0.12	0.16	0.16	E.verrucosa
								0.04	0.10	0.14	0.12	0.17	0.16	L.sarmentosa
									0.08	0.13	0.11	0.16	0.16	S.pallidaJQ241247
										0.16	0.14	0.18	0.17	P.clavata
											0.08	0.13	0.12	C.rubrum
												0.11	0.11	S.catenatum
													0.10	P.rubra

Table 3.1. Pairwise genetic *p*-distance (D_p) among Mediterranean species of MtMSH sequences.
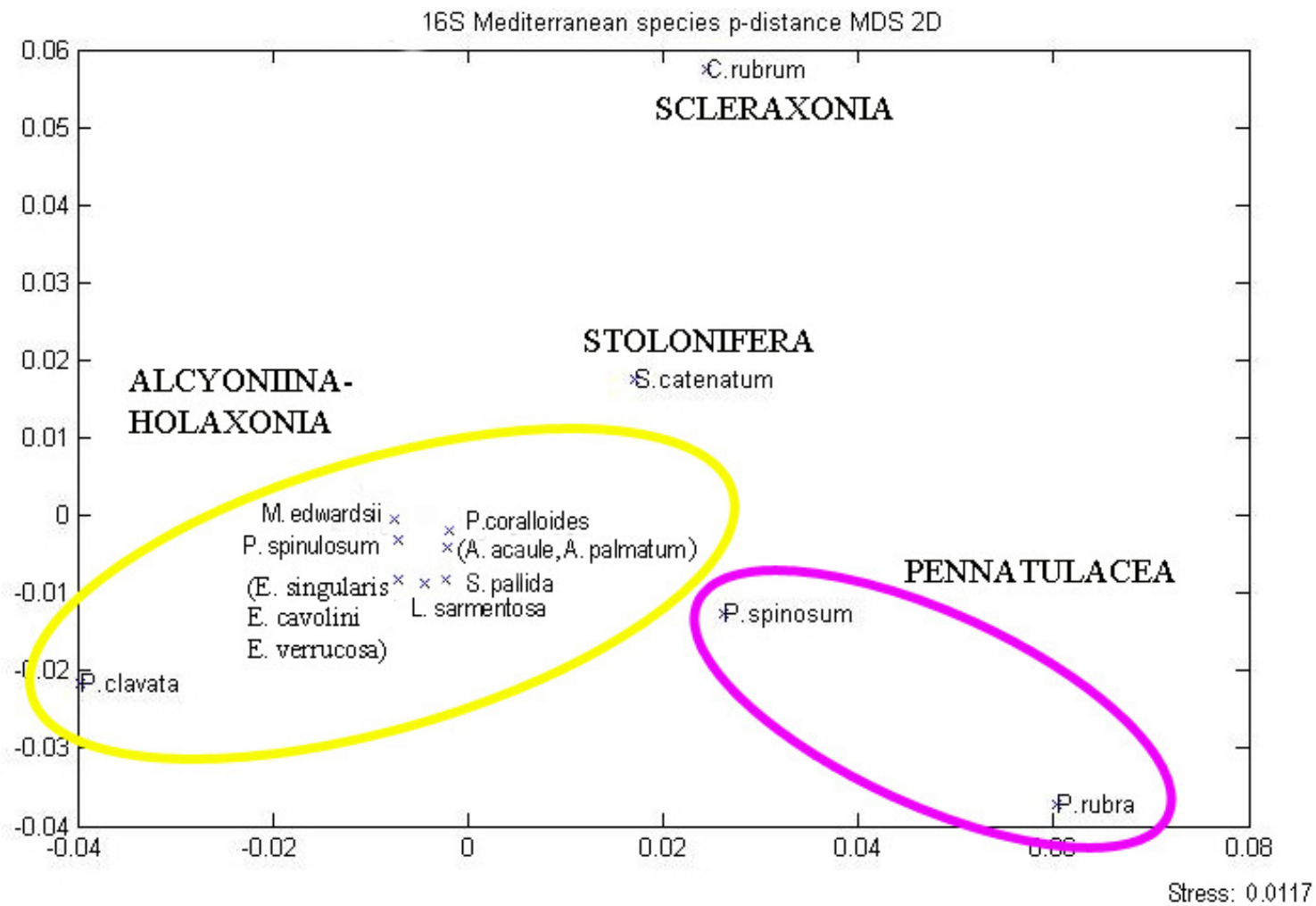
35

Figure 3.1.b. nMDS based on D_p of MtMSH Mediterranean octocorals sequences . Stress on 3D plot was of 0.0221.

3.2. Mediterranean 16S genetic variability

The 16S fragments length varied from 224 to 267 bp in the 15 Mediterranean taxa analysed. The length of the aligned sequences was 274 bp including the INDELS (see the following pragraph), exhibiting 219 homologues sites excluding gaps and missing data. Out of 274 pb only 37 were polymorphic sites (16.9%). The alignment presented a value of haplotype diversity of 0.952, related to a nucleotide diversity of 0.041. One single large insertions region in *S. catenatum* and *C. rubrum* represented the great source of variation between sequences length. It was localized between the homologues sites 148-201 (Figure 3.2.a).

Alcyonium acaule and *A. palmatum* exhibited no sequences divergence in the case 16S marker (D_p=0; Table 3.2 and Figure 3.2.b). The three sequences of the *Eunicella* species showed the identical nucleotide sequences (D_p=0; Table 3.2 and Figure 3.2.b). The greatest divergence among sequences (D_p>0.11) was observed between *Pennatula rubra* and *Paramuricea clavata* (Table 3.2 and Figure 3.2.b). As seen in MtMSH, *Corallium rubrum* showed higher genetic distance compared to all the other species, ranging between D_p=0.05 and D_p=0.10, particularly against *P. clavata* (D_p=0.10; Figure 3.1.b).

```
[                            1111111111122222222222333333333334444444444455555555556]
[                  12345678901234567890123456789012345678901234567890123456789 0]
A. acaule         CTAATTTTGAGTTAATAATTTTT-GTTGGTGGGACAGTTTAGTTGGGGCGACTACCTTTG
A. palmatum       ..........................-.....................................
P. coralloides    ..........................-......A..............................
M. edwardsii      ..G.......................-......A..............................
P. spinulosum     ..G.......................-......A..............................
E. cavolini       ....................T.....-......A..............................
E. singularis     ....................T.....-......A..............................
E. verrucosa      ....................T.....-......A..............................
L. sarmentosa     ..........................-......A..............................
S. pallida        ..G.......................-......A..............................
P. clavata        .......C.A.........AA...-......A..............................
C. rubrum         .......A.................-.....C.A..........................C..
S. catenatum      ......C.................-......A..............................
P. rubra          ......C.A.........A....-......A..............................
P. spinosum       ......C.A.........A....-......A..............................

[                                        1111111111111111111111]
[                  6666666666777777777788888888889999999999900000000000 11111111112]
[                  12345678901234567890123456789012345678901234567890123456789 0]
A. acaule         AATAAGAAACGAAGGCGAGCTTATGGTATACAA-AGCTAATCACATTAGCCTGACAGTGA
A. palmatum       ................................-...............................
P. coralloides    ................................-...............................
M. edwardsii      ................................-.......T......................
P. spinulosum     ................................-.......T......................
E. cavolini       ...........................T..-.......T......................
E. singularis     ...........................T..-.......T......................
E. verrucosa      ...........................T..-.......T......................
L. sarmentosa     ................................-.......T......................
S. pallida        ................................-.......T......................
P. clavata        ................................-.....TT.T...................
C. rubrum         .........G................-T.TT.A--....T......................
S. catenatum      .........................-T.TT.A--....T.............C........
P. rubra          .........................-TG.T.A--....T.............C........
P. spinosum       .........................-T..T.A--....T.T.....................

[                  111111111111111111111111111111111111111111111111111111111111]
[                  2222222222333333333334444444444455555555555666666666667777777777 8]
[                  12345678901234567890123456789012345678901234567890123456789 0]
A. acaule         GGGGGACACCCCTAG-CTGGCACAAGGA---CGAC--G--------------------
A. palmatum       ...............-..................-...........................
P. coralloides    ...............-..................---A..----------------------
M. edwardsii      ...............-..................---T..----------------------
P. spinulosum     ...............-..................---T..----------------------
E. cavolini       ...............-..................---T..----------------------
E. singularis     ...............-..................---T..----------------------
E. verrucosa      ...............-..................---T..----------------------
L. sarmentosa     ...............-..................---T.T--------------------
S. pallida        ...............-..................---T..----------------------
P. clavata        ...............-..................---T.G-.----------------
C. rubrum         ...............-..................--CTT..CCAT-T-ACA---------ACGTA
S. catenatum      ...............-..................CTT.C..--ATAGTACTTAGACATAGTACGTA
P. rubra          ........-....CGAG..A.......G-CC.CG--.AG--------------------
P. spinosum       ...................-...............-CC.CG--.-GG--------------------
```

```
[                    1111111111111111111222222222222222222222222222222222222222222]
[                    8888888889999999999900000000000111111111112222222222233333333334]
[                    123456789012345678901234567890123456789012345678901234567890]
A. acaule            - - - - - - - - - - - - - - - - - - TCCTATGTGGGCGATAATGACCCGATATGATTGTCCAAAA
A. palmatum          - - - - - - - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. coralloides       - - - - - - - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
M. edwardsii         - - - - - - - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . . . . . . . A. . . . . . . . . . .
P. spinulosum        - - - - - - - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
E. cavolini          - - - - - - - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
E. singularis        - - - - - - - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
E. verrucosa         - - - - - - - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
L. sarmentosa        - - - - - - - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . . . . . . . A. . . . . .
S. pallida           - - - - - - - - - - - - - - - - - - . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. clavata           - - - - - - - - - - - - - - - - - - . T. . . . . . . . . . A. . . . . . . . . . . . . . . . . . . . . .
C. rubrum            - - - - - - - - - - - ATGG- - GT. . . . . . . . . . . . . . . . . . . . . . . . . A. . A. . . T. . .
S. catenatum         GTGCGTAGCACGTAGTGGAT. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. rubra             - - - - - - - - - - - - - - - GGT. . . . . . . . . . . . . . . G. . . . . . . . . . T. . . A. . . . . .
P. spinosum          - - - - - - - - - - - - - - - G- T. . . . . . . . . . . . . . . G. . . . . . . . . . . . . A. . . . . .


[                    2222222222222222222222222222222222]
[                    4444444445555555555666666666677777]
[                    12345678901234567890123456789012 34]
A. acaule            TAAATATCGAAAGCGAATAAAAGCTACCGTAGCG
A. palmatum          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
P. coralloides       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
M. edwardsii         . . . . . . . . . . . . . T. . . . . . . . . . . . . . . . . . .
P. spinulosum        . . . . . . . . . . . . . . G. . . . . . . . . . . . . . ?. . .
E. cavolini          . . . . . . . . . . . . . . G. . . . . . . . . . . . . . . . . .
E. singularis        . . . . . . . . . . . . . . G. . . . . . . . . . . . . . . . . .
E. verrucosa         . . . . . . . . . . . . . . G. . . . . . . . . . . . . . . . . .
L. sarmentosa        . . . . . . . . . . . . . . G. . . . . . . . . . . . . . . . . .
S. pallida           . . . . . . . . . . . . . . G. . . . . . . . . . . . . . . . . .
P. clavata           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
C. rubrum            . . . . . . . . . . . . . . G. . . . . . . . . . . C. . . .
S. catenatum         . . . . . . . . . . . . . . G. . . . . . . . . . . . . . . . . .
P. rubra             G. . . . . . . . . . . . . G. . . . . . . . . . . . . . . . . .
P. spinosum          . . . . . . . . . . . . . . G. . . . . . . . . . . . . . . . . .
```

Figure 3.2.a. Alignment of the portion of the 16SrDNA gene for the 15 species analysed.

A.palmatum	P.coralloides	M.edwardsii	P.spinulosum	E.cavolini	E.singularis	E.verrucosa	L.sarmentosa	S.pallida	P.clavata	C.rubrum	S.catenatum	P.rubra	P.spinosum	
0.00	0.01	0.03	0.02	0.02	0.02	0.02	0.03	0.02	0.05	0.08	0.05	0.10	0.06	A.acaule
	0.01	0.03	0.02	0.02	0.02	0.02	0.03	0.02	0.05	0.08	0.05	0.10	0.06	A.palmatum
		0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.05	0.07	0.04	0.09	0.05	P.coralloides
			0.01	0.02	0.02	0.02	0.02	0.01	0.05	0.06	0.05	0.09	0.06	M.edwardsii
				0.01	0.01	0.01	0.01	0.00	0.05	0.06	0.04	0.09	0.05	P.spinulosum
					0.00	0.00	0.01	0.01	0.05	0.05	0.03	0.08	0.04	E.cavolini
						0.00	0.01	0.01	0.05	0.05	0.03	0.08	0.04	E.singularis
							0.01	0.01	0.05	0.05	0.03	0.08	0.04	E.verrucosa
								0.01	0.05	0.06	0.04	0.08	0.05	L.sarmentosa
									0.05	0.06	0.04	0.09	0.05	S.pallida
										0.10	0.08	0.11	0.06	P.clavata
											0.05	0.10	0.07	C.rubrum
												0.07	0.04	S.catenatum
													0.05	P.rubra

Table 3.2. Pairwise genetic *p*-distance among Mediterranean species of 16S sequences.

Figure 3.2.b. nMDS based on D_p of 16S Mediterranean octocorals sequences. Stress on 2D plot was of 0.0117.

3.3. Mediterranean phylogenetic analysis

3.3.1 MtMSH Mediterranean trees

The General Time Reversible model (GTR) with gamma distribution (+G) more invariant sites (+I) resulted to be the MtMSH dataset best substitution model. The shape of the gamma distribution was set to 0.667 and the proportion of invariant sites was 0.116. TRACER results on Bayesian tree show high value of ESS (>200) and AWTY post analyses highly supported the convergence of tree parameters.

Both Maximum-likelihood and Bayesian analyses recovered similar trees topologies (Figure 3.3.a and 3.3.b respectively). The out-group clade comprised the two sea pens as a monophyletic group (BT=99% and BP=100%; Figures 3.3). The main cluster (clade A; Figures 3.3) containing all the analysed species excepted the sea pens (out-group), was well supported by the maximum-likelihood bootstrap value (BT=99%) and Bayesian posterior probabilities (BP=100%).

At sub-order level within the Clade A, a large sub-clade (clade 1) included all members of the sea fan sub-order Holaxonia and the soft corals belonging to sub-ordinal group Alcyoniina, both linked as paraphyletic lineages (BT=100% and BP=100%; Figures 3.3). Moreover, analysed Stolonifera (*S. catenatum*) and Scleraxonia (*C. rubrum*) were linked at the base of the Clade A without clear relationships with the Clade 1.

At lower taxonomic level (family), the Clade 1 showed three monophyletic groups: Paralcyonidae, Alcyonidae and Gorgonidae family. The Paralcyonidae family appeared to be an independent lineage (BT=100% and BP=100%) and did not join with the other soft coral belonging to the Alcyonidae family. The Mediterranean Alcyoniadae species belonging to the two analysed *genera Alcyonium* and

Parerythropodium were associated to the same monophyletic group (BT=100% and BP=100%). The Gorgonidae family was split into two sub-groups corresponding to the two analysed *genera* (*Eunicella* and *Leptogorgia*).

Specifically at *genus* level, *Eunicella genus* is a well-defined group joining the three species analysed (BT=100% and BP=100%). *Eunicella spp.* was unlinked to the other co-familiar Gorgoniidae species (*Leptogorgia sarmentosa*), which was more related to *Swiftia pallida* (Plexauridae family; BT=80% and BP=99%). Additionally, *Alcyonium* and *Parerythropodium genera* were linked as paraphyletic lineages; in fact *Alcyonium palmatum* was clustered with *Alcyonium acaule* (BT=75% and BP=95%) instead of *Parerythropodium coralloides*. Finally, *Paramuricea clavata* (Plexauridae family) displayed unclear relationships with both the co-familiar species *Swiftia pallida* (Plexauridae family) and other Clade 1 species.
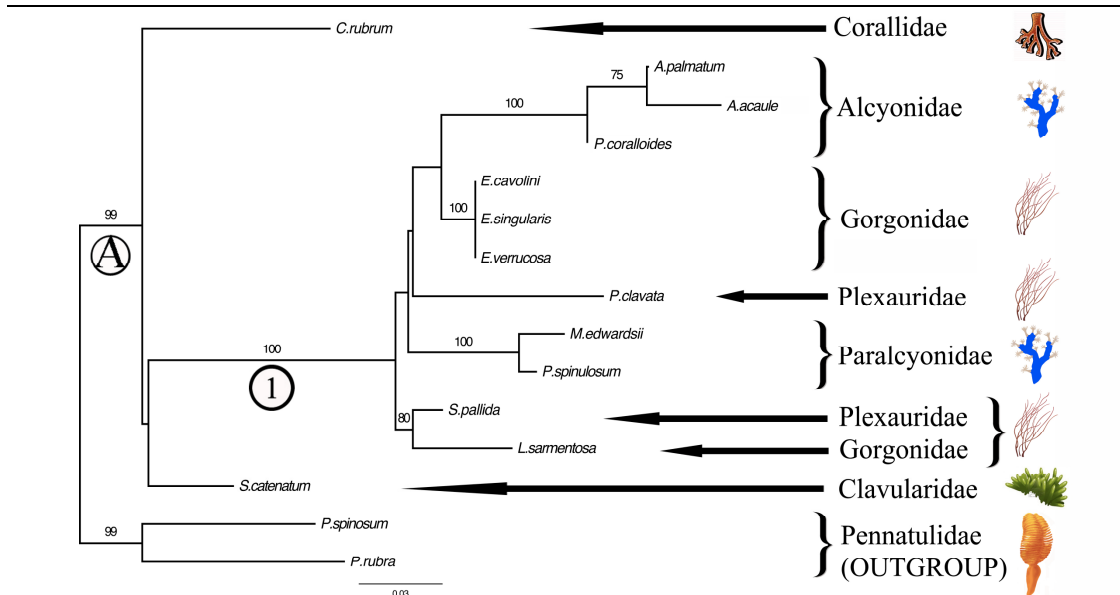
Figure 3.3.a. MtMSH ML tree of Mediterranean species (-lnL=2539). Bootstrap values evaluated on 150 permutation and values > 60% were shown. Figures on the right indicate the higher taxonomical level and were summarized in the legend 1.
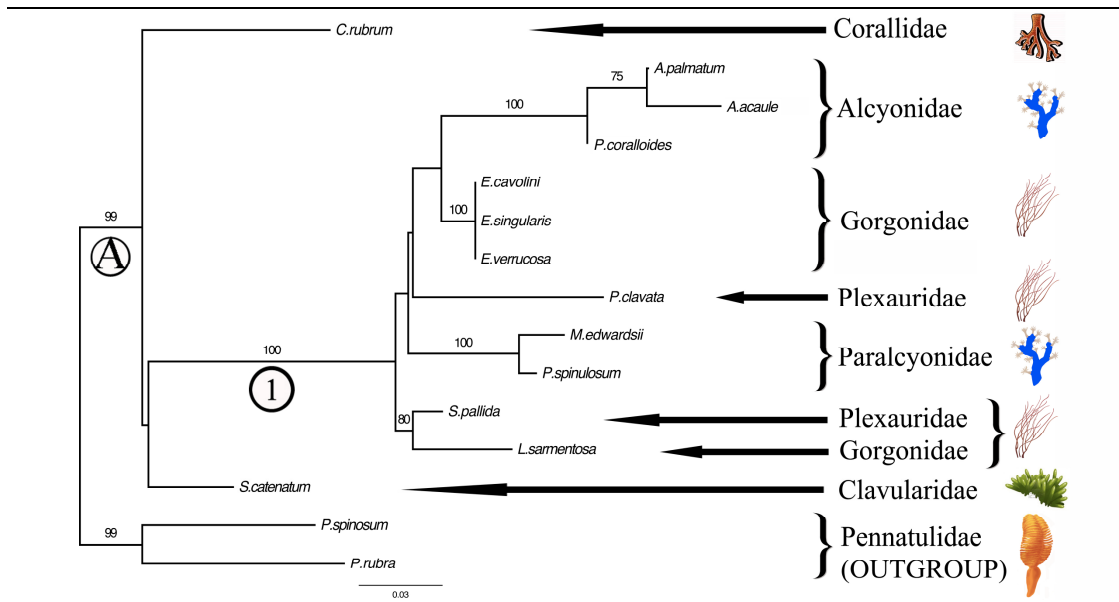
Figure 3.3.b. Bayesian MtMSH (50% majority rule) consensus tree of Mediterranean species. Bayesian posterior probability values > 60% were shown. Figures on the right indicate the higher taxonomical level and were summarized in the legend 1.

Legend 1. Icons of the order and sub-order of the Mediterranean octocorals.

3.3.2. 16S Mediterranean trees

The best substitution model regarding the 16S dataset was the general time reversible model (GTR) with gamma distribution (+G). <u>TRACER</u> results on Bayesian tree exhibited high value of ESS (>200), while <u>AWTY</u> post analyses supported the convergence of tree parameters.

Both Maximum-likelihood and Bayesian analyses recovered similar trees topologies, but showed different statistical support values of the nodes (Figure 3.4.a and 3.4.b respectively). The out-group clade comprised the two sea pens as a monophyletic group (BT=74% and BP=97%; Figures 3.3). The maximum-likelihood bootstrap value (BT<75%) did not support the main cluster (Clade A containing all the analysed species; Figures 3.4), nevertheless it was better identified in the Bayesian posterior probabilities (BP<97%).

At sub-order level within the Clade A, a large sub-clade (Clade 1) included all members of the sea fan sub-order Holaxonia and the soft corals belonging to the sub-order group Alcyoniina, linked as paraphyletic lineages. Additionally, the Clade 1 comprised also the Scleraxonia (*C. rubrum*), placed in an undefined tree position. However, this clade had low statistical support. The Bayesian tree better supported the monophyly of the Clade 1 (BP=59%; Figures 3.3.b) compared to the ML analysis (BT<60%; Figures 3.3.a). Moreover, Stolonifera (*S. catenatum*) was linked at the base of the Clade A without clear relationships with the Clade 1 species.

At lower taxonomic level (family), the Clade 1 exhibited two monophyletic groups: Paralcyonidae plus one Plexauridae species, and Alcyonidae family. For this marker, Paralcyonidae family seem to be a paraphyletic lineage and did not join the other Alcyonidae family soft coral. However, the Paralcyonidae plus Plexauridae

group had low statistical support (BT<60% and BP=62%). The Mediterranean Alcyoniadae *Alcyonium* and *Parerythropodium genera* were associated in the same monophyletic group through great statistical support (BT=88% and BP=99%). The other Gorgonidae and Plexauridae families were distributed without a clear phylogenetic pattern (BT<60% and BP<60%).

Purposely at *genus* level, *Eunicella genus* was not a defined group (BT<60% and BP<60%) and the three *Eunicelle spp.* were unlinked to the other co-familiar Gorgonidae species (*Leptogorgia sarmentosa*), which was placed in an undefined position within the Clade 1. *Alcyonium* and *Parerythropodium genera* were clearly associated as paraphyletic lineages, since *Alcyonium palmatum* was clustered to *Alcyonium acaule* (BT=88% and BP=95%) instead of *Parerythropodium coralloides*. Finally, *Paramuricea clavata* (Plexauridae family) despicted no clear relationships with the co-familiar specie *Swiftia pallida* (Plexauridae family).
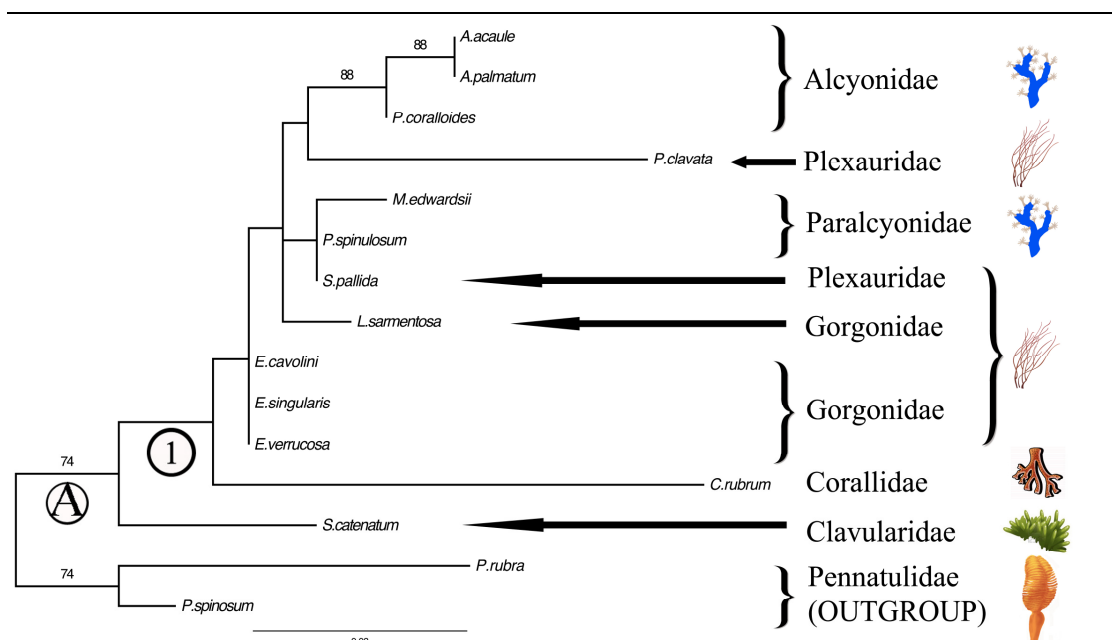
Figure 3.4.a. 16S ML tree of Mediterranean species (-lnL=680). Bootstrap values evaluated on 150 permutation and > 60% were shown. Figures on the right indicate the higher taxonomical level and were summarized in the legend 1.

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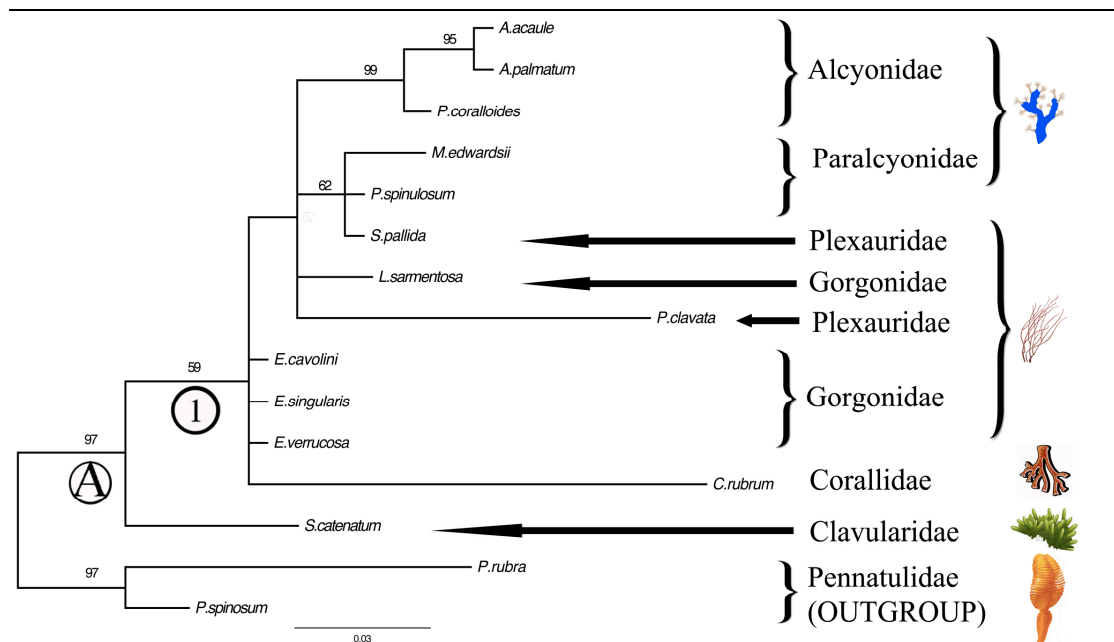
Figure 3.4.b. Bayesian 16S (50% majority rule) consensus tree of Mediterranean species. Bayesian posterior probability values > 60% were shown. Figures on the right indicate the higher taxonomical level and were summarized in the legend 1.

3.4. Mediterranean and Atlanto-Pacific species phylogenetic analysis

3.4.1. MtMSH trees

The selected extended MtMSH dataset substitution model was the general time reversible model (GTR) with gamma distribution (+G). TRACER results on Bayesian tree displayed high value of ESS (>200) and AWTY post analyses highly supported the tree parameters convergence.

The Maximum-likelihood (data not shown) and Bayesian analyses suggested similar trees topologies and nodes support values. Regarding the Bayesian tree, the out-group clade comprised all the sea pens (Order Pennatulacea) as a monophyletic group (BP=100%; Figure 3.5). The value of Bayesian posterior probabilities (BP=100%) strongly supported the main cluster involving all the analysed species (Clade A; Figure 3.5).

At sub-order level within clade A, two large sub-clades were highly statistically supported. The Clade 1 included all members of the sea fan sub-order Holaxonia and the soft corals belonging to the sub-ordinal group Alcyoniina related as paraphyletic lineages (BP=100%; Figures 3.5). The Clade 2 contained all the Scleraxonia species (BP=100%) as a monophyletic group. As in the previous analyses, the Stolonifera (*S. catenatum*) lied at the base of the Clade A without clear relationships with the other clades.

At lower taxonomic level (family), the Clade 1 was split into two main branches statistically highly supported. The Clade 1a included all the Alcyonidae family species (BP=100%). The Clade 1b encompassed five well-supported monophyletic groups, incorporating Paralcyonidae, Gorgonidae, Plexuridae and Plexuridae plus Gorgonidae. Specifically, the Paralcyonidae family was presented as monophyletic lineage (BP=100%) and its genetic pattern was greatly different respect to the other Alcyonidae family soft coral. Plexuridae and Gorgonidae families presented paraphyletic links to their *genera* within the Clade 1b. Chiefly, Plexauridae species belonging to *Paramuricea genus* constituted a monophyletic group (BP=100%), such as the Gorgonidae species of *Eunicella* (BP=100%). These also exhibited limited links (BP=73%) to the *Paramuricea* Plexuridae cluster. The remnant species of these two families were split into additional well-supported cluster (BP=100%) grouping the two *genera Swiftia* and *Leptogorgia,* belonging to the Plexuridae and Gorgonidae families respectively.

More specifically at *genus* level within the Clade 1, *Alcyonium* and *Parerythropodium genera* were linked as paraphyletic lineages within the Clade 1a. Mediterranean Alcyonidae species joined the same supported cluster within the Clade 1a (BP=100%). *Alcyonium palmatum* clustered with *Alcyonium acaule* (BP=100%)

instead of *Parerythropodium coralloides*. Moreover,the well-defined *Eunicella genus* group shared few contacts with the other co-familiar Gorgoniidae (*Leptogorgia*) *genus,* more linked to *Swiftia pallida* (Plexauridae family). *Paramuricea genus* (Plexauridae family) involved fewer relationships with the co-familiar species *Swiftia pallida* (Plexauridae family) than Eunicella *genus*.

At family level, the Clade 2 was strongly statistically recognized (BP=100%), and joined all the Corallidae family species as monophyletic group. Moreover, within this Clade, two well-supported lineages were identified. The Clade 2a included *Corallium laauense, Corallium ducale, Corallium niobe, Corallium elatius, Corallium rubrum* and *Paracorallium japonicum* (BP=100%). The clade 2b enclosed *Corallium secundum, Corallium konojoi* and *Corallium kishinouyei* (BP=100%).

More specifically at *genus* level, *Paracorallium* appeared to be nested within *Corallium genus.* A well-supported cluster (BP=100%) was observed within the Clade 2a, including *Corallium laauense, Corallium ducale, Corallium niobe*. *C.rubrum* showed strong link with *Corallium elatius* (BP=100%), while the *Paracorallium* species lied at the base of the Clade 2a.
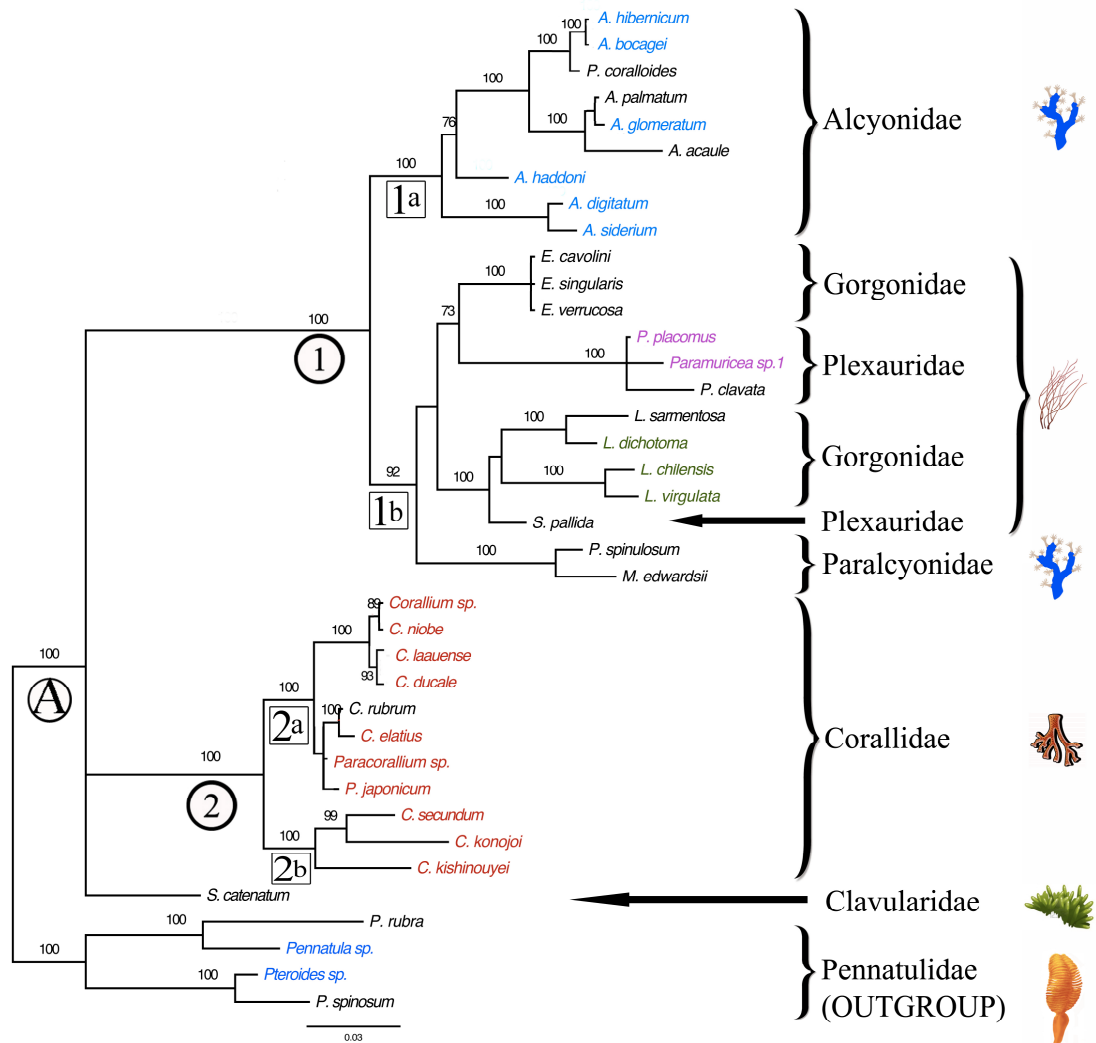
Figure 3.5. Bayesian MtMSH (50% majority rule) consensus tree of Mediterranean Octocorallia cogeners species. Atlanto/Pacific species belonging to the same *genus*/family were reported with the name of the same colour: pink= *Paramuricea;* green= *Leptogorgia*; light blue= Alcyonidae; red= Corallidae and purple= Pennatulidae. The Mediterranean species were reported in black. Figures on the right indicate the higher taxonomical level and were summarized in the legend 1.

3.4.2. 16S trees

The selected substitution model consisted in the general time reversible model (GTR) with gamma distribution (+G) for the extended 16S dataset.<u>TRACER</u> results on Bayesian tree showed high ESS value (>200), and <u>AWTY</u> post analyses supported the convergence of tree parameters.

The Maximum-likelihood (data not shown) and Bayesian analyses recovered similar trees topologies and node support values. Focusing on the Bayesian tree, the out-group clade comprised all the sea pens (Order Pennatulacea) as a monophyletic group (BP=99%; Figure 3.6). The main cluster (the Clade A containing all the analysed species) was well supported by the Bayesian posterior probabilities values (BP=99%; Figure 3.6).

At sub-ordinal level, within the Clade A, two large sub-clades were observed. The Clade 1 included all members of the sea fan Holaxonia sub-order together with the soft corals belonging to Alcyoniina sub-ordinal group. They are linked as paraphyletic lineages, but this node had low statistical support (BP<60%; Figures 3.6). Indeed, the Clade 2 clearly contained all the Scleraxonia species (BP=100%) as a monophyletic group. Moreover, Stolonifera (*S. catenatum*) lied at the base of the Clade A with unclear relationships with other clades.

At family level, the Clade 1 presented just one supported branch. The Clade 1a included all the Alcyoniina-Holaxonia sub-order species (BP=85%) excepted some belonging to *Eunicella genus* (Gorgonidae family), which lied at the base of the Clade 1. Within the Clade 1a, four well-supported groups were represented in Alcyoniade, Paralcyonidae plus one specie of Plexuridae, Gorgonidae and Plexuridae. Particularly, all the species of the Alcyonidae family merged into a monophyletic cluster

(BP=99%). Paralcyonidae family seemed to be a paraphyletic lineage not joining the other Alcyonidae family soft coral. The Paralcyonidae plus Plexauridae (*S. pallida*) group was statistically supported (BP=83%). Gorgonidae of *Leptogorgia genus* were observed as polyphyletic group consisting in two cluster. *Leptogorgia sarmentosa and L. capverdensis* clustered toghether (BP=86%) and were separated to *L. chilensis* plus *L. virgulata* group (BP=97%). Likewise, the Plexauridae group emerged as polyphyletic family, where only the *Paramuricea genus* appeared to be monophyletic.

More specifically at *genus* level, *Eunicella genus* was not a well-defined group (BT<60% and BP<60%). The three *Eunicelle spp.* had no relations with the other co-familiar Gorgonidae species, which was placed within the Clade 1a. Mediterranean Alcyonidae species joined the same well-supported cluster within the Clade 1a (BP=99%). *Alcyonium* and *Parerythropodium genera* were clearly linked as paraphyletic lineages, where *Alcyonium palmatum* clustered together to *Alcyonium acaule* (BP=100%) instead of *Parerythropodium coralloides*. Finally, *Swiftia pallida* (Plexauridae family) was associated to the Paralcyonidae family, while the other Swiftia sequences had no clear relationships with the Clade 1a groups.

At family level, the Clade 2 was recognized with high statistical support (BP=100%), and joined all the Corallidae family species as a monophyletic group. Likely to mtMSH, this marker identified two highly supported lineages. The Clade 2a included: *Corallium laauense, Corallium ducale, Corallium niobe, Corallium rubrum* and *Paracorallium japonicum* (BP=99%). The other clade 2b encompassed: *Corallium secundum, Corallium kishinouyei, Corallium konojoi* and *Corallium elatius* (BP=99%).

More specifically at *genus* level, *Paracorallium* seemed to be nested within the *Corallium genus*. Particularly, within the Clade 2a, the species of *Corallium laauense, Corallium ducale, Corallium niobe* were represented in a well-supported cluster (BP=100%), while *C.rubrum* and the *Paracorallium* species lied at the base of the Clade 2a. Although the handful of statistical support, also for this marker the red coral was more genetically closer to *Paracorallium* genera than the other *Corallium* species.
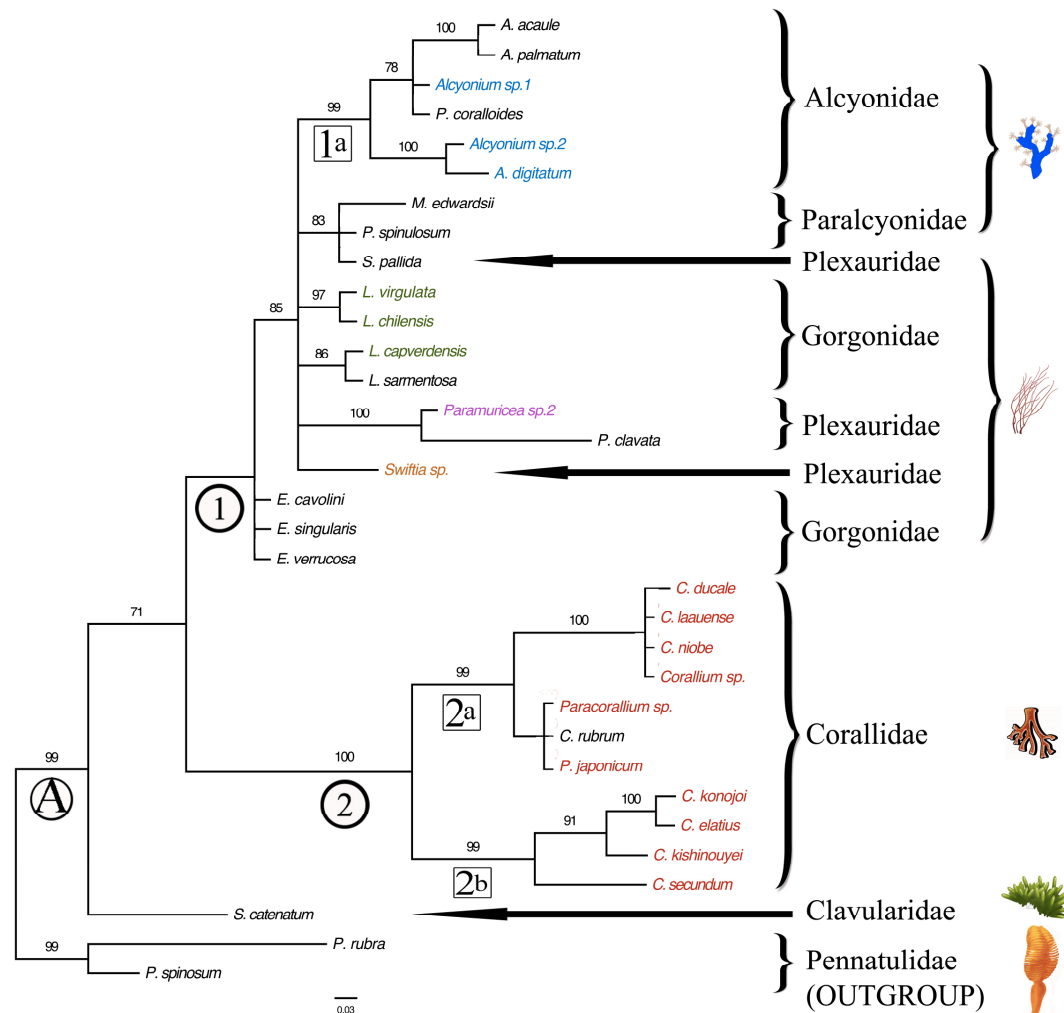
Figure 3.6. Bayesian 16S (50% majority rule) consensus tree of Mediterranean Octocorallia cogeners species. Atlanto/Pacific sSpecies belonging to the same genus/family were reported represented with the name of the same colour: pink= *Paramuricea;* green= *Leptogorgia*; light blue= Alcyonidae; red= Corallidae and purple= Pennatulidae. The Mediterranean species were reported in black. Figures on the right indicate the higher taxonomical level and were summarized in the legend 1.

3.4.3 MtMSH + 16S combined trees

IDL test was rejected due to significative p-value of 0.001 indicating the unfeasibility of combining MtMSH and 16S datasets. PARTINFINDER identified as the MtMSH + 16S partition best substitution model the general time reversible model (GTR) with gamma distribution (+G) more invariant sites (+I). The shape of the gamma distribution was set to 0.778 and the proportion of invariant sites was 0.222.

3.5. Divergence time

TRACER results in all 16S Bayesian tree presented low value of ESS (<200), therefore the convergence of tree parameters was not supported (data not showed). Consequently, only divergence times on MtMSH cogeners tree were estimated using two dating methods. The substitution model for the Bayesian molecular dating was the same of the MtMSH cogeners dataset (see Paragraph 3.4.1). The divergence time trees was rooted with Pennatulacea. These trees revealed similar tree topology as the Ottocorallia MtMSH tree (see Paragraph 3.4.1), and the same main clades of previous analysis conducted with MR.BAYES were recovered, with high nodes statistical support (Paragraph 3.4.1; Figure 3.5).

3.5.1 Fix mutation rate estimate

The estimation of the Mediterranean Ottocorallia cogeneric species divergence times by Bayesian inference with fix mutation rate set the split of the two main orders of Alcyonacea and Pennatulacea in the middle of Paleozoic, nearly 400 MYA (Figure 3.8). The Clade A of Alcyonacea started to diversify into the five sub-

orders analysed nearly in the same period, and both displayed a large confidence range (Figure 3.8).

At sub-ordinal level, within the Clade A, both the two large monophyletic branches of the Alcyoniina-Holaxonia Clade 1 and the Scleraxonia Clade 2 begun to diversify themself around the middle of Mesozoic, nearly 206 MYA and 134 MYA respectively. Moreover, Stolonifera (*S. catenatum*) had deeper relations in time with the other two main clades. It separated from the Clade 2 meanly 290 MYA with a huge confidence range.

At family level within the Clade 1, the Alcyonidae family (the Clade 1a) started to spread the two *genera* analysed in the central Mesozoic, nearly 130 MYA. Paralcyonidae was formerly isolated within the Clade 1b, around 177 MYA. Likewise, the Plexauride-Gorgonidae complex begun to differentiated into their *genera* in the same geological era, nearly 160 MYA, both in the Jurassic.

More specifically, the majority of the Clade 1 lineages diverged from their Atlanto/Pacific cogeners species before the MSC about 5 MYA (Figure 3.8). Analysing this dataset, *P clavata* and *L. sarmentosa* were separated to their cogeners MRCA very early in time, before 40 MYA. However, this estimation could be bias due to the sample size; in fact, these *genera* had the lowest number of sequences. Other species such as *M.edwardsii, P.spinulosum*, the three *Eunicella* species and *S.pallida* had no cogeners to compare, so the estimates of divergence times were not feasible. However, the *Alcyonium-Paraerythropodium* group had the higher resolution between species and the best representative estimated divergence times (Figure 3.8). Within the Clade 1a, *A. acaule* and *P. coralloides* were separated from their MRCA more than 5 MYA. Divergence time means were 38.5 MYA and 18.16 MYA

respectively with 95% high probability density not including 5 MYA. Indeed, *A. palmatum* diverged from its MRCA *A. glomeratum* meanly 7.54 millions of years ago with 95% high probability density ranged between 17.75 and 0.24 millions of years.

At family level, within the Clade 2 (Figure 3.8) the diversification of the Corallidae family species from Clavularidae was set about 290.21 MYA at the end of Paleozoic. The two main clusters observed within the Clade 2 were separated from 130.65 MYA in the middle of the Mesozoic. The clade 2b (C. *secundum, C. konojoi* and *C. kishinouyei*) seemed had begun to diversify his lineages earlier than clade 2a, estimated on 96.45 MYA and 52.41 MYA respectively.

Within the Clade 2a, *C. rubrum* was highly linked to *C. elatius* and with *P. japonicum*. They were split into the other Clade 2a species about 52.09 millions of years ago. Moreover, the estimated mean divergence time of *C. rubrum* from the *C. elatius* (MRCA lineage) was set to 8.07 millions of years ago. The 95% high probability density of the mean ranged between 16,86 and 0,80 millions of years ago including the end of MSC.
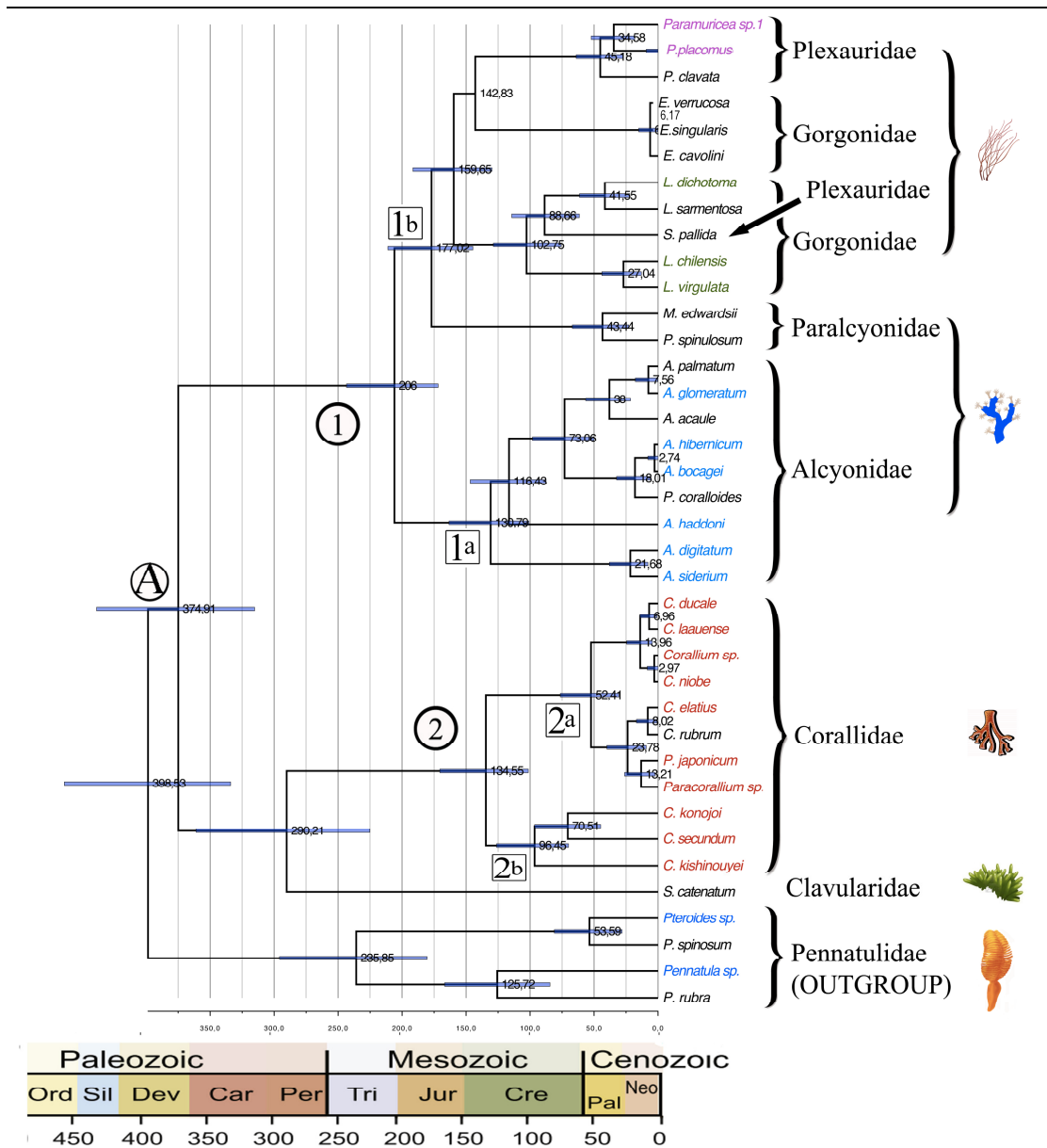
Figure 3.8. Bayesian dated phylogeny by the mutation rate based on MtMSH (50% majority rule) consensus tree of Mediterranean cogeners species. Species belonging to the same *genus*/family were reported with the name of the same colour: pink= *Paramuricea;* green= *Leptogorgia*; light blue= Alcyonidae; red= Corallidae and purple= Pennatulidae. The Mediterranean species were reported in black.

3.5.2 Calibrated point estimation

The Bayesian Mediterranean species estimate of the divergence times implementing the calibration point yielded different results in the mutation rate estimation (Figure 3.9). The majority of the lineages of the Mediterranean octocorals were divided also in this analysis from the Atlanto/Pacific cogeners species before the MSC, about 5 MYA. However, the estimated means of divergence times of the Mediterranean species, as *A. palmatum* and *C. rubrum,* was generally more closer to the end of MSC crisis.

At sub-ordinal level, within the Clade A, both the two large monophyletic branches of the Alcyoniina-Holaxonia Clade 1 and the Scleraxonia Clade 2 begun to diversify themselves around the end of the Mesozoic, nearly 115.01 MYA and 83.47 MYA respectively. Moreover, Stolonifera (*S. catenatum*) had older relations with the other two main clades; in fact, its detached from the Clade 2 nearly 162.82 MYA with a huge confidence range.

At family level within the Clade 1, the Alcyonidae family (Clade 1a) started to spread into the two analysed *genera* from the end of the Cretaceous, nearly 74.21 MYA. Paralcyonidae was previously isolated within the Clade 1b, around 93.89 MYA; also the Plexauride-Gorgonidae complex begun to diversify their *genera* at the same geological era, nearly 83.57 MYA, both in the end of the Cretaceous.

More specifically, the majority of the Clade 1 lineages were divided from their Atlanto/Pacific cogeners species before the MSC about 5 MYA (Figure 3.9). However, also this estimate could be bias due to the sample size; in fact various

genera had few number of sequences. Species such *P clavata* and *L. sarmentosa* were separated to their cogeners early in time, before 20 MYA. Moreover, other species such as *M.edwardsii, P.spinulosum*, the three *Eunicella* species and *S.pallida* had no cogeners to compare; subsequently, the estimates of divergence times for these organisms were not feasible.

Within the Clade 1, the *Alcyonium-Paraerythropodium* group (clade 1a) had the higher resolution between species and the best representative estimated divergence times (Figure 3.9). Within the Clade 1a, both *A. acaule* and *P. coralloides* were separated from their MRCA before 5 MYA. Divergence time means were 18.14 MYA and 10.63 MYA respectively with 95% high probability density that include the 5 MYA. Indeed, *A. palmatum* diverged from its MRCA *A. glomeratum* meanly 3.87 millions of years ago with 95% high probability density ranged between 12.45 MYA and present days.

On the other side, at family level within the Clade 2 (Figure 3.8), the diversification of Corallidae family species from Clavulariidae was set about 162.82 MYA in the middle of the Mesozoic. The two main clusters observed within the Clade 2 were separated from 83.47 MYA at the end of the Mesozoic according to fossil records. Inside Corallidae, the clade 2b (C. *secundum, C. konojoi* and *C. kishinouyei*) begun to diversify his lineages earlier than the Clade 2a, estimated on 55.34 and 30.89 MYA respectively.

Within the Clade 2a, *C. rubrum* was strongly linked to *C. elatius* and *P. japonicum*. They were split to the other clade 2a species about 30.89 MYA. More specifically, the estimated mean divergence time of *C. rubrum* from the *C. elatius*

(MRCA lineage) was set meanly on 4.04 MYA. The 95% high probability density of the mean ranged between 11.73 and 0,29 millions of years ago including the end of MSC. This estimate of the cladogenesis of *C. rubrum* was correlated with the end of MSC.
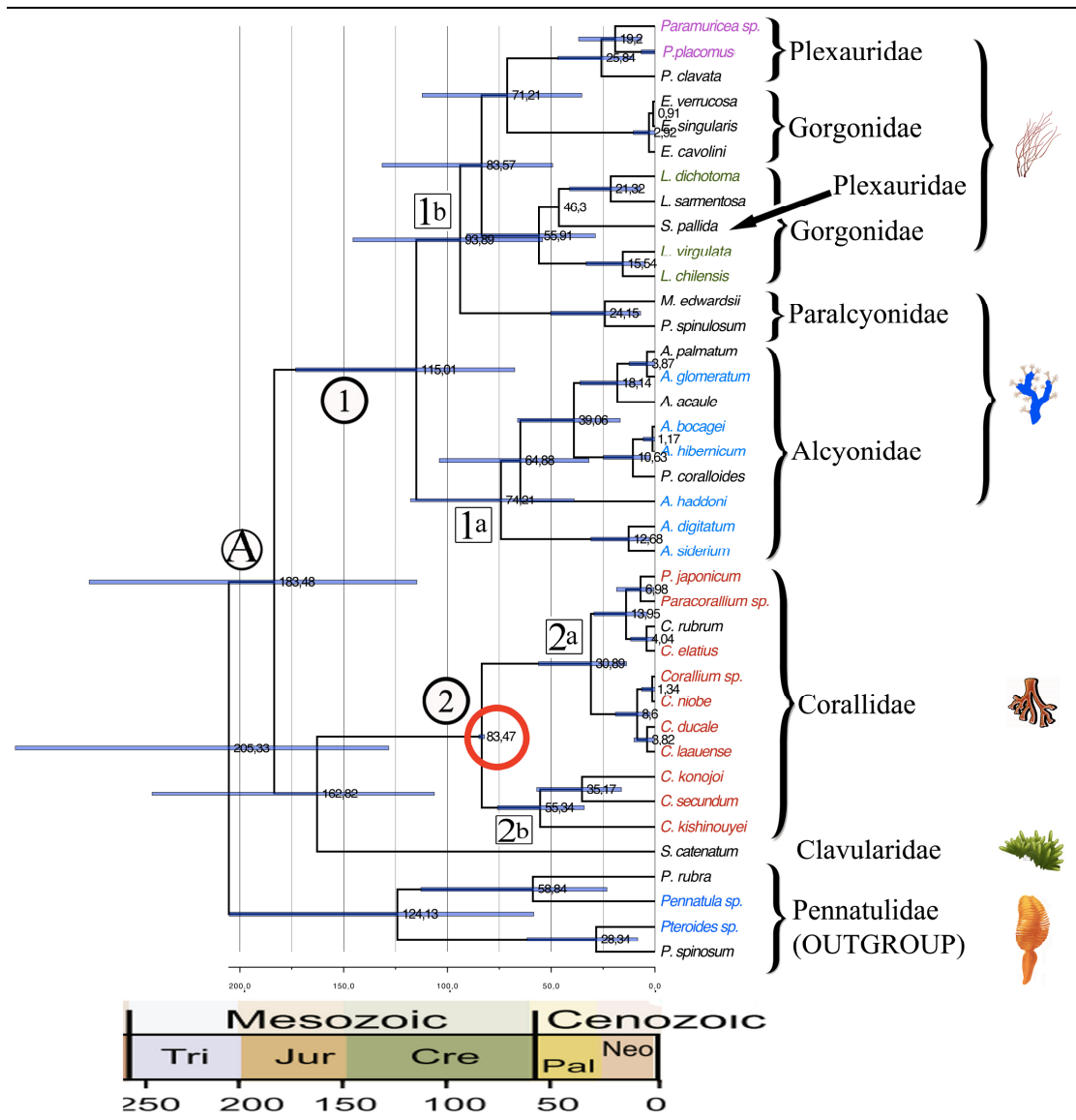
Figure 3.9. Bayesian dated phylogeny by the calibrate point based on MtMSH (50% majority rule) consensus tree of Mediterranean cogeners species. Species belonging to the same *genus*/family were reported with the name of the same colour: pink= *Paramuricea;* green= *Leptogorgia*; light blue= Alcyonidae; red= Corallidae and purple= Pennatulidae. The Mediterranean species were reported in black. Red circle indicate the calibration point used.

4 Discussion

Phylogenetic studies conducted to date provided a good knowledge on the evolutionary processes in Atlanto/Pacific octocorals (see McFadden et al. 2010 for review). The present study is one of the firsts on molecular phylogenesis of Mediterranean octocorals, addressing timeline of origin and diversification of the species. The major findings of this study are:

1) molecular and morphological phylogenies of octocorals provides consistent or conflicting results, depending on the taxonomical level considered;

2) Mediterranean octocorals fit well in the molecular phylogeny trees of Altanto/Pacific congeneric species;

3) the divergence time among Mediterranean and Atlanto/Pacific octocoral species varies depending on the analysed taxa.

4.1 Phylogeny of the Mediterranean octocorals

Both mtMSH and 16S mitochondrial markers gave similar results concenring the phylogeny of Mediterranean octocorals. However, the two markers differ in resolution power, due to the lower mutation rate of 16S compare to mtMSH (Sanchez et al 2003a). Moreover, they differ in length. 16S marker was shorter and had a low substitution rate, therefore it was not able to reveal phylogenetic relationships among Mediterranean octocorals. Conversely, mtMSH marker yielded a more representative

phylogeny. It is known that inclusion of a large number of genomic regions should improve the quality of the inferences in the phylogenetic analyses (Cummings et al., 1995). However, the gene trees obtained by combining mtMSH and 16Sresults did not improve our understanding on Mediterranean octocorals phylogeny due to the inconsistent genetic signals provided by the two markers.

The Clade A recovered in our analyses unifies all the Mediterranean species belonging to the order Alcyonacea, but it seems to be a phylogenetic artefact than a monophyletic group, due to the sample design and chosen out-group.

The out-group clade comprised all the sea pens (Order Pennatulacea) as a monophyletic clade. Despite both, morphological and molecular synapomorphies, support the monophyly of Pennatulidae family (McFadden et al. 2010 and references therein), many studies recovered a well-supported clade of octocorals including deep-water families of Pennatulacea, Calcaxonia (Chrysogorgiidae, Isididae, Primnoidae), Scleraxonia (Coralliidae, Paragorgiidae) and Alcyoniina (genus Anthomastus; McFadden et al. 2006 and references therein).

Within the Clade A, a large paraphyletic sub-clade (Clade 1) was observed. It included all the sea fan of the sub-order Holaxonia and the soft corals belonging to the sub-order Alcyoniina. The Clade 1 corresponded to the Berntson et al. (2001) Clade C, to the Sánchez et al. (2003a) "Alcyoniina–Holaxonia" Clade and to the McFadden et al. (2006) Clade 1. When the Mediterranean species were included in the analysis, morphological differences between the two suborders were not supported (McFadden et al. 2006). Moreover within Clade A, the Clade 1 was clearly distinct in the two species *Sarcodiction catenatum* and *Corallium rubrum,* belonging respectively to the suborders Stolonifera and Scleraxonia. The two species got a basal position within the

Clade A in the Mediterranean phylogeny. McFadden et al. (2006) and Berntson et al. (2001) suggested that some Scleraxonia and Stolonifera species lay at/close to the base of Octocorallia. McFadden et al. (2006) hypothesised that the morphological characters typical of the stoloniferan, as narrow stolons from which small polyps arise singularly, evolved several time within the octocorals. However, this hypothesis needs further experimental support.

Despite the phylogeny of Mediterranean species did not support the current taxonomic classification at suborder level (Bayer et al. 1981), at family/genus level phylogenetic clades were largely congruent to taxonomical classification. All the Mediterranean species joined in each *genus* rank as excepted; however some paraphyletic cluster remain unresolved, such as *Alcyonium-Parerythropodium*.

The main difference between this and the McFadden (2006) analysis regarded the root of trees. The selection of Pennatulacea as out-group (as Sanchez et al. 2003a) was in this thesis influenced by the taxonomical point of view, while the mid-point rooting better fitted the species genetic diversity.

The phylogeny integrated the Mediterranean species in the Atlanto/Pacific congeners octocorals confirming previous patterns. Increasing the number of species for both markers, the improved he resolution power of the trees. Particularly, the distinctiveness of the Scleraxonia species (the Clade 2; see afterwards) respected to the other two main clades (the Alcyoniina-Holaxonia Clade 1 and the Pennatulacea out-group Clade) became more robust (PB = 100%). The results of this study supported previously studies (France et al 1996; Sánchez et al. 2003a; McFadden et al. 2006; Herrera et al. 2010; McFadden et al. 2010) on the phylogenetic relationships among widely distributed species.

At family level, inferences regarding the relationships of Ottocorallia families were unavoidably limited due to number of taxa sampled. Corallidae (the clade 2; see afterwards) and Alcyonidae (within the Clade 1) families were the most representative groups with 10 and 6 congeners sequences, respectively, corresponding to 9 different species within both families. Moreover, the Clade 1a and 2 were monophyletic groups. These, together with the Pennatulidae family (the out-group clade), were correlated to their taxonomic ranks.

At *genus* level, all Acyonidae species were included in the *Alcyonium-Parerythropodium* cluster, which were linked as paraphyletic lineages. All the Mediterranean Alcyonidae species joined the same cluster within the Alcyonidae monophyletic clade (the Clade 1a in the MtMSH trees). Intra-genus relationship of *Alcyonium* species was largely confirmed by COI+igr1+mtMSH phylogenetic analysis of the 10 species of the northern hemisphere (McFadden et al. 2011 and references therein). In this survey, the authors recovered a Mediterranean plus Atlantic species cluster, which supported the allopatric speciation of the Mediterranean species from North-Atlantic after the end of the Mediterranean salinity crisis.

Regarding the other Alcyonacea, the Mediterranean Gorgoniidae (*Eunicella spp.*) and Plexauridae (*Paramuricea spp.*) species were strictly related. Phylogenetic relationships between these families had been further examined using data from msh1, ND2, and ND6 (Sànchez et al. 2003b, Wirshing et al. 2005). These works indicated that the Plexauridae family was clustered with the Gorgoniidae within the Holaxonia suborder. However, *Paramuricea genus* was highly distinctive in both Mediterranean phylogenies, as found in an extensive Plexauridae and Gorgonidae phylogenetic

analysis (Wirshing et al. 2005). Moreover, some other *genera* of the same family (e.g. *Echinomuricea, Placogorgia, Villogorgia and Paracis*) presented the same thorn-scale sclerites (Bayer 1981). Hence, these features supported the Bayer's hypothesis that Plexauridae may ''require further subdivision into a restricted Paramuriceidae sub-family including only the genera with thorn-scales or comparable sclerites''(Bayer 1961). Finally, both MtMSH and 16S genes did not discriminate among the different *Eunicella* species, as in Gori et al. 2012. A similar result was previously obtained for other genetic markers, such as the COI sequences of *E. singularis* and *E. cavolini* which were identical, or the identity of the ITS2 sequence from *E. singularis* and one of the *E. cavolinii* haplotypes (Calderòn et al. 2006). This could be a consequence of phenotypic variation within a single species of *Eunicella* (Gori et al. 2012). Otherwise, more liable alternatives regarded the recent divergence process or the low rate of mitochondrial evolution found in octocorals (France and Hoover, 2002).

In both markers the Clade 2 jointly comprised *C.rubrum* and all the species of the Corallidae family as monophyletic group. The clade included all of the sea pens (Pennatulacea), while the Corallidae clade corresponded to the "Calcaxonia" clade of Sánchez et al. (2003a), and to Berntson et al.'s (2001) 18S phylogeny A+B clades. The results of this study were in agreement with McFadden et al.'s (2006) phylogeny, in which Pennatulacea and *Corallium* were split in two separate clades. Also Herrera et al. (2010) merged *Corallium* and *Paracorallium* in one monophyletic clade. Within the inferred monophyly of the Coralliidae family, the *Paracorallium* genus appeared to be nested within *Corallium*, suggesting the paraphyly of the latter as in Herrera et al. (2010). Two well supported lineages were identified in the Clade 2 in both markers. The clade 2a comprised *C. laauense, C. ducale,* and *C. niobe,* while the clade 2b

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encompassed *C. secundum, C. konojoi* and *C. kishinouyei.* The other three species presented some differences between markers. Specifically, *C. elatius* acquired an undefined position within the Clade 2. Contrarily, the relationships between *C.rubrum*, *P. japonicum* and the Clade 2a (*C. laauense, C. ducale, C. niobe*) were supported in both mtMSH and 16S phylogenies. The Clade 2a contained one Atlantic species (*C. niobe)*, while all the other live in the Pacific. Phylogeny based on mtMSH indicated the red coral as placed near the *C. elatius* within the Clade 3. However, *Paracorallium japonicum* (red Japanese coral; Uda et al 2011), *C. elatius* (pink coral; Uda et al. 2011) and *C. rubrum* group were not well defined, depicting an incongruent evolutionary signal. Further studies implementing other molecular and taxonomical traits are required to understand the relationships within the Clade 2.

Many taxonomical traits correlated the Corallidae species (Bayer 1964; Daly et al. 2007)*.* In the case of *Paracorallium,* two features characterize the *genus:* the presence of autozooids seated in axial deep pits with beaded margins, and longitudinal grooves on the surface of the axis (Uda et al. 2011; Bayer 1996). Nevertheless, the pits considered as distinctive of this genus were not observed in *P. japonicum* inhabiting the waters off Kochi (Japan) and in *Corallium* species (Kishinouye, 1903; Bayer 1996). Additionally, *C. rubrum* and *P. japonicum* species were associated also by some ecological features, such as the shallower water preference compared to other Corallidae species (Tsounis et al. 2007). Hence, the integration of molecular, morphological and ecological markers indicated that the association of *Paracorallium japonicum*, *C. elatius* and *C. rubrum* species was highly likely. Although the red coral is endemic of the Mediterranean Sea, the other 2 species (*Paracorallium japonicum* and *C. elatius*) are restricted to North Western Pacific waters (Tsounis et al. 2010) and they are 17000 km far from the Mediterranean congeneric species. Similar pattern of

"disgiunct" speciation between Mediterranean and West Pacific species was recovered also in *Posidonia oceanica,* or in *P. australis* and *P. ostenfeldii complexes* inhabiting Australian tropical and sub-tropical waters (Gobert et al. 2006; Aires et al. 2011).

4.2 The origin of Mediterranean octocorals

Several studies on molecular dating investigated whether recent history of the Mediterranean Sea has promoted allopatric speciation, such as in teleost fishes (Meynard et al. 2012) or in intertidal Gasteropoda (Trochidae; Donald et al. 2012). Regarding the Mediterranean octocorals phylogeny, monophyly was largely confirmed for the Mediterranean species within the Atlanto/Pacific genera as reported in McFadden et al. (2006), indicating high similarities between all the congeneric Ottocorallia species. This result was crucial to support the allopatric speciation hypothesis of the Mediterranean octocorals from the Atlantic congeneric spesies. However, we were able to analyse just two clades (the Clade 1a and the Clade 2 encompassing Alcyonidae and Corallidae families respectively) with a high level of confidence for the MtMSH marker. The relationships and the divergence times estimates for all the other Mediterranean species should be taken with prudence due to the limited number of sequences published for the two analysed markers.

Considering the Bayesian divergence times using the estimated mutation rate, the majority of the Mediterranean lineages within the Clade A were divided from their Atlanto/Pacific most recent common ancestors (MRCA hereafter) before 15 MYA. *Alcyonum palmatum* and *C. rubrum* were the single two species presenting an estimate mean divergence time during the last 10 MYA. Moreover, the mutation rate

implemented in this method was evaluated across many *Leptogorgia* species (Lepard 2003) and subsequently, it was widely applied in phylogenetic analyses of octocoral species (McFadden et al. 2007; Thoma et al. 2009). Phylogeny dated using Lepard (2003) mutation rate set the Alcyonacea diversification origin comparatively deep in the past during Paleozoic (more than 300 MYA). This result was not supported by Park et al. (2012). Hence, the efficacy of the Bayesian divergence times implementing the estimated mutation rate of Lepard (2003) was restricted to the investigation of the Alcyonacea species origin in the Clade 1a.

Resuming, *A. palmatum* was the single species recently diverging within the Clade 1a. It split from its MRCA (shared with *A. glomeratum*) nearly 7.54 MYA with 95% higher probability density including the end of MSC. The results regarding the Mediterranean *A. acaule* and *P. coralloides* phylogeny and their NE-Atlantic distribution did not support the hypothesis that the recent Mediterranean Sea history events contributed to the cladogenesis of these octocorals. Consequently, earlier multiple factors such as the continental breakup, expansion of the Tethys Sea and of shallow habitats, changes in ocean temperature and chemistry, predation pressure, reproductive traits, early origin and burst adaptive radiation should provide the main framework to understand the biodiversity of these two species (Park et al. 2012 and references therein). Indeed, *A. palmatum* displayed a more recent genesis, as red coral (see afterwards), that is slightly correlated with the end of MSC. Therefore, the allopatric speciation hypothesis seems to better clarify the origin of this species. However, its wide spatial distribution in the North Eastern Atlantic Ocean and the Mediterranean Sea (McFadden et al. 2011) is in conflict with the allopatric hypothesis.

The results concerning the few Mediterranean endemic species indicated that the speciation processes of *Maasella edwardsii*, *Eunicella cavolini* and *E. singularis* were likely to be occurred before MSC. Accordingly, these three evolutionary lineages should have colonized the Mediterranean Sea from the great Atlantic income (the Zanclean flood) after MSC, about 5 MYA (Patarnello et al. 2007). Following this scenario, the species that are nowadays called "endemic" likely originated due to migration, rather than allopatric speciation within the Mediterranean. However these results should be prudently considered due to Ottocorallia features as long-lived, slow growing (Tsounis et al. 2010) and high dispersal capabilities resulting in slower speciation rates (Thoma et al. 2009).

The Bayesian divergence time based on the estimated mutation rate set the origin of the Corallidae species 130 MYA, in the central Mesozoic. This result was in contrast with the first Corallidae species hard skeleton fossil record discovered in Europe (Schlagintweit and Gawlick, 2009), which delayed the origin nearly 50 MYA. So, the Bayesian divergence time employing a calibration point based on this fossil record yielded a highly representative time scale assessment. Aurelle et al. (2011) suggested that *C. rubrum* populations might have persisted in the Mediterranean area through the last Quaternary. The mtMSH estimation of *C. rubrum* point-calibrated mean divergence time from the MRCA shared with *C. elatius* lineage was highly correlated to the end of MSC, around 4 MYA.

As for red coral, Donald et al. (2012) reported similar allopatric speciation pattern between Mediterranean and Atlantic Gasteropoda (Trochidae) species of *Osilinus* and *Phorcus* genera. Moreover, many other benthic invertebrates species do not occur either in the Atlantic Ocean and the Mediterranean Sea; their geographic

ranges do not overlap in the area adjacent to the Strait of Gibraltar, confirming the importance of this strait and the nearby Alboran front as biogeographic breaks (Patarnello et al., 2007). For instance, species-level differentiation across the strait was observed in limpets (*Patella caerulea* and *P. depressa*; Côrte-Real et al., 1996), barnacles (Pannacciulli et al., 1997) and crabs (*Carcinus aestuarii* and *C. maenas*; *Xantho granudicarpos* and *X. incusus*; Almaça, 1985). This strait apparently acts as barrier in organisms with short larval stages, such as *P. lineatus*, whose lecithotrophic veliger remains in the water column for 6–7 days maximum (Desai, 1966; Hickman, 1992; Crothers, 2001). In the case of octocorals, the different sexual reproductive strategies, together with asexual ones, have important ecological as well as genetic consequences (Knowlton and Jackson 1993). Specifically, *C. rubrum* lives in caves, an habitat characterized by particular conditions with relatively little water movement. This species is gonochoric brooder with internal fertilization that releases lecitotrophic larvae during late summer (Tsounis et al. 2010). Hence, the main process responsible of the *C. rubrum* phylogenetic pattern should be the limited dispersal distance of red coral larvae, determining genetic differences among populations even over short distances of less than 3 km (Calderon et al. 2006, Costantini et al. 2007a,b).

Finally, the *C. rubrum* evolutionary history was not clearly identified; in fact, these results should be prudently evaluated due to the small number of Corallidae specimens and molecular markers. The implementation of a specific sampling design would be required to elucidate the internal relationships of this family (Herrera et al. 2010).

4.3 Conclusion

Understanding the interplay between phylogenetic diversity and environmental gradients at large biogeographic scales may help us understanding the mechanisms that are behind this diversity (Meynard et al. 2011). The importance of this kind of information is becoming particularly relevant in the Mediterranean Sea, where the biodiversity may be at high risk under the rates of current global changes (Meynard 2012 and references therein).

Regarding the Mediterranean endemic species *C. rubrum*, information concerning its evolutionary history are still limited. In fact, the gene trees of the Corallidae family are far form describing the phylogenetic tree of these precious corals. Further precious corals specimens and new molecular markers are needed to reach a strong phylogenetic hypothesis of red coral speciation. Moreover, the large-scale genomic coverage offered by the emerging ''next-generation sequencing'' technology might be effective to improve the octocorals phylogeny (McFadden et al. 2010). Other important enhancements might be gained through the development of under-selection markers McFadden et al. (2010).

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